

THE UNIVERSITY OF MANITOBA

STUDIES IN TUMOR IMMUNOLOGY

BY

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SUMMARY

Two model systems for the development of cancer immunotherapy have been investigated:

- (i) In vivo destruction of tumor cells by an indirect cell-mediated immune mechanism

Polyoma cells coated with anti-tumor rabbit antibodies were administered in lethal doses to C57Bl/6J mice. The growth of the tumor cells was partially or completely inhibited in vivo by syngeneic lymphoid cells sensitized to rabbit immunoglobulins. The phenomenon was immunologically specific.

- (ii) Cytotoxic response in vitro against tumor cells

The in vitro cultures of a methylcholanthrene induced sarcoma, MC-D, from inbred strain 13 guinea pigs with syngeneic lymphoid cells resulted in the production of lymphoblast like cells capable of destroying monolayers of the tumor. Similar cytotoxic cells emerged from some primary tumor cell cultures without the addition of lymphoid cells to the system. The cytotoxic cells possessed some of the characteristics of activated (thymus derived) lymphocytes. It is suggested that the cells obtained from primary tumor cultures were the progeny of tumor-sensitive host lymphocytes, which had infiltrated the tumor tissue in vivo. The cytotoxic cells produced in vitro were capable of partially inhibiting the growth of tumor cells in vivo upon conjoint subcutaneous application.

" NEITHER THE PRESTIGE OF YOUR SUBJECT AND
 THE POWER OF YOUR INSTRUMENTS
 NOR THE EXTENT OF YOUR BEARNEDNESS AND
 THE PRECISION OF YOUR PLANNING
 CAN SUBSTITUTE FOR
 THE ORIGINALITY OF YOUR APPROACH AND
 THE KEENNESS OF YOUR OBSERVATION."

(Hans Selye)

This message expresses Dr. Selye's scientific phylosophy and serves also as a motto for his Institute. Dr. Selye's spirit, inexhaustible ambition and original approaches to scientific problems made a lasting impression on me while being his pupil at the University of Montreal, and helped greatly to complete this work. I want to thank him for everything.

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Abbreviations frequently used in the text

AFP		Alpha-fetoprotein
ALS		Anti-lymphocyte serum
BaP		Benz(a)pyrene
B cell		Bone marrow (or bursa) derived lymphocyte
BCG		Bacillus Calmette-Guerin
BL		Burkitt lymphoma
BSA		Bovine serum albumin
B16		B16 melanoma of C57 mice
C ⁶		Complement
CBH		Cutaneous basophil hypersensitivity
CBuLA		Chicken bursa derived lymphocyte antigen
CEA		Carcinoembryonic antigen
CFA		Complete Freund's adjuvant
CGG		Chicken gamma globulin
CMEM		Complemented minimal essential medium (Eagle)
Con-A		Concanavalin A
CTLA		Chicken thymus derived lymphocyte antigen
DMBA		7, 12-dimethylbenz(a)anthracene
DNA		deoxyribonucleic acid
DNCB		Dinitrochlorobenzene
DNFB		Dinitrofluorobenzene
DNP		Dinitrophenol
EB virus		Epstein-Barr virus
FCS		Fetal calf serum
Fer-Con-A		Ferritin conjugated concanavalin A
FITC		Fluorescein isothiocyanate

FMR		Friend-Moloney-Rauscher (leukemia viruses)
G		Gross (leukemia virus)
GCSA		Gross (virus specified) cell surface antigen
Gp		Guinea pig (13 - strain 13; H - Heston strain; Ha - Hartley strain)
gs		Group specific antigen of RNA viruses
GVHR		Graft-versus-host reaction
GTX		Gross (virus specified) surface antigen
HS		Hanks solution (-L supplemented with 5% lactalbumin hydrolysate)
HSV		hypertrophic spleen virus
Ig		immunoglobulin
LBC		Lymphoblast like cell
LNC		Lymph node cell
LT		Lymphotoxin
MBLA		Mouse bone marrow derived lymphocyte antigen
MC, MCA		3 - methylcholanthrene
MC-D		3 - methylcholanthrene induced sarcoma (line D) of strain 13 guinea pigs
MEM		minimal essential medium (Eagle)
MIF		migration inhibitory factor
MLC		mixed lymphocyte culture
MLV		murine leukemia virus (-F - Friend, -R - Rauscher)
mRNA		messenger ribonucleic acid
MSV		murine sarcoma virus (-H -Harvey, -M -Moloney)
MTV		mammary tumor virus
ML99		medium 199

NDV		Newcastle disease virus
NIP		4-hydroxy-3-iodo-5-nitrophenyl
NLC		normal lymphoid cell
OA		ovalbumin
Θ		(theta) thymic alloantigen of mice
PBS		phosphate buffered saline
PC		plasma cell antigen of mice
PCA		passive cutaneous anaphylaxis
PFC		hemolytic plaque forming cell
PHA		phytohemagglutinin
Po		polyoma virus induced tumor of C57 mice
PoA		polyoma antigen
Po-Ab		polyoma tumor cell coated with antibody
poly-dA·dT		poly d adenine · d thymidine
poly-dG·dC		poly d guanine · d cytosine
poly A:U		poly adenylic - poly uridylic acid
poly I:C		poly inosinic - poly cytidylic acid
Po-RIg		polyoma cells coated with rabbit antibodies
PPD		purified protein derivative of Mycobacterium tuberculosis
RES		reticuloendothelial system
RGG		rabbit gamma globulins
RNA		ribonucleic acid
RIg		rabbit immunoglobulin
RLV		radiation leukemia virus
RSV		Rous sarcoma virus of chickens

S		surface (tumor) antigen
SLC		sensitized lymphoid cell
SMEM		spinner - modified minimal essential medium (Eagle)
SPC		spleen cells
SRBC		sheep red blood cells (-RIg - rabbit immunoglobulin coated, -Ab - antibody coated)
T		nuclear tumor antigen induced by DNA viruses
TA		tumor antigen
TAA		tumor associated antigen
T cells		thymus derived lymphocyte
TL		thymus - leukemia antigen
Tla		gene locus determining TL
ts		type specific antigen of RNA tumor viruses
TSTA		tumor specific transplantation antigen
UV		ultraviolet light
VCN		Vibrio cholerae neuraminidase
WBC		white blood cell

Chapter I

INTRODUCTION *Foreword

From a survey of the vast literature dealing with different aspects of research in oncology, the conclusion emerges that the majority of experimental and human tumors are antigenic. In the context of the concept of immunological specificity, one may distinguish between tumors possessing cross-reacting antigens (e.g. virus-dependent or embryonic antigens) and tumors characterized by individually specific antigens. Viral tumors have been shown to possess common antigenic determinants as long as identical or closely related viruses are involved in the neoplastic transformations and only in some infrequent cases have cross-reactions been demonstrated among chemically-induced tumors. In addition, individual, non-crossreacting antigens have been discovered for both types of tumor, which might originate as a consequence of random cell injuries; thus, "spontaneous" tumors may belong to either category. It is likely that most antigenic tumors have a number of different antigenic determinants both on the cell membrane and intracellularly, the pathological and immunological significance of which is hardly known. The fact that some of the antigens, including those of viral origin which are manifested on tumors, may also be present in (extremely) low concentration on normally functioning tissues is

* The bibliographical references have been listed in alphabetical order at the end of the thesis and are identified by numbers in brackets.

most perplexing. It is conceivable that certain of these antigens, referred to broadly as tumor associated antigen (TAA) will eventually prove to be characteristic of cancer cells and the elucidation of the mechanism underlying their appearance in higher concentration on tumor cells will obviously contribute to the understanding of oncogenesis and may facilitate the fight against neoplastic disease.

Host immune responses, both cell-mediated and humoral can be detected to most antigenic tumors. The weight of experimental evidence supports the generalization that resistance to solid tumors may be transferred with lymphoid cells, rather than with serum, while dispersed tumors of the hemopoietic and lymphoid system can be suppressed more readily with antibodies. An absolute general rule, however, has as yet not been established and it is likely that, in various phases of the tumor-host relationship, host resistance is represented by different factors. Thus, for example, it has been recently reported that solid tumors induced by polyoma virus in rats regressed when serum containing "unblocking" antibodies was transferred from preimmunized animals to tumor bearing hosts (56). There are also other examples of humoral protection against solid neoplasms as discussed later. On the other hand, humoral antibody response to I2C leukemia of strain 2 guinea pigs could not be detected with a variety of methods; yet, immunization was possible and even a significant therapeutic effect could be achieved by adoptive transfer of immunity with sensitized lymphoid cells (264). Stimulation of lymphocytes by autologous leukemic cells from patients with acute leukemia in a

mixed lymphocyte-type culture (307) may indicate the presence of a cell-mediated defence mechanism against lymphoproliferative malignancies also in human. From these few examples, it is obvious that it is hazardous to generalize at this stage of our incomplete knowledge of the immunobiology of tumor-host relationships on possible immunological strategies for tumor destruction. Nevertheless, it seems reasonable to suggest that antigenic tumors must find a way to overcome destruction by the host's defence mechanisms in order to succeed. Natural or induced immunodeficiency states of the host, immunological tolerance and enhancement, a decrease in the density of tumor antigens or production of a non-antigenic coat on the malignant cell surface, as well as immunosuppression by factors elaborated by the tumor may be considered as possible escape mechanisms.

On rare occasions tumors may reach a considerable size and then spontaneously regress (274; 555; 763). Thus, although the mechanism underlying these regressions is virtually unknown, it is to be noted that among 176 adequately documented cases in this category, there were 31 adenocarcinomas of the kidney, 29 neuroblastomas, 19 malignant melanomas, 19 choriocarcinomas, 13 bladder carcinomas, 11 soft tissue sarcomas, 8 osteosarcomas, 7 carcinomas of the colon and rectum and the rest represents other neoplasms (274). All tumors associated with the highest frequency of spontaneous regression have been shown to be strongly antigenic, which is indirect, but convincing, evidence for the possible involvement of

immunological mechanisms in regression. Therefore, the hope, that immunotherapy for certain types of malignancy will become feasible in the foreseeable future is not entirely unfounded. However, a better understanding of the immunobiology of tumor-host relationship is urgently needed for the development of rational methods of cancer immunotherapy in man.

In Chapter II, an attempt has been made to survey the rapidly exploding literature of the complex field of tumor immunology. Because of apparent contradictions and the lack of uniformity of the conclusions of many different studies, and because of the widely differing approaches used in different laboratories, it has been a difficult task to present a cohesive overview of the field in a reasonably concise manner. Consequently, in parts, this Chapter may appear as a tabulation of experimental data and theories, rather than a critical condensation of the subject matter. However, in an attempt to provide as complete a picture as possible of the relevant literature, the author has taken the risk of incurring this criticism rather than exclude some important information which has, as yet, not reached the status of a "classical" study.

Since the central theme of this study is the possible immunological basis for a rational approach to the therapy of tumors, the author felt that it was appropriate to include a chapter on modern immunological concepts and methods, as they may relate to tumor immunology. However, in view of the explosive growth of

knowledge also in this area in recent years, and in particular in cellular immunology which appears to have a direct bearing on destruction of tumor (target) cells, the author has encountered similar difficulties in trying to summarize in an objective and fair manner the different and, in some cases, conflicting studies. For this reason, the chapter on Basic Immunology has grown to a greater length than originally anticipated. With consideration to the reader not interested in or completely familiar with the basic aspects of immunology, this topic has been included as Appendix I.

The scope of the present study

This study was undertaken with a view to obtaining more information about the immunological aspects of tumor-host relationships and in the expectation that potential models for cancer immunotherapy may be developed. All investigations performed are related to two basic models: (a) in vivo destruction of tumor cells by an indirect cell-mediated immune mechanism, and (b) immune response in vitro against tumor cells.

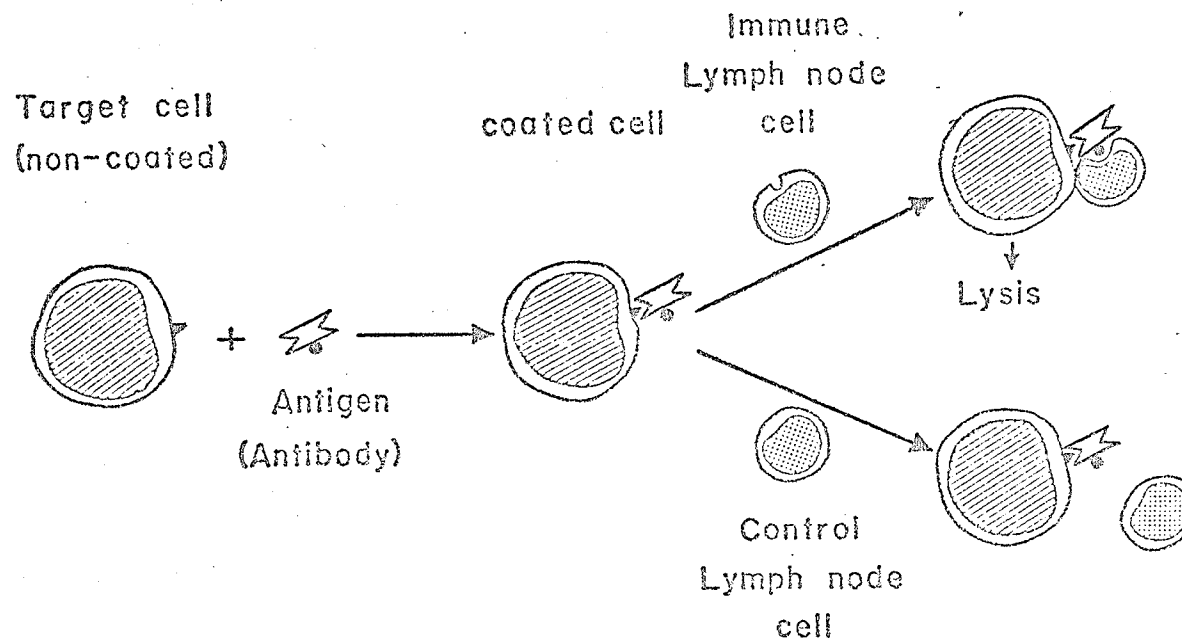
(a) In vivo destruction of tumor cells by an indirect cell-mediated immune mechanism

Edmund Klein (486) observed that regression of cutaneous human tumors resulted if a strong inflammatory ("delayed hypersensitivity") reaction was induced in the tumor foci by presumably immunological means, using trenimon (2,3,5-tri-ethylene-imino-(1,4)-benzoguino) or dinitrochlorobenzene (DNCB) as contact sensitizers, known to elicit adoptively transferable cell-mediated immunity. With this indirect immunotherapeutic method the

regression of as many as 110 macroscopically visible basal cell carcinomas could be induced in patients that were resistant to conventional treatment. Furthermore, this "immunotherapeutic" method proved to be equally effective as the best chemotherapeutic procedure, i.e., over 95% cures were obtained with both therapies (941). However, it must be emphasized that topical chemotherapy is relatively easy, because highly effective drugs may be used locally, that could not be administered systemically because of their high toxicity.

The dramatic, clinical success of the therapeutic procedure developed by Klein (941), strongly suggesting an immunological mechanism as its basis, provided the inspiration in the present study for the development of a model which would permit the induction of local delayed hypersensitivity reactions in tumor foci located in deep tissues. The basic principle of the model is illustrated schematically in Figure 1. As indicated, immunoglobulin molecules in this scheme play the dual role of (i) antibody with respect to the antigen(s) on the tumor (target) cells, and (ii) antigen with respect to the immune 'killer' lymphoid cells, i.e., sensitized lymphocytes capable of unleashing a cell-mediated immune reaction. The killer lymphocytes required for this model could be produced by sensitizing syngeneic animals either to heterologous immunoglobulins isotypic with tumor-specific antibodies or to hapten (e.g. phenylarsonate or dinitrophenyl-) conjugates of immunoglobulins, isotypic with isologous anti-tumor (enhancing) antibodies.

Figure 1. PRINCIPLE OF TUMOR CELL DESTRUCTION BY AN INDIRECT
CELL MEDIATED IMMUNE MECHANISM



In practical terms, it was envisaged that (i) tumor specific antibodies would localize in the tumor tissue and (ii) killer lymphoid cells transferred from donors sensitized to immunoglobulins isotypic with these antitumor antibodies would "home" onto the antibody-coated tumor cells and would lead on contact with the latter to a local inflammatory, cytotoxic reaction resulting in the destruction of the tumor cells.

It must be pointed out that, although, within the context of this model, the cell-mediated immune response is considered to be the most important mechanism for the destruction of malignant cells, one must not overlook the possibility of transferring along with lymphoid cells responsible for this manifestation of the immune response also the cellular elements capable of producing the (whole) spectrum of antibodies participating in the humoral response. Clearly, the immune response, particularly in relation to malignant cells, is far more complex than presented with the simple framework of this working hypothesis, which had been conceived prior to the articles published within the last year which suggest as an alternate immunotherapeutic model the production in the host of the, as yet not clearly defined, "unblocking" factors (56; 403). Nevertheless, as shown in Chapter III, promising results have been obtained in this study for the partial, in vivo immune suppression of a virus-induced polyoma in the mouse, supporting thus the validity of the proposed model.

(b) Immune response in vitro against tumor cells

A number of investigators have demonstrated that both humoral and cell-mediated immune reactions may be induced in vitro using various tissue culture systems (44; 133; 338; 722; 789; 912). More recently it has been also reported that the mixed lymphocyte reaction, which had been previously regarded as an immunologically specific proliferation of lymphocytes in vitro in response to foreign histocompatibility antigens, produces effector cells capable of cytotoxic action on target cells (391; 421; 823). Similar proliferative lymphocyte responses have been described in mixed cultures of peripheral lymphocytes from human patients stimulated by autochthonous tumor cells, as revealed by the incorporation of H^3 -thymidine (307; 638; 843).

These recent reports prompted the second part of this study in the expectation that methods could be developed for the in vitro production of lymphocytes immune to tumor antigens, which would be able to destroy tumor cells. This working hypothesis was also predicted on the assumption that under appropriate tissue culture conditions, immunocompetent lymphocytes would proliferate to give a massive population of sensitized cells which would be cytotoxic on contact with the antigenic tumor. Clearly, if one could establish the conditions for the development of the proposed cell-mediated cytotoxic reaction, this system would lend itself to the investigation of various immunological factors involved in tumor-host relationship. Moreover, this would open up the possibility of cancer therapy by immune lymphoid cells which

can be, in principle, produced in vitro in large quantities by modern tissue culture methods. The hope was also entertained that immune lymphocytes could emerge from cultures of peripheral leucocytes and tumor cells of the same patient, since one could thus produce a population of killer cells without the usual complications of clinical experiments of this nature due to histo-incompatibility between the sensitized cells and the host's tumor cells.

As demonstrated in Chapter IV, evidence was obtained for the possibility of mounting an effective in vitro reaction against neoplastic cells, using as a model a methylcholanthrene-induced tumor in inbred Sewall-Wright strain 13 guinea pigs and the corresponding syngeneic lymphoid cells.

Chapter II

TUMOR IMMUNOLOGYIntroduction

The existence of a cancer microbe was postulated as early as 1872 by Nepveu, and this assumption was again advanced in 1881 by Nedopil and Cripps, but these authors offered no empirical evidence to support their contention. Thus started a wide search for infectious agents causing cancer and by 1905 a number of microorganisms had been suggested to have an etiological role in neoplasia (896). Subsequent research, however, did not substantiate these hypotheses. Ellermann (1908) demonstrated first a filtrable agent in chickens capable of producing "systemic" leukemia as well as solid tumors of lymphoid cells. Later Rous undertook a systematic search for viruses in various solid tumors and leukemias in chickens (315) and Bittner (1940) discovered a "milk factor" causing mammary tumors in mice (315). Schottler and Schottler (1934) presented epidemiological evidence in bovine leukemia in support of an infectious etiology. It was realized, however, that genetic factors might also have a role in the disease.

An overwhelming body of evidence accumulated for over 50 years supports the view that a great variety of animal tumors are caused by viruses and there is strong indication that a number of human tumors also have viral etiology (316; 367; 748).

Both RNA and DNA viruses are able to induce animal and human tumors, as summarized below (385).

- (i) RNA viruses are the causative agents of avian and murine leukemias and sarcomas.

Type C virus particles have been derived fromavian, cricetan, bovine, feline, canine, and human leukemias and sarcomas. In human leukemias and lymphomas type C particles have been often detected in various tissues. Similar particles were observed in rhabdomyosarcoma, osteosarcoma, giant cell tumors and liposarcoma (236; 626). It was also described that when a 16 year old girl with acute lymphoblastic leukemia received a bone marrow graft from her HL-A matched brother after total body irradiation (1000 rad), the recurrent leukemia, evident 62 days after the marrow graft, was of male origin with XY chromosomes. Obviously a neoplastic transformation of donated lymphoid cells took place in vivo, possibly by a leukemogenic agent, such as virus, which could be present in susceptible donor cells, or transferred from host to donor cells (291). Cell free extracts of human osteosarcomas were able to induce osteosarcomas, fibrosarcomas and benign bone tumors in Syrian hamsters by inoculating them at birth. Human sera with positive reaction against human osteosarcomas reacted positively also with four of five of such tumors induced in hamsters. These findings indicate that the hamster tumors were caused by a human virus (716).

- (ii) Among the DNA viruses with oncogenic property may be listed:

Papova viruses: polyoma virus; Shope papilloma, simian virus 40 (SV40).

Adenoviruses: subgroup A (types 12, 18, 31): subgroup B (types 3, 7, 14, 21); subgroup C (types 1, 2, 5).

Herpes viruses: the virus of Lucke's frog kidney carcinoma; Epstein - Barr virus of Burkitt's lymphoma and infectious mononucleosis in human; the virus of Marek's neurolymphomatosis in chicken; herpesvirus hominis type 3 was recently incriminated in the causation of human uterine cervical carcinoma.

Epstein - Barr (EB) virus is widespread in human populations.

Infection in youth seems to result only in seroconversion, and the antibodies acquired are associated with subsequent immunity to infectious mononucleosis. In contrast, 15% of those who remain seronegative as young adults develop the disease with characteristic lympho- proliferative lesions. In Africa and New Guinea, where Burkitt's lymphoma is very common, hyperendemic malaria superimposed on a ubiquitous virus, like EB virus, might be an important environmental factor. Furthermore, EB virus is able to transform human lymphoid cells in vitro with the following characteristics:

- a) normal cell morphology is altered to a blastoid form,
- b) infection confers the power of unlimited proliferation,
- c) the proliferating infected cells seem to lose contact inhibition since they frequently grow in relatively large clumps.

- d) when infected, the cells acquire what has been described as a specific chromosome marker,
- e) the infected cells acquire virus- determined surface neo-antigens (269).

Samples of 249 sera derived from African donors suffering from a variety of malignant diseases were examined for EB-virus-related antibody levels. When divided into 3 major subgroups: lymphoproliferative tumors, carcinoma and sarcoma, none of the mean titers approached the high levels characteristic for Burkitt's lymphoma or nasopharyngeal carcinoma. On breaking down the material further into specific diagnostic or pathological subgroups, similar low mean values were found, except for small groups of plasma cell tumors and carcinomas of the palate (with high titers) and osteogenic sarcoma (with moderately high titers). The data do not support a regular association of high anti-EB-virus titers with malignant disease in general and thus reaffirm the special position of Burkitt's lymphoma and nasopharyngeal carcinoma in this respect (796). Another study of similar nature revealed that EB virus does not replicate opportunistically against a background of any lymphoproliferative disease (349).

Moreover, because of the impressive success of vaccination against many infectious disease, and assuming that such agents are involved in the etiology of cancer, many workers have attempted to cure cancer by recourse to immunological procedures. Thus, patients with advanced neoplasia were immunized with autologous or heterologous tumor tissue or with their extracts. Passive immunotherapy has

been attempted with heterologous antisera produced against human cancer tissues in animals (dog, donkey, goat, rabbit, sheep, horses), or with fresh whole blood from leukemia and melanoma patients, and more recently with large doses of commercial pooled human gamma globulins, and even by intratumoral injection of γ -globulin fractions prepared from sera of patients who had been inoculated with autologous vaccine. For the nonspecific stimulation of the host's defence, extracts of spleen and other viscera, anti-reticuloendothelial serum, BCG, various neutral polysaccharides ("Coley's toxins", "Shear's polysaccharides"), zymosan, etc. were also used. Administration of nonspecific defence factors such as complement, properdin or "fresh cells" (bone marrow) was also attempted. Although in a few patients objective documentation of tumor regression was reported, rarely, if ever was it possible to conclude that the responses were due to immunologic mechanisms (825).

The discovery of histocompatibility antigens revealed that most of the earlier experiments suggesting the immune destruction of tumors in animals could be interpreted as having been due to immunity against transplantation antigens. This resulted in complete discredit of tumor immunology until Foley (298) demonstrated immunity in inbred C3H-He mice to methylcholanthrene (MC) induced sarcomas. Moreover, Prehn and Main (710) pointed out that skin grafts were readily accepted, while MC-sarcomas were rejected after preimmunization with syngeneic tumor tissue in various inbred

strains of mice. Usually, different lines of MC-sarcomas did not crossreact; however, some degree of crossreaction was observed in later transplant generations. The next historical milestone is represented by the work of Klein et al. (493) who showed that immunity to murine MC-sarcomas could be elicited in autochthonous hosts and that resistance against a given sarcoma did not lead to cross-resistance against different sarcomas induced in the same inbred strain of mice by MC. Based on a great deal of evidence, there is general consensus today that independent of the etiological agent most (if not all) tumors are antigenic and therefore increasing efforts are being made to exploit this antigenicity for therapeutic purposes.

Tumor antigens

The subject of tumor antigens has been reviewed repeatedly over the past decade (355; 372; 385; 489; 490; 491; 518; 660). Initially the antigenicity of various tumors was established by transplantation methods and hence this effect was attributed to "tumor specific transplantation antigens" (TSTA), by analogy to transplantation antigens. More recently, however, as reviewed later, a number of tumor antigens have been shown to crossreact (if not to be identical) with antigens of normal tissues present during embryonic or even in extrauterine life and hence the term "tumor associated antigen" has been introduced. However, since these antigens enable in a majority of cases the host to recognize the tumor as nonself, they will be referred to throughout this thesis as tumor antigens(TA).

Tumor antigens are usually classified according to the etiology of the tumor, e.g. antigens of chemically induced tumors, and TA present on tumors of viral origin. As a rule, different tumors induced by the same virus crossreact antigenically, even when the transformed cells belong to various animal species, while chemically induced tumors tend to have individually distinct, and only occasionally crossreacting, TA. There is a variation regarding the specificity and strength of TA even within one group of tumors induced by the same chemical carcinogen (866). Spontaneous tumors of animals and man may belong to either category, including those induced by physical agents, such as x-irradiation, ultraviolet radiation, etc.

I. Viral and virus induced tumor antigens

RNA oncogenic viruses are usually released continuously by the neoplastic cells and, therefore, virion antigens may be found inside the cells where they are produced, on the cell membrane (budding of virus particles), and also in the serum and/or other biological fluids of tumor bearing animals (Bittner's milk factor). Two major groups of antigens may be distinguished: structural virus antigens which are part of the mature virus and non-viral antigens.

DNA oncogenic viruses are cytotoxic in certain cell types, and the virus particles are reproduced. In other systems the replication of these viruses suffers an early arrest. Whereas the full genome of the virus is needed for coding for the synthesis of infectious virus particles, only part of the viral genome is necessary for the induction of malignant transformation. The viral genome is integrated into the nucleus of transformed cells and, as a rule, Papovavirus and Adenovirus induced tumors do not produce virus, while the Herpes virus transformed

chicken lymphoma (Marek's disease) and Burkitt's lymphoma cells may release virus. Therefore, Papovavirus and Adenovirus induced tumors do not contain virion antigens while Herpes virus induced tumors may do so (8; 385; 491).

A. Antigens of RNA-virus induced tumors

(i) Group specific (gs) antigen are derived from the internal ribonucleoprotein of the virus particles and may be released from concentrated virus particles by tween-ether treatment or sodium dodecyl sulphate. The gs antigen of Gross virus has an estimated molecular weight of 35-40,000 and a sedimentation coefficient of 2.7S. By immunoelectrophoresis two immunologically identical components with mobilities corresponding to those of α - and β -globulins may be separated. The antigen can be resolved into at least 2 molecular species by isoelectric focusing and into 3 by polyacrylamide gel electrophoresis. The antigen contains 2.5-4% nucleic acid (364). Two major components of group specific antigens, gs-a and gs-b, have been isolated from avian myeloblastosis virus. They are single polypeptide chains; the molecular weight of gs-a is 20,000 and of gs-b is 11,000. The two antigens have been compared in terms of their amino acid analyses, tryptic peptide fingerprinting, and N-terminal sequence determinations (Figure 2), and found dissimilar. There was micro-heterogeneity in the structure of gs-b at residue 6; threonine and glutamic acid were found. When residues 14-20 of gs-a are compared with 11-19 of gs-b, some similarity occurs. Full evaluation of possible homology between the two proteins must await elucidation of their complete sequences. The virus used to prepare the proteins studied is known to consist of a mixture of viruses with different subgroup antigens and different oncogenic properties. The structural heterogeneity of gs-b could reflect

either of these factors, or some other form of viral variation not yet identified (651). The gs antigen of avian Rous sarcoma virus (RSV) has a sedimentation constant of 2.5 (238).

The major representatives of murine leukemia viruses (Gross, Friend, Moloney and Rauscher) share gs antigens (385), which are present in the plasma of mice infected at birth, as well as in naturally or artificially infected cells of various histological

Amino-terminal sequence of gs-a.
 pro-val-val-ile-lys-thr-glu-gly-
 pro-ala-trp-thr-pro-leu-glu-pro-
 lys-leu-ile-thr-arg-leu-ala-
Amino-terminal sequence of gs-b.
 leu-ala-met-thr-met- $\left\{ \begin{array}{l} \text{glu} \\ \text{thr} \end{array} \right\}$ -his-lys-
 asp-arg-pro-leu-val-arg-val-ile-
 leu-thr-ser-thr-gly-ser-his-pro-val-

FIG. 2. Amino terminal sequences of group specific antigens, *gs-a* (23 residues) and *gs-b* (25 residues), obtained by automated Edman degradation. Both threonine and glutamic acid were present at residue 6 of *gs-b*.

Niall et al., Proc. Nat. Acad. Sci. USA 67:1804, 1970.

origin (387; 679). Similarly the gs antigen is common to all members of the avian leukosis/sarcoma virus group. Gs is found also in RSV-induced tumors of hamsters, guinea pigs, and chickens. RSV-induced mammalian tumors usually do not contain significant quantities of infectious virus. Although the small amounts of virus found in primary tumors seldom persist in transplanted tumors, these tumors contain also gs antigen. It is detectable in the nucleus, cytoplasm and cell surface of infected cells. It is of interest that a large proportion of chickens that are free of avian leukosis virus contain an antigen identical to the gs antigen (238).

Gs 3 antigen of murine leukemia viruses was also present in cat and hamster leukemia viruses as detected by Ouchterlony test. The major antigens of murine leukemia viruses, gs 1 and gs 2, were absent from the cat, hamster and rat viruses. No serological crossreaction between mammalian and avian leukemia viruses has been observed (320).

When cell cultures were infected with one of the following strains of oncogenic RNA viruses: Rauscher, Moloney, Friend leukemia virus of mice, murine sarcoma virus (Moloney), reticulum cell sarcoma virus of SJL/J strain of mice, or feline lymphoma virus, all cultures gave a positive mixed hemadsorption reaction with titer of 1:128 or higher with anti-Rauscher leukemia virus (RLV) serum. Sera from 6 cats with leukemia or lymphoma (spontaneous or induced) reduced two-fold the titer between feline or murine cultures and the anti-RLV serum. Similar effects were observed

using sera of mice with Friend leukemia and of a hamster with sarcoma induced by murine sarcoma virus (Moloney). Sera from normal mice or hamsters had no effect. Electron microscopy demonstrated type C virus particles and budding in all cell lines showing a significantly high titer. These findings suggest common antigen(s) in the cultures containing type C virus particles derived from animals of different species (578). On the other hand the gs antigen of mouse, hamster, cat and rat C-type viruses each carries species-specific antigenic determinants.

A human cell line, ESP-1 lymphoma, which contained C-type virus, also possessed an antigen with determinants identical to those detected on the group-specific antigen of murine C-type viruses. Immunological identity of mouse and human C-type virus gs antigens was suggested as one possible explanation of these data (334).

Purified mammary tumor virus (MTV) of mice was incubated with sera of women with breast cancer and of normal individuals, then injected into mice susceptible to MTV. The mean incidence of MTV infection in mice inoculated with the control serum-virus mixture was 87% and only 69% with sera of tumor bearing patients ($P=0.02$). Consequently, it was concluded that these results indicated a viral etiology for the disease and that human and murine viruses were closely related (152).

In another study autologous and homologous serum-cell systems from patients with different types of leukemia, lymphoma, osteogenic sarcoma and breast carcinoma gave positive reaction in a number of cases even in autologous system. Sera from some

of the patients and some of their relatives gave positive results with cultures from leukemia, reticulum cell sarcoma and bone tumors containing type C particles and derived from mice, rats, hamsters and cats. The same sera were negative with embryo cell cultures derived from animals of different species in which C particles could not be found (237).

A shared antigen of murine and feline leukemia viruses, designated as interspecies antigen, was also detected by complement fixation using broadly reactive rat anti-murine leukemia virus sera (786).

All mice, cats and virtually all chickens seem to be completely refractory to developing antibody to the group-specific antigens characteristic of the RNA tumor viruses of their own species (434). A recent investigation revealed, however, that antibody against feline gs antigen could be stimulated in the cat (656).

(ii) Type specific (ts) antigens are derived from proteins of the viral envelope (387). Ts viral envelope antigens are represented at the membrane of transformed cells; subunits of the envelope are present in the blood and may be adsorbed onto surfaces of normal cells. Ts antigens are specific for the various strains of oncogenic RNA viruses although some degree of crossreactivity may be present. Fine differences between the Friend, Graffi, Moloney, Rauscher and Rich strains of murine leukemia viruses can be demonstrated by virus neutralization, immune diffusion, and fluorescent antibody tests with anti-ts sera.

Cells carrying envelope antigens of murine leukemia viruses react with cytotoxic antibodies in the presence of complement and undergo lysis. The cytotoxic test reveals no significant difference between leukemic cells induced by the Friend, Moloney or Rauscher viruses. Leukemic cells positive for the Gross leukemia virus antigen fail to absorb cytotoxic antibodies from sera reacting with Friend, Moloney or Rauscher leukemia cells, but the latter may absorb some cytotoxic antibody from sera reacting with Gross leukemia cells. It is possible that the Gross virus is ubiquitous in mice and that leukemic cells or extracts of different origin carry various degrees of contamination with the Gross virus. Absorption of immune sera with pellets of Rauscher or Friend virus particles removed the virus neutralizing antibodies without significantly altering the titer of cytotoxic antibodies. Absorption of these sera with leukemic cells resulted in the loss of both virus neutralizing and cytotoxic antibody activities.

Ts antigens of murine leukemia viruses may be of G-type (Gross) or FMR-type (Crossreacting antigens of Friend, Moloney and Rauscher viruses) (385; 387; 679).

Neutralization experiments have also demonstrated complex antigenic relationships between various avian leukosis and sarcoma viruses. Three major subgroups may be distinguished: A, B and C. Within each subgroup the viruses share common ts antigens, exhibit reciprocal patterns of interference and have the same host range

in genetically susceptible strains of chicken cells. Some strains are the mixture of 2 types (e.g. A+B). Considerable antigenic diversity is seen between viruses within any one subgroup. More than 3 subgroups may exist (238).

Many of the biological properties of avian leukosis-sarcoma viruses depend directly on their antigenic structure, particularly that of type specific. Type specificity determines receptor specificity on the cell surface as revealed by interference, which is related to genetic susceptibility or resistance of chickens to certain strains of viruses (238).

(iii) Viruses with hybrid antigens

A defective virus, which does not have the genetic information for the synthesis of viral envelope, although it may induce tumors, is not released from such neoplastic cells. Frequently the genome of the defective virus combines with the genome of a helper virus, and in these cases the surface antigens of the neoplastic cells will display the antigenicity of the envelope antigens possessed by the helper virus. For avian, murine leukemia and mouse mammary tumor viruses the helper virus is one of the avian and murine leukemia viruses, respectively (385).

(iv) Non-viral surface (S) antigens of RNA virus induced tumors.

For a long time it was doubtful, whether such tumors have antigens other than structural constituents of virions. However, non-producing tumors induced by defective viruses were detected; they were able to induce transplantation immunity, although no virion antigens were present. The Moloney virus induced XM-1 hemangiosarcoma

in mice did not release virus but induced transplantation immunity (385). A nonproducer tumor induced by murine sarcoma virus Harvey (MSV-H) in inbred hamsters behaved similarly. This tumor grew better in x-rayed than in unirradiated hosts and killed 14/14 and 5/12 challenged hamsters, respectively. Hamsters immunized with irradiated nonproducer tumor cells resisted challenge with viable cells; 13/15 normal controls and 0/11 preimmunized hamsters developed tumors. Immunization of hamsters with the infectious MSV-H per se or a producer tumor also induced resistance to the transplantation of a nonproducer line. Finally, no MSV-H neutralizing antibodies were detected in sera of hamsters immunized and resisting challenge with the nonproducer line, but were seen in the sera of hamsters immunized with the producer line or MSV-H. The results suggest that the nonproducing MSV-H induced tumor possesses a new tumor antigen(s) which is not an MSV-H virion antigen but can be induced by MSV-H (588). On the other hand the nonproducer BALB/3T3 cell line, transformed in vitro by Rauscher leukemia virus, was unable to induce any detectable transplantation immunity in mice (835).

The Gross virus in addition to gs and ts induces another "Gross cell surface antigen" (GCSA) and also a soluble antigen is present in infected animals which is a nonvirion antigen possibly derived from GCSA. Another cell surface antigen induced by the Gross virus is G_{IX} (G= Gross, IX=the determining gene is located in linkage group IX), which exhibits mendelian inheritance but also appears de novo in cells that become productively infected with

Gross leukemia virus. GCSA is always associated with virus infection, G_{IX} sometimes occurs independently of productive infection. The structural gene coding for G_{IX} is common to all mice, like Tla. It is not yet certain whether either of the two segregation genes (G_{IX}, Tla) belong to the murine leukemia virus genome rather than to the cellular genome (845).

Pretreatment of mice free of mammary tumor virus (MTV) with mammary carcinomas results in tumor immunity. In these hosts the tumors are invariably crossreactive and the principal tumor antigens recognized are probably those of the inducing virus and virus associated products. In neonatally MTV-infected C3H hosts, which are tolerant of the virus and its associated antigens, only a relatively small fraction of mammary carcinomas (10/42) were immunogenic. Of 6 immunogenic tumors tested for crossreactivity, only one pair crossreacted and only with each other. These individually distinct tumor antigens are probably not virus associated and represent a previously undetectable group of antigens for virus induced tumors (905).

(v) RNA viral antigens of normal tissues and chemically induced tumors

Infection with murine leukemia virus (Gross) which is the wild-type leukemia virus of the mouse, in one form or another, is virtually ubiquitous among mice. Mice with high incidence of spontaneous leukemia (AKR, C58) have demonstrable murine leukemia virus associated antigens in their tissues throughout life; other mice show this sign of infection only in later life (C3Hf/Bi) and still other mice (C57Bl/6, BALB/c) rarely or never do so, even in

later life, but their tumors, induced or spontaneous, and also cultures established from normal tissues of these strains produce virions and antigens. Murine leukemia virus antigen is demonstrable probably in all mouse embryos at some stage of development. A similar situation exists with RSV in chickens. These viruses may be integrated into the genome and gs can be inherited as a mendelian dominant (237; 845).

Antisera from hamsters with tumors induced by the Schmidt Ruppin strain of RSV reacted in complement fixation tests with antigens extracted from organs of chick embryos and young chickens. Between 50 and 90% of the embryos from different flocks had the antigen in liver, brain, intestine, kidney, lung and yolk sac. Embryos that were congenitally infected with avian leukosis virus, as well as embryos that were free of infectious virus, all contained approximately the same amount and distribution of the antigen (239).

Virus-related antigens of tumors induced by 3-methylcholanthrene (MC) were observed in 16 genotypically different strains of mice having different natural expressions of C-type RNA tumor virus genomes. The MC-induced tumors (a total of 302), which were nearly all subcutaneous sarcomas of mesenchymal origin, contained complement-fixing antigens reactive with rat antisera selected for high-titered reactions to the group specific antigen of the C-type RNA tumor virus. Since normal mesenchymal tissues (muscle and subcutaneous) did not reveal gs antigen, the concurrent activation or derepression of phenotypic expression of viral or

viral-related antigens in many of the tumors suggest that oncogenes of C-type RNA virus genomes have served as specific determinants of the induced cancers. Because other studies showed that the virogenes and oncogenes of the C-type RNA viral genome are vertically transmitted as repressed genomes, probably as part of the natural gene inheritance, the authors hypothesized that the carcinogenic action of MC is achieved by directly or indirectly derepressing the endogenous RNA tumor virus oncogenes that must be in all mouse cells. Since endogenous C-type RNA viruses were also demonstrated in hamsters, rats, cats and chickens, the hypothesis should apply to induced carcinogenesis in other vertebrates as well (949). Similarly, activation of mouse sarcoma virus-related antigen was also observed in rat embryo cells transformed by polyoma virus (746).

B. Antigens of DNA virus induced tumors

(i) Tumor (T) antigen is induced by members of Papova- and Adenoviruses. It is localized in the nucleus of transformed cells. Identical short lived intracellular antigens are also synthesized early during the infectious cycle of virus replication. In malignant cells these antigens persist and remain specifically identifiable. They are specific for the inducer virus and not for the cell type or species in which the malignant transformation occurs. Adenoviruses crossreact with their T antigens. T antigens are not identical with components of the virion nor with enzymes of DNA metabolism. The formation of T antigen in lytic infection systems can be prevented by inhibitors of RNA and protein synthesis,

but not by DNA inhibitors. T inducing capacity of the virus is more resistant to UV irradiation than virus infectivity. Although T antigen is characteristically found within the nucleus, where it seems to be associated with RNA, it is also observed in the cytoplasm. By electron microscopy it has been discerned in the form of groups of fibers. T antigen is heat labile and destroyed by trypsin. In tumor bearing animals antibodies to T antigen have been detected; these antibodies neutralized a thymidine kinase activity, which could be the function of the T antigen (372; 385; 452; 731). Adenovirus 12 and Adenovirus 31 T antigens have sedimentation coefficient of 2.4S and 2.93S, respectively. The isoelectric point of the former antigen is 5.0.

(ii) Surface antigens (S,TSTA): At least some of the surface antigens present on cells transformed by Papova and Adenoviruses are specified by the virus and, therefore, identical if induced by the same strain of virus and independent of the origin of the cell type converted. Synthesis requires DNA transcription and protein synthesis. The antigens, which are able to induce increased resistance to the tumor are often called "tumor specific transplantation antigens" (TSTA), while other surface antigens which are host-specified are symbolized with S. Presently it is not possible to decide, whether S and TSTA are identical, similar in part, or completely different. There is some suggestion that they are not identical. SV40 oncogenic cell lines were described which expressed either S antigen, T antigen and TSTA, or S antigen only. A direct correlation was found between the absence of T antigen and TSTA and the absence

of detectable viral mRNA and viral DNA, either active or inactive. Some revertants of polyoma virus transformed cells, however, that apparently lose their TSTA still express T antigen and contain detectable mRNA. Furthermore, SV40 S-antigen may be detected in enzyme-treated non-SV40-transformed cells. SV40 and polyoma transformed cells were shown to crossreact with embryonic antigens (731; 880). SV40 TSTA could not be found in nuclear or soluble cytoplasmic fractions (820).

A soluble TSTA which was prepared from Adenovirus-12-tumors of mice by homogenization, or freezing and thawing, followed by centrifugation at 100,000g for 1 hr, could immunize mice against the tumor. The immunogenicity of this extract was not affected by heating at 37°C for 1 hr or at 56°C for 30 min, or by treatment with DNAase and RNAase, but it was destroyed by treatment with 0.5% trypsin, lipase or phospholipase. Treatment with 0.5% sodium deoxycholate, 0.5% sodium dodecyl sulphate or 1.0% Tween 80 destroyed the immunogenicity of the extract as well but ether did not. TSTA was precipitated by dialysis at pH 5.0-6.0, and was not recoverable from the precipitate following redialysis at pH 7.4 (114).

The highly oncogenic group A and weakly oncogenic group B adenovirus types all induce a common TSTA. Another TSTA is induced by the group C viruses, while no evidence has been obtained for the existence of any TSTA induced by group D virus type (26; 27).

(iii) Tumor antigens induced by Herpes viruses

Lucke's frog kidney carcinoma, Marek's neurolymphomatosis in chickens and Burkitt's lymphoma of man are tumors caused by Herpes viruses. Transformed lymphoid cells of both humans and chickens were found to produce virus. Virus positive cells in human may vary from zero to more than 1% among the malignant cells obtained from various patients when cultured in vitro. From 14 newly established lines only the lines carrying relatively high EB-virus loads showed positive membrane antigen reactivity. The biopsies from which the positive lines were derived were membrane positive, but EB-virus negative as a rule. This suggests that the production of the virus is suppressed in the tumor cells in vivo. Repeated establishment of parallel lines from the same patient, derived from successive biopsies, led to lines with fairly similar EB-virus levels, whereas lines derived from different patients were quite different, suggesting that Burkitt's lymphoma has a clonal origin.

Intracellular and membrane antigens are present in transformed cells. In virus producing cells virion antigens may be found in both sites. The "early antigen" is an intracellular EB-virus determined antigen, that appears during an acute productive or abortive infection. In addition an intracellular nucleocapsid complex may be detected which is antigenically different from the EB-virus determined membrane antigen, which is a non-viral antigen. Both the membrane and intracellular antigens must be regarded as antigen complexes with several distinct subcomponents. Soluble EB-virus associated antigens may also be detected in patients with

progressive tumor growth, which are frequently absent from patients with regression (8; 408; 491).

(iv) Oncogenic DNA viruses with hybrid antigens

An oncogenic hybrid of adenovirus type 7 and SV40 has been described. In the transformed cells an SV40 T antigen was present, but the hybrid virus could not be neutralized by SV40 antiserum. Furthermore, nononcogenic adenovirus type 2 was observed to acquire oncogenic potency by association with the SV40 virus genome (385).

C. Antigenic conversion

This term was first used in reference to the acquisition by an established transplanted leukemia of a new specific cellular antigen as a consequence of infection by an unrelated leukemogenic virus. A leukemia line which had originally been induced by Gross virus became permanently susceptible to cytotoxic Rauscher antiserum after passage in mice infected with Rauscher leukemia virus. Polyoma antigen can also be introduced into MC-induced sarcomas or Moloney virus-induced lymphomas by superinfection with polyoma virus. Antigens characteristic of murine leukemia agents can appear on the surface of carcinoma or sarcoma cells of other, independent origin, and even on normal cells, which clearly demonstrates that the phenomenon is an event separate from malignant transformation. Collateral evidence will have to be sought, often on indirect lines, in order to distinguish between these "passenger" viruses and the real oncogenic agents (488; 660).

II. Antigens of chemically induced tumors

Most chemically induced tumors and even tumors induced by physical means, such as ultraviolet radiation or by implantation of cellophane films, possess neoantigens capable of inducing resistance of variable strength in syngeneic hosts, as demonstrated by sensitive assay systems with graded inoculum doses. As a rule, chemically induced tumors show an extreme, individual antigenic specificity. Thus, several tumors induced in one host by the same chemical carcinogen may show individually specific neoantigens. In addition a number of "spontaneous" tumors, where a viral etiology could not be demonstrated, were found to be antigenic both in animals and human (490; 595).

The antigenicity of methylcholanthrene induced sarcomas may be graded as high, moderate, or low (866), and although they predominantly possess antigens with individual specificity, crossreactivity was repeatedly demonstrated (710; 738; 739). Five out of six hepatomas induced by diethylnitrosamine in strain 2 guinea pigs were shown to be antigenic and two of these five tumors shared common antigens (967). Lung adenomas induced by urethan in immunosuppressed mice had also an in vitro detectable crossreacting antigen. Tumors induced by urethan in mice with normal immunological reactivity did not show antigenicity (189).

Hepatomas and ear duct carcinomas induced by 2-acetylaminofluorene in rats were shown to be generally lacking immunogenicity as assessed by their capacity to induce resistance against transplanted tumor cells in syngeneic rats. Where resistance could be induced, it was only weak, as measured by the maximum tumor cell inoculum which was rejected. The low immunogenicity of these tumors was

also reflected by their inability to reproducibly induce tumor-specific humoral antibody responses detectable by membrane immunofluorescence reactions (47; 49).

Two out of seven mammary carcinomas showed antigenicity following repeated implantation of irradiated (15,000r) tumor grafts into syngeneic rats. Immunity could also be established by excision of primary tumors. Induced resistance was specific so that no protection was afforded against other mammary carcinomas (48).

A cell line from C3H mouse prostate was treated in vitro with MC and transformed to malignancy. Seventeen clones of transformed cells were tested for their immunogenicity in C3H mice. Mice were immunized by production of fibrosarcomas after injection of the individual clones; the tumors were ligated and regressed. Cells from the same clone were used for challenge, and the numbers of tumors produced at different cell doses were compared with those induced in nontreated control mice. Eleven of these clones were found definitely antigenic and only one was nonantigenic. No crossreactivity was found within seven pairs of clones obtained from the same dish or within three clones derived from different dishes. Immunization of mice with the normal malignant control cells did not prevent the production of tumors on induction of transformed cells (620).

The "spontaneous" malignant conversion of C3H mouse cells in vitro was accompanied or followed by considerable antigenic

modifications. The development of isoimmunizing properties was demonstrated in vivo by transplantation tests and in vitro by cytotoxicity of antisera and sensitized lymphoid cells. Crossreactions indicating the development of common antigens were observed in transplantation experiments with L-929 fibroblasts of 4 cell lines converted "spontaneously" to malignancy in vitro, and 4 tumors were derived from these cultures. In vitro tests revealed crossreactions among 3 of these cell lines. No evidence was obtained supporting the suspicion that the development of common antigenic properties was due to murine leukemia virus, mammary tumor virus or mycoplasma (479).

Tumor antigens from single cell suspensions of two MC-induced sarcomas in guinea pigs were solubilized by sonication, and were shown to induce transplantation resistance to the corresponding tumor in syngeneic guinea pigs (426).

Antigens of aminoazo dye-induced hepatomas of rat could be solubilized by extraction with distilled water or by limited papain digestion. After DEAE cellulose fractionation the material was still too heterogeneous to allow chemical characterization. Treatment of hepatoma cells with either papain lipase or β glucosidase resulted in the removal or inactivation of the tumor specific antigen(s). However, α glucosidase, β galactosidase, hyaluronidase, neuraminidase and ficin did not affect the antigenicity. These results indicate that the antigen is associated with glycoprotein and lipid components of the cell membrane (51; 52).

III. Onco-fetal antigens

A research assistant of Paul Ehrlich, G. Schone, reported in 1906 that mice, which had been injected with fetal tissue, acquired the capacity to reject transplants of tumor tissue which otherwise killed non-immunized animals; adult tissue did not evoke this response (10). Although it was suspected since then that fetal and neoplastic tissues have common characteristics, convincing evidence supporting this view has been provided only by recent research demonstrating that various tumors and fetal tissues share common antigens. The number of these antigens is rapidly increasing and it is not unlikely that eventually most tumors will be demonstrated to possess some kind of fetal antigen. In accordance with Alexander (10) the term onco-fetal antigens will be adapted in this survey to describe tumor antigens of embryonic specificity (10; 344; 345).

(i) Carcinoembryonic antigen (CEA). Human colonic carcinomas were found by Gold and Freedman (346) to have antigens distinct from those of the normal colonic tissue, as detected by specific antisera produced in rabbits. This antigen could be found in adenocarcinomas of the entodermally derived digestive system epithelium (esophagus, stomach, small intestine, colon, rectum, pancreas and liver) and it was also a normal constituent of fetal digestive tissues in the first two trimesters of gestation. The term "carcinoembryonic antigen" refers to the fact, that this antigen, which is manifested only on cells of digestive tract cancers in adults

is present during fetal life in the same organ system. Antibody response to CEA could also be detected by hemagglutination. The concentration of CEA is greater in lower bowel malignancies and the antigen was absent from primary cancers of all other tissues. Immuno-electron microscopic examination revealed that CEA is located, to a large extent, in the glycocalyx of the colonic cancer cell surface (347). Patients harboring cancer tissue of either colonic and rectal origin were found to have detectable levels of circulating CEA by a radioimmunoassay (344).

Chemically, CEA is a large water soluble glyco-protein with a sedimentation coefficient of 6-7S or 10.1S having the electrophoretic mobility of β globulin (186; 304). The carbohydrate moiety may be of primary importance in the antigenic determinant group.

According to the original findings, 199 of 200 patients with cancer of either colonic or rectal origin had circulating CEA detectable by radioimmunoassay (345). Because of the great potential of these observations in the diagnosis of digestive system malignancies, these experiments were repeated by several other workers. LoGerfo et al. (553; 554) confirmed the presence of elevated levels of CEA in sera of patients with cancers of entodermal origin, but found this antigen also in patients with breast carcinoma, lung carcinoma and prostatic carcinoma, and also in patients with non-malignant diseases. Furthermore CEA was found by others in ulcerative colitis, chronic liver disease, and even in normal adult colon in low concentrations (252; 254). CEA

was also detected by the Ouchterlony method in the feces of 5 of 10 healthy volunteers, 12 of 18 patients suffering from gastrointestinal cancer (including 10 of 11 cases of colonic cancer) and 3 of 13 patients suffering from non-neoplastic disease (303). Zamcheck et al. (962) found 72-97% positivity in sera of patients with cancer of the colon. (Serum threshold of positivity was taken at 2.5 ng CEA/ml.). The approximate positivity was 45% in early stages, when the tumor was limited to the bowel wall, 55% for locally invasive cases and 88-96% in patients with distantly metastatic tumors. In a series of 26 patients with carcinoma of the pancreas there was 88% positivity. In severe active alcoholic cirrhosis and alcoholic pancreatitis 50% of patients had detectable levels of CEA. On the other hand none of 40 young persons selected as healthy individuals had detectable CEA in more than 200 assays. On the basis of these studies, it was concluded, that the test was not "specific" for colonic cancer alone. However, taking an overview of all the studies to date, it may be concluded that they support the promise of a clinically useful test for the diagnosis of digestive-tract cancer (744; 962).

(ii) Alpha - fetoprotein. During embryonic development a protein containing traces of carbohydrate and possessing electrophoretic mobility slightly lower than that of albumin is synthesized and secreted into the blood by cells of hepatic parenchyma and yolk sac. This α -globulin is the first to appear in mammalian sera, and represents a dominant serum protein in early embryonic life; hence it was designated as α -fetoprotein (3) (AFP). After birth, during

the early postnatal period, synthesis is carried out by liver cells only. AFP's have been reported for 18 mammalian species; AFP's from different animal species exhibit immunological crossreactions. The molecular weight of AFP's are in range of 65,000-70,000. The electrophoretic mobilities of AFP's differ slightly from species to species: AFP's of man, monkey and rat have mobilities close to that of albumin (postalbumin). Mouse AFP is located in the α_2 globulin fraction and dog AFP in the α_3 -fraction (3; 774).

Abelev (3) found that hepatocellular carcinomas of the mouse synthesized α -fetoprotein which was secreted into the blood. Rat hepatomas were found to secrete it as well. Using the agar gel immunoprecipitin technique, Tatarinov (1964) detected AFP in patients with hepatocellular carcinoma and by 1966 he described six such cases. It was also detected in patients with malignant teratoblastomas of the testis and ovary, but not in other tumors or in nonneoplastic liver disease (3).

However, recent investigations employing more sensitive methods (aggregate hemagglutination, immunoradioautography and radioimmunoassay) revealed that the specificity of the AFP test for liver cancer was far from being absolute. Low levels of AFP were detected during pregnancy, indian childhood cirrhosis, hepatitis or in other nonneoplastic hepatopathies predominantly in young children (up to 12 months of age), and trace amount were also detected in sera of normal individuals. Thus, the fetal serum level of AFP is taken as 100% (2.8mg/ml), the level of normal

individuals corresponds to 0.001-0.002% (4-10.5ng/ml). On the same basis the level during regeneration of the liver (animal experiments) may be up to 0.5%. In hepatocellular carcinomas or germinal tumors with elements of embryonic cancer it may rise to 100-200% (may be as high as 70mg/ml) being produced by the tumor itself. However, in some hepatomas and teratocarcinomas AFP has not been detected (3; 644; 903).

In rats receiving orally 4'dimethylaminoazobenzene (0.06% in semisynthetic diet) AFP appeared as early as the 3rd week of feeding and 76% (31 out of 41) had detectable levels (0.02-0.04 mg/ml) at the 6th week. Subsequently AFP levels decreased and by the 11th to 12th week AFP disappeared from the serum. After 13 weeks, AFP re-appeared (0.6-1.0 mg/ml) in 27 of 33 rats (the late stage of appearance) and 26 of the positive rats developed hepatomas (925).

There is considerable difference in the incidence of "seropositive" hepatomas in various parts of the globe, i.e. South African Union (82%), Senegal (79%), Congo (77%), Mozambique (67%), Indonesia (87%), Uganda (64%), USSR (64%), Astrakhan (96%), Siberia (82%), England, France, Greece (50-60%), USA (42%) higher in negroes (3; 817); it may be concluded that the incidence of AFP positive hepatomas is higher in areas where hepatocellular cancer is endemic. The reason for this difference is unknown. In animal experiments certain carcinogens (O-amino-azotoluene, dimethylaminobenzene, nitrosoamine) induced AFP positive tumors, while aflatoxin, a most probable carcinogen for man, induced AFP negative hepatomas in rats. It would, therefore, seem that other

factors, such as the biological characteristics of the tumor, the stage of oncogenesis, the age of the patient may determine AFP production (3). Nevertheless, the detection of AFP in serum is a useful diagnostic test when hepatomas, teratocarcinomas, or germinal cell tumors are suspected. It also appears to serve a useful purpose in the follow up of some patients receiving treatment for germinal cell carcinomas of the testis or ovary (817).

(iii) Alpha₂ ferroprotein is related to ferritin and is produced by the liver in fetal and postnatal life up to the age of 2 years (10; 344; 345). It is found in the sera of children suffering from a variety of cancerous conditions, e.g. nephroblastoma, neuroblastoma, teratoma, hepatoma, lymphosarcoma, reticulum cell sarcoma, osteogenic sarcoma, cerebral tumors. Of 334 cancerous children, 81% had a positive serum test for alpha₂-ferroprotein; this test was positive in only 8% of 122 children with other diseases.

(iv) Beta globulin is another human fetal antigen, which appears frequently in the sera of patients with neoplasms of nonhepatic origin or with primary liver cancer, and also with other non-neoplastic liver disorders such as hepatitis and some form of cirrhosis. It is also considered to be associated with liver regeneration (903).

(v) Gamma fetal protein-2: A fetal antigen with electrophoretic mobility of γ -globulins has been found in saline extracts of many human tumors, including carcinomas of the breast, colon, ovary, stomach, various sarcomas and leukemias. Antibody to this protein

was found in sera of a minority of cancer patients but not in sera of normal donors or of patients with nonneoplastic disease. On the basis of these data, it has been suggested that this antigen may be considered to be the product of a cell with malignant or near malignant physiology (256).

(vi) Fetal sulphoglycoprotein possessing blood group substance A activity is present in the fetal gut. It has been found in the gastric juice of nearly all patients with carcinoma of the stomach (75-78%), occasionally also in the absence of cancer (14/148) and more frequently in older patients (10; 344; 345; 375).

(vii) Placental alkaline phosphatase (Regan iso-enzyme). In 4-5% of patients with various malignancies an enzyme is produced which is biologically and immunologically indistinguishable from placental alkaline phosphatase(10).

(viii) Heterophile fetal antigen is present in the serum of human, bovine, porcine, canine and feline fetuses (344; 345); its electrophoretic mobility corresponds to that of γ -globulins. An antibody has been detected in sera of 8 out of 1030 patients with various types of cancers that was capable of precipitating this antigen from saline extracts of (82/115) malignant solid tumors of the breast, colon, ovary kidney, parotid gland, muscle, bone, nerve and of some benign tumors (12/16). The antigen was also present in some sera (17/200) from patients with solid tumors and leukemias.

Crossreaction of undefined tumor antigens with embryos

A rabbit antiserum to whole mouse embryo extracts was reacted with antigens extracted from the respective normal embryonal, neonatal and adult tissues, as well as with 72 extracts of malignant cells or tissues from cancerous animals. Cancer tissue was obtained from 18 different mouse strains, which arose in 12 diverse tissues. Viral, chemically induced tumors, 29 "spontaneous" and 2 tumors which arose following x-irradiation were examined. A composite extract of five organs (heart, lung, spleen, liver and kidney) from both male and female adult mice was used to absorb the rabbit antiserum. A serum absorbed against 19 day old embryo extracts produced precipitin lines with the extracts of all 72 cancers in the Ouchterlony test, but failed to react with extracts of mouse L-cells and with extracts of any of the normal adult tissues except skin, small intestine and regenerating liver. On the other hand an antiserum against 9 day old embryo extracts, after absorption with adult antigen (composite extract), was found to interact only with extracts of adult skin, beside reacting with extracts of the 72 tumors and with embryonal and neonatal extracts. It was, therefore, concluded that embryo-specific murine antigens reappeared in adult cells following malignant transformation (847).

Aminoazo-dye-induced rat hepatomas were investigated by immunofluorescence for embryonic antigens with sera from multiparous female rats. Most of the sera reacted with more than one tumor, suggesting that the embryonic antigens were common tumor components (51).

A rabbit antiserum was produced against a 6-7 week old intact human fetus and absorbed with an excess of a mixture of adult myocardial, pulmonic, hepatic, splenic, renal and intestinal tissue extract. Precipitin reactions were obtained in Ouchterlony tests with this absorbed antiserum with extracts of two colon carcinomas, a breast carcinoma, a hepatoma, a squamous cell carcinoma and a clear carcinoma of the kidney. Immunofluorescent microscopy revealed that the absorbed rabbit antiserum, after incubation with various tissues, was bound to the cells of the six types of carcinomas and to the epidermis of the adult skin. It did not bind to other normal adult tissues. These findings indicate that in man carcinomas corresponding to all three germinal layers contain antigens which crossreact with embryonic tissues (485).

A rabbit antiserum against a partially purified human leukemia antigen, after suitable absorption, reacted in the Ouchterlony test with a circulating antigen present in about one third of patients with leukemia. Five of six fetal sera tested were also positive. Negative results were obtained with 22 sera from healthy volunteers, solubilized extracts of normal spleens, buffy coat, or with hemolysed group A red cells. Similarly, solubilized extracts of rapidly growing established lymphoid cell lines failed to react. In addition whole cells of these lines did not absorb out leukemia specific antibodies (386).

Finally, in a series of experiments, Coggin and coworkers were able to immunize hamsters against SV40-induced tumor cells with 9-12 day old irradiated hamster fetal cells or with mouse fetal cells. Furthermore SV40 oncogenesis could be interrupted in neonatally infected hamsters, if immunization was carried out

during the latent period with either hamster or human embryo cells treated by x-irradiation (19; 170; 171).

IV. Antigenic changes and neoplasia

A. Expression of organospecific, allo- and xeno-(Forssmann) antigens by tumors.

It has been well established that tumors originated from non-endocrine tissues produce various hormones in an active form (102). One might, therefore expect that such tumors should express at least some of the specific antigens of the corresponding endocrine organ; however, this problem has not been examined so far. Some other examples of similar "derepression" of antigens in neoplastic cells are well known, which will be discussed here.

(i) TL (thymus-leukemia) antigen. TL may be considered as an organospecific alloantigen. Although the gene determining TL (Tla locus) is present in all mouse strains, it is never expressed in certain strains under normal conditions, while others do express it. TL may occur, however, on leukemias of TL negative mouse strains: of 160 leukemias in TL negative strains 70 were TL positive (105). TL positive lymphocytes never appear in the circulation under normal conditions but they do so in thymic leukemias. Thymus cells did maintain TL on their surface for 2 days in the lymph nodes of recipients after i.v. injection. The absence of TL-positive cells outside the thymus in healthy mice, therefore, reflects a mechanism whereby thymus holds back such cells. This confining mechanism may break down in leukemia (792). The

presence of TL on leukemias of positive mouse strains probably reflects an arrest of differentiation, while in negative strains gene derepression occurs.

(ii) MM lymphatic alloantigen. A new lymphatic cell antigen of some strains of mice which also occurs as a tumor antigen on ascitic mammary tumors (MM102) of C3H mice has been discovered. This antigen is not present in normal cells of C3H/He mice and is, therefore, capable of eliciting tumor-specific immune reactions in this host carrying the MM102 mammary tumor. On the other hand, many of the 24 strains of mice examined expressed it, e.g. C57Bl/6, AKR, C58, 129, C57L, H-2H, 101, RF, SJL/J and Swiss mice. This antigen appears to be regulated by a dominant gene independent of other known alloantigens (151).

(iii) Forssmann antigen. An antigenic reactivity characteristic of the Forssman antigen was demonstrated by immunofluorescence technique with heterologous antiserum on the surface of SV40 and polyoma transformed hamster cells and in hamster cells cultured in vitro. No Forssmann reactivity was detected with adenovirus 12 transformed hamster cells (297; 758).

(iv) Hetero-Organic antigens. A total of 55 rat tumors were studied for the appearance of organo-specific antigens: 16 myogenic tumors induced in vitro by 100rad or 5x100rads; 27 hepatocellular induced by the oral administration of N-diethylnitrosamine, and 12 kidney tumors induced by the administration of 2mg N-dimethylnitrosamine through gastric tube for 5 days. Sera specific for

muscle, liver and kidney were tested for possible crossreactions with extracts of the tumors. Nine of the 27 hepatomas gave precipitating complexes with anti-kidney sera and 3 of 27 with anti-muscle sera. Myogenic tumors did not react with anti-liver sera, and there was one weak reaction out of 10 with anti-kidney sera. Two of 12 kidney tumors reacted with anti-muscle sera, but there was no reaction with anti-liver sera. Absorption studies revealed that not all the antigens of 7 hepatocellular tumors with kidney hetero-organic activity were identical - there was a mosaic like pattern. These data were interpreted to indicate that in the tumor cells there occurred a derepression of genes controlling the synthesis of the proteins tested (665).

Immunofluorescent tracing by rabbit antiserum with specific reactivity against human intestinal mucosa revealed cross-reaction with human ovarian cystadenoma epithelium and not with epithelium of any of several other human mucous epithelia. The mucinous cystadenoma epithelium apparently contained at least one but not all the specific intestinal mucosal antigens (642).

An antiserum, produced against one-celled unfertilized mouse eggs was cytotoxic at a high dilution to SV40-transformed mouse cells but not to normal cells or to SV40-transformed cells of other species. This anti-egg serum was cytotoxic to mouse cells transformed by viruses other than SV40 only at low dilutions. The cytotoxic effect of this serum against mouse eggs was eliminated by absorption with SV40 transformed mouse cells and not by mouse

cells transformed by other viruses. An antiserum against SV40-transformed cells was also cytotoxic for mouse eggs, and this cytotoxic effect could not be absorbed out with mouse spleen cells. Antispleen serum had no effect on mouse eggs and reacted against SV40 transformed cells only at a low dilution. These results indicate immunologic crossreactivity between antigen(s) of unfertilized mouse eggs and mouse cells transformed by SV40 (502).

B. Antigenic deletion

It is frequently observed that parallel with the appearance of tumor antigens on malignantly transformed cells, other antigens which were normally present on these cells before neoplastic conversion took place completely disappear or are found only at a lower density. This phenomenon is known as antigenic deletion.

Each of five MC-induced sarcomas differed in the concentration of (high, intermediate, or low) surface antigens of the seven H-2 specificities tested. The tumor-specific immunogenicity was inversely related to the H-2 antigenicity, in as much as highly immunogenic tumors possessed fewer H-2 antigens on their surface and vice versa. For example, early metastases to the lung was associated with low levels of tumor-specific immunogenicity and high levels of H-2 antigenicity (393).

Absorption experiments showed that a subline of polyoma virus-transformed C-3H/HeV cells, with decreased oncogenicity in adult C3H mice, contained 8.8 times more tumor antigen(s) and

2.5-45 times less H-2 specificities than the original line, and both cell lines had much less H-2 than normal C3H spleen cells (885). A considerable decrease in H-2 antigen(s) could also be demonstrated by electron microscopy on the surface of Gross virus-induced E σ^1 G2 leukemia cells (831).

Mouse tumors derived from 3 cultured cell lines, which had undergone "spontaneous" neoplastic transformation in vitro, were transplantable into allogeneic hosts, even after these hosts were immunized with the originator normal C3H tissue, indicating that neoplastic transformation caused a loss or dilution of normal H-2 antigens (479).

In a patient with lymphoma most HL-A antigens on the lymphocytes specified by the patient's genotype could not be detected in cytotoxicity reactions with HL-A antiserum as the malignant lymphoma with normal peripheral lymphocytes progressed to disease with circulating neoplastic lymphocytes. Tissue culture monolayers of skin fibroblasts from the patient did not exhibit any change in HL-A content (803). A similar case was detected in acute leukemia by leukoagglutination using antisera from multiparous women (646).

Blood group antigens A, B and H were present in benign neoplasms originating from tissues having these antigens. In carcinoma in situ of the cervix the isoantigens were significantly decreased in amount and irregular and spotty in distribution. Negatively reacting "windows" corresponding to highly anaplastic

cells were seen by mixed hemadsorption test (219). Furthermore colonic autoantigens were not present in four cases of colonic carcinoma (501) and the concentration of brain specific antigens was reported to decrease in human brain tumors (231).

The urea extractable antigens of normal mouse epidermis were present in much smaller amounts in the corresponding extracts of papillomas and squamous-cell carcinomas induced by MC. Papillomas and carcinomas had several urea extractable antigens in common and both the benign and malignant growths had at least one dominant antigen. Moreover, the papilloma and carcinoma antigens were present in normal epidermis, but in smaller amount than in the tumors (138). Induction of dysplastic and neoplastic lesions in the skin of hairless mice by MC or by daily UV irradiation was associated with a loss of intercellular and cell membrane antigens of normal skin, as demonstrated by indirect immunofluorescence. Similarly, the development of squamous cell carcinoma and invasion of the medullary dermis was associated with a reduction or loss of intercellular and cell membrane antigens in malignant areas. Invading tumor cells were always totally depleted of normal skin antigens while adjacent areas had differentiated with normal intercellular and cell membrane staining (633). Similarly, deletion of liver-specific cell surface antigens from aminoazo dye-induced rat hepatomas was demonstrated by immunofluorescence; the event was paralleled by the appearance of new tumor-specific antigens (50).

C. Changes in antigenicity of transformed cells

(i) Antigenic modulation

In TL positive leukemias of TL negative mouse strains, TL antigen can be completely suppressed by passage of the leukemia cells in hosts immunized against TL positive leukemias, but it is not suppressed in hosts immunized against TL negative leukemias. Furthermore, the antigen is fully restored by subsequent passage in non-immunized hosts. A substantial body of experimental evidence favours the view that an adaptational change affecting the whole cell population, rather than selection of variants or the presence of blocking antibody, is the basis of this antigenic modulation (105).

A similar change of H-2 antigens was observed during the passage of F_1 hybrid tumors in parental mouse strains. By this method variants could be selected which grew well in parental strains. Two rules emerged during the selection of sublines: (a) the transplantation behaviour selected for was irreversible, as it was maintained after retransplantation to the nonselective F_1 hybrid, and (b) each variant had a higher degree of antigenic specificity compared to that of the original tumor, as it did not grow at all in the other parental strain. The variant sublines absorbed only hemagglutinins directed against the H-2 type of their compatible strain, but not those of the other parental strain in which the tumor had been passaged. Thus, antigens of group K of the H-2 locus could be lost without affecting D antigens, while loss of D antigens was always accompanied by the loss of K antigens.

Therefore, it is evident that the maintenance of some H-2 components is essential for cell viability and/or proliferative capacity. All attempts to obtain zero variants for H-2 failed, though sometimes the tumor responded by growing in any strain of mice, a reduced surface concentration of H-2 antigens was present. The loss of one parental H-2 antigens is a genetic, inherited process (487).

(ii) Antigenic chance in relation to drug resistance

DBA/2 mice were immunised against L1210 leukemia and to a subline resistant to methylglyoxal-bis (guanylhyazone). Using paired labelled γ -globulin mixtures containing ^{131}I -normal and ^{125}I -immune γ -globulins it was shown that above a certain level of crossreactivity the specific uptake of immune γ -globulins was 4-10 times higher by the cell type used for immunization of the serum donors than by the other cell type. Furthermore, complete absorption of antibodies was only possible with the cell type used for immunization. These data indicate that resistant leukemic cells are more immunogenic than L1210 cells in DBA/2 mice, and that the two cell lines share common antigens and that, in addition, each line has different antigenic components (481).

Tumor fluid from a patient with cystic malignant melanoma contained a homogeneous protein, which induced in vitro stimulation of DNA synthesis in autologous lymphocytes. The protein was believed to be a soluble tumor antigen since, following therapy with dimethyl triarzeno imidazole carboximide, this antigen disappeared from the tumor fluid (643).

The loss of T antigen was observed from cells derived from a hamster tumor induced by adenovirus 12 after storage in liquid nitrogen with 10% dimethylsulphoxide. However, the morphological and growth characteristics, as well as the tumorigenicity of the cells were retained (914).

(iii) Antigenic change in relation to cell cycle

It was observed that periodic changes occurred in the sensitivity of a Moloney virus induced murine lymphoma (YCAB) when exposed to anti-H-2 and anti-Moloney cytotoxic antisera. Similar phenomena were detected with cell lines of Burkitt lymphoma and the corresponding anti-tumor sera. With synchronized cells, the highest antigen expression was demonstrated to occur in the G_1 phase of the cell cycle and it was lower in S and G_2 phases (161). In another study with the YCAB mouse lymphoma, it was shown that the viral antigen was accessible to antibody and was able to activate complement in the presence of antibody throughout all cellular growth phases, while cytotoxicity was confined to the G_1 phase of the cell growth. The inability to lyse the cells in the presence of specific anti-viral antibody and complement, during the logarithmic phase of cell growth, was not due to the lack of expression of Moloney virus antigen(s) on the cell surface, or to inaccessibility of this surface antigen(s) to antibody, or to failure to activate the complement effector system (532).

D. Time lapse of antigenic expression and malignant transformation

As mentioned earlier, antigens of oncogenic viruses may be present on normal tissues without any sign of malignancy. Some related observations will be presented here, which illustrate that neo-antigens may occur long before the manifestation of malignancy.

Celada et al. (145) infected mice at birth with Moloney virus and stimulated them later with SRBC during the incubation period, before the outbreak of leukemia. Incubation of spleen cells with anti-Moloney serum plus complement caused a decrease in number of plaque forming cells in one fourth of the infected mice, indicating that their antibody producing cells contained virus specific surface antigen. Other investigators observed also in murine viral leukemia that transformed cells develop antigens before becoming malignant (370).

Three outgrowths of preneoplastic hyperplastic alveolar nodules of mammary tissue and six tumors were found to be immunogenic in syngeneic BALB/c hosts, despite the absence of the viral agent. Normal lactating mammary gland and mixture of heart and kidney tissue fragments also elicited immunogenic effects, as revealed by challenge with mammary carcinomas and preneoplastic tissues. Although heightened resistance was occasionally demonstrated in response to immunization, the more common effect was enhancement (935).

Pieces of skin taken from mice at the site where dimethylbenzanthracene (DMBA) had been injected 5 to 30 days previously, were all rejected by syngeneic recipients as incompatible grafts. The rejection was significantly delayed if the recipients were treated by immunosuppressive drugs, such as cyclophosphamide and methylhydrazine. At this time no neoplastic change occurred in the skin either macroscopically or microscopically. Animals that had previously rejected DMBA skin grafts failed to reject DMBA treated syngeneic skin grafts in a second set fashion from the same or other donors (573; 574). In another series of experiments

pieces of skin from mouse strains sensitive and also from strains not sensitive to the leukemogenic effect of Friend or Rauscher virus, taken 5 to 30 days after injection of the virus, were frequently rejected as incompatible grafts when transplanted to syngeneic recipients. The skin from the sensitive mouse strains was able to transmit the leukemogenic virus, but not from the non-sensitive strains. A second skin graft was rejected in an accelerated fashion, i.e. second set rejection. Faster rejection also occurred in primary skin grafts transplanted onto mice pre-immunized with leukemic cells (574).

V. Antigenicity of human tumors

Immune reactions in humans have been demonstrated by a variety of methods to practically all classes of autochthonous tumors (141; 385; 400; 596; 625; 836; 911).

The demonstration of antigens of some human tumors has already been mentioned in appropriate sections of this chapter, as the antigens of EB-virus induced Burkitt lymphoma and onco-fetal antigens. Obviously, the possibility of detecting crossreacting antigens among human tumors is an important and complicating feature. If fetal and "passenger" virus-induced antigens can be excluded, crossreactivity would suggest a viral etiology.

Peripheral blood lymphocytes from a total of 373 tumor patients were tested for cell-mediated immunity against human neoplasms of various histological types with the colony inhibition or cytotoxicity test. Evidence indicating antigenic crossreactivity among tumors of the same histological type was obtained with malignant melanomas, carcinomas of the colon, breast, testis, endometrium

and ovary, and with various sarcomas (405). Blood lymphocytes from children, who were either cured of neuroblastomas or had persistent tumors, decreased the plating efficiency of cultured autochthonous or allogeneic neuroblastoma cells. Lymphocytes from 4 of 5 mothers whose children had neuroblastomas inhibited tumor cells of the mother's own child and from other children to the same extent. Plasma from some neuroblastoma patients and from some mothers decreased the plating efficiency of neuroblastoma cells, if active complement was added; however, skin fibroblasts from the neuroblastoma patients were not inhibited in growth and lymphocytes of healthy children and of their mothers did not inhibit the growth of neuroblastoma cells nor the fibroblasts (402).

A common antigen was demonstrated in different histologic types of human sarcomas with antibodies obtained from sarcoma patients. A high incidence of antisarcoma antibodies could also be detected in close family members of sarcoma patients (625).

In patients immunized against malignant melanoma, two types of antibodies occurred: one reacted with surface antigens and was specific for each tumor, the other was active against cytoplasmic antigens present in most or all melanoma cells (277; 544).

Thirty one of 46 sera from patients with breast carcinoma showed positive reaction in the indirect immunofluorescence test with tissue culture cells derived from one breast carcinoma, one case of fibrocystic disease and two osteosarcomas. None of the sera tested reacted with the following cultures: giant cell tumor, rhabdomyosarcoma, Levine-3 cell line, HEK-1-HRLV (human embryo

kidney producing Rauscher leukemia virus), normal human embryo, and human adult skin. All sera from blood bank donors were negative with cells of all cultures tested. Absorption experiments with some of the positive sera indicated that this fluorescence reaction may have been due to tumor antigens (715).

Fluorescence studies with sera from ten meningioma patients showed that four of them reacted against cell cultures from both homologous and autologous meningiomas. Fluorescence staining was strictly limited to the cytoplasm and tended to be concentrated in the perinuclear region. One out of four cell lines, established from other tumors, demonstrated fluorescence staining. Two meningioma cell lines were stained with 12 sera (at 1:8 dilution) of normal blood donors and cytoplasmic fluorescence was present in two cases. Other cell lines from cerebral tissue did not react with sera from normal and meningioma patients. Imprints of meningiomas reacted similarly to that of cultured cells (141). Cytotoxicity tests with peripheral lymphocytes from patients with brain tumors suggested antigenic crossreactivity between glioblastoma cells from different patients and between glioblastoma and melanoma cells (540).

Results obtained with the microcytotoxicity test indicated that human urinary bladder tumor cells possessed tumor antigens which crossreacted with each other (119).

Solubilization of human leukemia specific antigens from the cell membrane was achieved by papain and these were fractionated

by chromatography on Sephadex G-75 and G-200 columns. Individual antisera were prepared against 4 of these antigenic preparations in rabbits. Successive absorptions with normal human serum, a soluble HL-A antigen pool and papain removed the reactivity of these antisera against a panel of soluble HL-A preparations obtained from spleens from 21 non-leukemic individuals. The antisera, after absorption, were tested against the five soluble leukemic preparations and although all gave positive reactions with the original material, each produced also a different pattern of precipitation lines with the other preparations. Although the small amount of soluble material available prevented extensive studies necessary to identify all the lines completely, the data showed that some lines were common to leukemias of different types: 2 acute myeloblastic, 1 sub-acute myeloblastic, chronic myeloid and 1 chronic lymphatic leukemia was examined. There was no reaction with pregnant sera, human ascitic fluid and soluble antigen from human thymuses, suggesting that no embryonic or TL-type antigen was involved in these reactions. During further purification by chromatography on a DEAE-cellulose ion exchange column, activity was found only in fractions containing also transplantation antigens (916).

VI. Theories on neoplastic transformation and appearance of tumor antigens

It has been established that different families of DNA molecules are present in the mammalian cell DNA genome, which are characterized by the presence or absence of poly-d G.dC or poly dA.dT sequences, respectively. These repetitive sequences

constitute more than one third of the cellular genome, while the rest of the genome is made of unique nucleotide sequences which are the cellular genes. The eukaryotic cell DNA contains 10^3 to 10^4 sequences of 200 to 400 repetitive nucleotides (68).

It is also well known that the mammalian cell progresses through defined phases after cell division: the S-phase is characterized by active DNA synthesis which is accompanied by the synthesis of histone mRNA and proteins. The G₂-phase of the cell cycle is characterized by active RNA and protein synthesis. During the mitotic phase (M) only mitochondrial RNA is synthesized, while the chromosomes are regrouped into two nuclei and the cell divides. During the G₁ phase mRNA molecules which were synthesized during previous phases still function and enzymes which participate in DNA synthesis appear in the cell.

According to a recent theory of the organization of the mammalian cell genome (68), the transcription of RNA molecules from different DNA classes might be controlled by independent processes involving different RNA polymerases. Different genes are considered to be situated in the chromosomal DNA always adjacent to the promoter sites made of different polynucleotide sequences, which, although not part of the genetic information, have a major importance in the regulation of the transcription process in the nucleus. It is suggested that the genes which control the synthesis of cellular DNA and the synthesis of the proteins for this process are situated adjacent to poly-dG.dC sequences, while

all the genes which contain information for the cellular structural proteins are adjacent to poly-dA.dT. Irreversible changes occurring in the poly-dG.dC sequences, might lead to activation of the adjacent genes with consequent DNA synthesis and cell transformation.

The viral DNA genome is considered to resemble in its organization the cell DNA and it contains poly-dG.dC and poly-dA.dT sequences. Cells infected with a tumorigenic DNA virus might enable the interaction of the poly-dG.dC sequences present in the viral DNA genome with the poly-dG.dC regions in the chromosomal DNA. After one division cycle the viral DNA is integrated into the host DNA in the cellular chromosomal DNA which contains poly-dG.dC regions. The cellular RNA polymerase which transcribes the chromosomal DNA with the poly-dG.dC sequences can also transcribe mRNA from the viral genes adjacent to the poly-dG.dC sequences in the integrated viral DNA, but not from the genes which are initiated at poly-dA.dT. Recent investigations on Rous sarcoma and other RNA tumor viruses showed that they are able to code for DNA with the aid of an RNA-dependent DNA polymerase and probably virus specific DNA is integrated into the genome. This DNA product of RNA tumor viruses might have poly-dG.dC sequences which might participate in the integration of the viral DNA into the genome (68).

Data on the action of carcinogens and x-irradiation which cause cell transformation demonstrated that most of these agents either interact with or effect guanine residues in the DNA. Therefore, carcinogens need not act as mutagenic agents but rather as compounds which, by interaction with the guanine residues, upset

the control mechanism of the cell and activate adjacent nuclear genes which regulate DNA synthesis. This suggestion indicates that (a) not all cells are susceptible to chemical carcinogens, (b) only cells which have poly-dG.dC sequences in a conformation which allows interaction with a carcinogen might be susceptible to transformation, and (c) it is also possible that part of the affected cells can overcome the carcinogenic effect by inducing synthesis of endonucleases capable of degrading the affected region in the DNA. Only cells which lack these enzymes might be transformed (68).

In considering the possible mechanisms of antigenic alteration of chemically induced tumors, McKhann and Haywood (595) suggested that it was not likely that mutations of structural genes would be responsible for antigenic change, since occasional single point mutations would not induce a marked change in cell structure. The mammalian genome contains an enormous amount of repressed genes and, therefore, as suggested by Burnet, each cell has the potential of being antigenically different from most or all other cells. Hence the change causing the antigenic diversity of chemically induced tumors might easily occur at the genetic control level affecting repressors, inducers and operators. If histones uncover DNA, the gene is derepressed, mRNA and protein synthesis occurs resulting in the production of neo-antigens. Furthermore changes in mRNA and protein synthesis by itself could easily accommodate alterations in the surface

concentrations of normal or tumor antigens, such as spacial arrangement on the membrane, uncovering of deeper formerly "unrecognized" layers, or rearrangement of the antigenic mosaic.

Gold (344; 345) also proposes, that the appearance of embryonic antigens on tumors is mediated by the derepression of certain genes during neoplasia, which are also active during the embryonic period but repressed in later life. The processes of gene activation and repression in mammalian cells might be essentially the same as those in bacteria, which have been studied in depth.

Boyse et al. (107) consider that tumor antigens may arise as a result of activation of three types of silent genes: (a) activation of genes that are normally functional at another time, e.g., during embryogenesis; (b) activation of genes which are not expressed in the tissue that has undergone malignant transformation, but which are normally expressed in some other tissue; (c) activation of genes which are normally expressed neither at any time nor in any tissue in the individual whose malignant cells carry the new antigen.

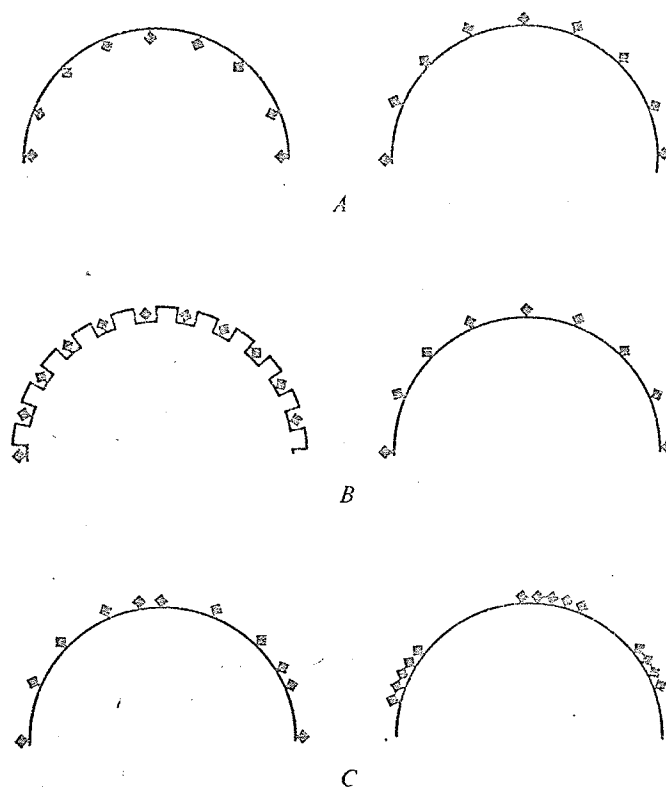
According to Black et al. (90) altered surface properties of neoplastic cells may be due to the appearance of specific antigens coded for by viral genes contained within the transformed cell or to nonspecific antigens resulting from derepressed host genetic information. This latter change may be manifested by exposure of cryptic membrane sites through failure to synthesise membrane

materials, in loss of organospecific or blood group antigen (A, B, H). For example, the appearance of a new heterophile (Forssmann) antigen may be due to the loss of a sugar normally covering the antigen. Transformed cells may often be agglutinated by plant agglutinins reacting with their altered surface specificities. Concanavalin A (Con-A), for instance, agglutinates cells transformed by viruses, chemicals, x-rays and this agglutination is mediated by alpha-methyl-D-glucopyranosid or -mannopyranoside sites on the cell surface which is uncovered by trypsin treatment of normal cells.

Theoretical models for structural and metabolic changes explaining cell agglutinability by plant agglutinins, that accompany malignant transformations are represented on Figure 3 A, B, C, D (445; 652). A change of a cell surface may be due to exposure of cryptic sites or semi-cryptic sites, a concentration of exposed sites by a decrease in cell size or a rearrangement of the exposed sites. For example, the agglutinability of transformed 3T3 and hamster cell lines by Con-A directly correlates with malignancy, but not with wheat germ and soybean agglutinins (445).

3T3 fibroblasts of BALB/C mice transformed by SV40 virus with (SV3T3) investigated by Electron microscopic analysis of ferritin-conjugated Con-A (Fer-ConA) revealed that while 3T3 cells had an essentially random distribution of Fer-Con A, the SV3T3 cells showed Fer-Con A almost exclusively in large, randomly dispersed clusters. Sometimes this dense packing of Fer-Con A into clusters on SV3T3 was not seen over the entire surface of the cell membrane, but was restricted to a large region on the membrane surface which was associated with villus-like structures. There

Figure 3. THEORETICAL MODELS OF SURFACE CHANGES ON MALIGNANT CELLS



Three mechanisms that have been proposed to explain the changes in cell agglutinability with plant agglutinins that accompany malignant transformation. *A*, The mechanism of "cryptic sites" presented by Burger (Fig. 5 of ref. 2). The agglutinin sites are buried near the cell surface, but can be exposed by proteolysis or malignant transformation. *B*, The mechanism of "semi-cryptic sites" presented by Sela, Lis, Sharon and Sachs (Fig. 2 of ref. 10). The agglutinin sites are not buried as in *A* and can bind agglutinin, but cell agglutination is prevented due to the presence of surface structures which can be removed by proteolysis or malignant transformation. *C*, The redistribution mechanism presented by Singer and Nicolson (Fig. 7 of ref. 19). The agglutinin-binding sites are normally present in a distribution that does not favour agglutination (that is they are dispersed) but after proteolysis or malignant transformation a redistribution (clustering) of agglutinin sites occurs on the membrane surface that favours cell agglutination when compared with the normal cell and its dispersed sites.

² Burger, M. M., *Proc. US Nat. Acad. Sci.*, 62, 994 (1969).

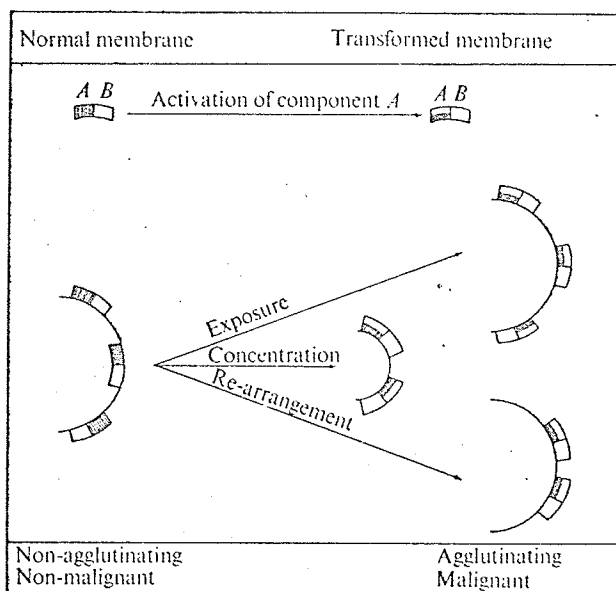
¹⁰ Sela, B., Lis, H., Sharon, N., and Sachs, L., *Biochim. Biophys. Acta* (in the press).

¹⁹ Singer, S. J., and Nicolson, G. L., *Science* (in the press).

Nicolson, G. L., *Nature-New Biol.*
233:244, 1971.

Figure 3. (continued)

D.



Model of the structural and metabolic changes of concanavalin A sites resulting in agglutinability and malignancy.
 , , Component A; , component B; white, active; , inactive.

Inbar, M. et al., Nature-New Biol.
 236:3, 1972.

was approximately a 3-3.5 times increase in the density of Fer-Con A on SV3T3 cells by comparison with 3T3. It is postulated that the more clustered distribution of the Con A sites on SV3T3 cells is responsible for the increased agglutinability of these cells by Con A in contrast to the parent 3T3 line (652).

It is reasonable to assume that neoplastic cells are specially injured cells which are unable to coordinate and stay in homeostasis with the body. Cell injury in general may lead to three kind of events: (a) cell death, (b) full regeneration, and (c) irreversibly damaged but viable cells. The malignant cell belongs to category (c) and does not respond any longer to signals of homeostasis as normal cells would, and confers its unregulated growth pattern onto its daughter cells.

It is well established that coordination in the body is achieved by direct cell interactions and by neuro-hormonal regulation, both of which are mediated by special surface receptors of the responding cells. Cyclic adenosine monophosphate (3',5'-AMP) may be regarded as an intracellular hormone involved in transmission of receptor signals. Although a great deal of the details of intracellular signal pathways is unknown, it is clear that gene activation can take place through these pathways, in accordance with homeostasis, induced either by contact with other cells or hormones (732). It follows that neoplastic cells must be defective in some point of the normal regulatory pathway. Theoretically the

defect may be located on the cell surface (receptor defect), in the cytoplasm (signal transmitting metabolic pathways) or in the nucleus (DNA genetic code). Because the change must be inherited, a defective genome seems obligatory for cancer cells. Although most tumor cells were shown to exhibit surface changes recognized by the host as antigens, according to this hypothesis, cells with a perfectly normal surface, but with intracellular defects, will behave also as neoplastic and will not be antigenic. Indeed in spite of great efforts, some tumors have not been shown to be antigenic. However, lack of antigenicity can never be demonstrated with absolute certainty in vivo.

The widely demonstrated, unique antigenicity of chemically induced tumors and of those caused by other non-viral agents indicates that they cause cell injury at random and a great variety of changes may be inherited in malignantly injured cells. Therefore, any agent capable of inducing cell damage may induce neoplasia.

Oncogenic viruses have genetic codes for neoplastic conversion of cells, which may be regarded as a special, non-random-type cell damage. It is interesting to note in this context, however, that virus transformed cells were demonstrated to have noncrossreactive antigens as well as crossreactive antigens at different concentrations, indicating that random events may also be involved. Furthermore DNA templates of RNA viruses may be integrated into the genome of normally functioning mammalian cells, as it was shown recently, and in this case it is conceivable that additional damaging factors are necessary for neoplastic conversion.

Damage of genetic material in the nucleus may lead to activation of silent genes by (a) destroying control mechanisms, (b) alteration of genes (possibly by partial inactivation), or (c) complete inactivation of active genes. Studies on the antigenic patterns of neoplastic cells indicate that a number of these changes may happen during neoplastic conversion, the exact mechanism of which is largely obscure.

Host Immunity to Tumors

A large body of evidence indicates that most transplantable animal and autochthonous human tumors elicit immune reactions in the host (385; 405; 489; 490; 629; 866). Some of these may involve (i) complement-dependent cytotoxic antibodies against dispersed malignant cells such as leukemia or against tumor cells circulating in the blood, (ii) immune lymphocytes capable of incapacitating cells of solid tumors by direct contact, or by triggering other cells (macrophages) for cytotoxicity, (iii) phagocytosis of tumor cells grown in ascitic form by macrophages, (iv) 7S antibodies that interfering with cell-mediated immunity directed against solid tumors.

A. Cell-mediated immunity

Pathologists observed first that tumors evoke a lymphocyte response of the host. Lymphocytes and histiocytes were found in the tumor, when transplants failed to establish themselves. It was assumed that in these cases "allergic cell death" occurs which involves the mutual destruction of cancer cells and lymphocytes. A good deal of morphological evidence indicates that lymphocytes,

which are often seen in transplanted and autochthonous cancers, play some essential part in an immune process (478).

Inoculation of mice with Moloney sarcoma virus (MSV) resulted in the appearance of foci of cells having characteristics of neoplastic cells, yet the predominant component of the lesion was an inflammatory reaction better fitted to the designation of "atypical granuloma". Lesions of similar type were frequently observed in the spleen, adrenal glands, liver, internal genitalia, peritoneal surfaces, and retroperitoneal tissue. Some lesions persisted for long periods or recurred at the sites of injection (783). In similar studies it was shown that the regression of MSV induced tumors is age and viral-dose dependent and related to the immune status of the host at the time of tumor growth. Cortisone pretreatment delayed the time of regression, the morphological sequence of tumor regression being characterized by lymphocytic infiltration, edema, neutrophilic infiltration and then complete reabsorption (747).

Focal microscopic lesions exhibiting mononuclear cell infiltration were commonly found only in immunologically competent mice during the latent period of methylcholanthrene-induced papilloma development. Sublethally irradiated or antilymphocyte serum treated hosts developed macroscopic papillomas without the appearance of microscopic lesions except in one documented case. Thus, common histological features of microscopic inflammatory foci and known papilloma regression sites included dermal keratin debris, lymphocyte exocytosis, and mast cell degranulation associated with epithelial pyknosis (514).

Two transplantable hamster lymphomas one which metastasizes (ML), while the other only grows at the site of implantation (NML), were examined in the electron microscope. The tumor cells of the two lymphomas were found to be identical except for a slightly greater amount of rough-surfaced endoplasmic reticulum on ML; both contained the typical ultra structural features of experimental tumor cells, including virus particles. The difference between the two tumors was found to be in the invading macrophages. The macrophages in NML were large cells containing phagosomes filled with tumor cells in varying stages of digestion. ML also contained macrophages but these were small cells without phagosomes, which accounts for their not being found in earlier light microscope studies. That these latter cells were macrophages was evident from their membrane processes and the presence of primary lysosomes. It was concluded that the difference between ML and NML lies not in the ability of the host macrophages to invade ML, but rather in the inability of the latter macrophages to become activated (88).

The in vitro agar culture system for growing hemopoietic colonies has been used to determine levels of macrophage precursors in the bone marrow and spleen of mice with primary and transplanted tumors. The tumor bearing state generally resulted in an increased number of precursors in the bone marrow, and partially in the spleen. Consistent rises were also observed in blood monocytes and granulocytes and in the serum level of the factor stimulating bone marrow colony growth in vitro. The proliferation of these precursor cells in response to tumors may represent an important component of the immune response to tumor cells (415).

Delayed hypersensitivity skin reactions are readily inducible in animals that are immune to syngenic tumors either with tumor extracts or whole tumor cells injected intradermally; delayed reactions were demonstrated in mice (378; 431; 507; 658; 675; 922).

Southam et al. (826) showed that about half of leukocytes of cancer patients inhibited the growth of autologous tumor cells when mixed and injected conjointly with the latter cells back into the patient. This was the first clear-cut demonstration of the existence of cell-mediated immunity to autochthonous human tumors. Subsequently a number of investigators induced tumor specific delayed hypersensitivity skin reactions in patients with a variety of neoplasms, e.g. melanoma (94; 795). Burkitt lymphoma (95; 278), colon carcinomas (424), breast cancer (839) and to various other carcinomas, sarcomas and lymphoproliferative disorders (667; 836). Stewart (837) concluded that delayed skin reactions are in good correlation with the degree of stromal infiltrations of tumor by lymphocytes. In later experiments, however, it was found that repeated skin testing with autologous extracts of patients with breast cancer resulted in weaker reactions, as followed up to 34 months after the first skin testing. Shortened survival was seen in those patients with breast cancer who had delayed reactivity to their own tumor (839).

Berg (77) concludes on reviewing the morphological evidence available for the immune response to breast cancer that most woman will develop some manifestations of resistance, the significance of which is presently not entirely clear. Morphological estimates

of the immune response can be made, and as they are refined these estimates should continue to be useful complements to other tests of host resistance.

Cell-mediated immunity has been demonstrated *in vitro* to a variety of tumors by inhibition of macrophage migration in animals (160; 442; 509; 510; 834), and by the inhibition of peripheral leukocyte migration in humans (22; 802; 950; 951). Furthermore in the majority of cases, human peripheral lymphocytes have been found to be stimulated by autologous tumor cells in mixed cultures (307; 638; 842; 843; 911). Autologous tumor extracts were also reported to stimulate lymphocytes in culture (457; 787). No significant response was obtained, however, to carcinoembryonic antigen with lymphocytes from patients with gastrointestinal cancer, pregnant women, or normal individuals (530).

Other manifestations of interactions of autochthonous lymphocytes with cancer cells have been considered to be due to cell-mediated immunity in human, viz. clustering, congregation and emperipolesis (753), growth or colony inhibition (331; 403; 404; 405; 541) cytotoxicity and lymphocyte attack (640; 641).

In a recent study of tumor-specific resistance to autochthonous MC-induced tumors in rats a direct relationship was demonstrated between in vivo transplantation resistance and in vitro cytotoxicity of autologous immunocytes (480).

As revealed by the colony growth inhibition test, peritoneal cells from mice at the beginning of tumor growth showed some cell-mediated immunological activity which disappeared in

mice bearing tumors which were 4 weeks old or more. This state extended at least 7 days and, in some experiments, up to 3 weeks after surgical removal of the tumors. However, later the mice that had surgery recovered entirely their specific immunological potential in the colony inhibition test, i.e. their peritoneal cells became as active as those sampled from mice immunized with x-irradiated cells. Several experiments produced evidence that reinoculation of tumor cells into x-irradiated mice, 7 and 14 days after tumor removal, accelerated significantly the recovery of their specific immunological potential (59).

Spleen cells from some tumor-bearing mice inhibited the growth of autochthonous tumor cells when cell mixtures were injected into untreated mice (959). A number of experiments have demonstrated that resistance to solid tumors may be transferred adoptively by lymphoid cells in highly inbred animals, but not with serum (385; 492). Recent transfer experiments in guinea pigs gave similar results. When immune peritoneal cells were fractionated, fractions containing lymphocytes and neutrophils could initiate delayed skin reactions to tumor antigens and suppress tumor growth. Cells in the fraction rich in macrophages lacked the ability to initiate tumor suppression, but were able in some tests to initiate delayed hypersensitivity; this was probably due to the presence of a small number of lymphocytes. Moreover, suppression of tumor growth and induction of delayed hypersensitivity by sensitized cells was antigenically specific (964).

In a group of tumor-bearing mice additional inocula of antigenically related tumor cells grew in 0 to 8% of animals at all intervals after initial tumor transplantation except day 1. When the same tumor cell doses were injected into normal animals tumors

developed in 87 to 100%. Mice which received spleen cells from tumor-bearing animals manifested a significant delay in tumor development and/or decrease in tumor incidence if spleen cells were given 14 or 21 days before challenge with tumor cells. These results indicate that concomitant immunity is present in tumor-bearing animals (229; 882).

Purified immune lymphocytes from mice did not exhibit target cell destruction in vitro but were able to prevent the growth of L5178Y lymphoma cells in vivo, when transferred to normal recipients. It was suggested that an immunologically specific cellular cooperation with macrophages was responsible for this difference (271). Further experiments showed that confluent monolayers of macrophages may be "armed" if supernatants of immune spleen cells, incubated for 24 hours with the lymphoma cells used for immunization, were added. Spleen cells from immunized mice incubated with L5178Y on day 7 after immunization released a factor conferring on normal macrophages the capacity to inhibit in an immunologically specific way the growth of L5178Y lymphoma cells. Spleen cells removed up to 15 days after immunization were active in this respect, but not later. The cytotoxic action was also exhibited by macrophages from the peritoneal cavity of immunized animals or by normal macrophages after contact with hyperimmunized spleen cells. However, after the macrophages from immunized mice had been exposed to the specific antigen, they acquired the capacity to kill unrelated target cells. An identical pattern of behaviour was shown by macrophages which had been "armed" in vitro

with spleen cells from immunized mice. By exposing macrophages to very small amounts of double-stranded RNA, endotoxin or the lipid A fraction of endotoxin they were also shown to become activated and thus acquire the capacity to kill nonspecifically. It has been also shown that if macrophages are collected too soon after immunization, they may be "activated" (by contact with the antigen in vivo), and the resultant macrophage monolayer will be non-specifically cytotoxic (272; 273). Another group of investigators demonstrated that normal macrophage monolayers were overgrown with seemingly healthy L cells, but L cells added to activated macrophages obtained from Toxoplasma infected mice were almost totally destroyed. No cytopathic effect was noted when supernatant media, taken from cultures in which activated macrophages had mediated target cell destruction, were added to L cell monolayers. Cell free extracts, however, prepared from normal and activated macrophages by freezing and thawing were equally cytotoxic. This macrophage mediated cytotoxicity did not seem to be a complement dependent reaction. At no time were toxoplasma organisms detected in any monolayer. Nonadherent peritoneal cells from normal or toxoplasma-infected mice did not produce a cytopathic effect. Aggregation of activated macrophages around target cells and early degenerative changes in target cells occurred approximately 2 hours after contact. Phagocytosis of target cells by macrophages, however, was not observed. Syngeneic sarcoma and allogeneic tumor cells were destroyed by activated macrophages as well, but macrophages

had slight or no cytopathic effect on syngeneic or allogeneic mouse embryo cells. Other intracellular protozoa (*Besnoitia jellisoni*) and bacteria (*Listeria monocytogenes*) are able to activate macrophages, but inflammatory stimulants, used to produce peritoneal fluid, are not (416).

The importance of cell-mediated immunity in tumor-host relationships is illustrated by a number of experiments in which reduced resistance was seen towards chemical and viral carcinogenesis, as well as to transplanted histocompatible tumors, in the absence of thymus (517; 888). The induction of thymic lymphomas may also be facilitated if cell-mediated immunity is suppressed by ALS instead of thymectomy (517).

Neonatal thymectomy in mice enhanced the carcinogenic effect of 3,4-benzopyrene, and 3-methylcholantrene (568; 602), and greatly increased susceptibility to oncogenesis due to adenovirus 12 (483). Neonatally thymectomized mice could not be immunized with virus against a subsequent challenge with a syngeneic polyoma-virus-induced tumor. Thymus grafting did restore transplantation resistance of thymectomized mice, provided virus inoculation was given 4 weeks after grafting (887). During an attempt to transmit human leprosy to mice, polyoma-type tumors arose in all mice thymectomized as adults and treated with anti-lymphocyte globulin (318). Experiments on thymectomized rats showed that resistance to oncogenesis by polyoma virus is not the result of resistance to virus infection, but is primarily the result of an immunologic

response of the host to the tumor antigen induced by the virus in the tumor cell (910). Thymectomized rats were inoculated with the Schmidt-Ruppin strain of Rous sarcoma virus. In rats without palpable sarcomas and those carrying small tumors, respectively, cell-mediated immunity could be demonstrated in about the same frequency (around 50%) both in thymectomized and sham operated animals. Thymectomized rats with large tumors disclosed such an immunity in a lower fraction than sham operated animals. It was concluded that neonatal thymectomy in rats does not prevent the development of cell-mediated immunity to the Rous-virus induced tumor antigens (101).

Growth of transplanted tumors in DBA/2 mice was accelerated by thymectomy, which could be restored to normal by thymus graft or thymic tissue implanted in Millipore chambers (1). Finally, the incidence of spontaneous lung adenomas in groups of neonatally thymectomized mice was significantly higher than in intact mice (894).

Thymectomy affects virus (MTV) induced mammary carcinomas in mice in an unexpected way. Removal of the thymus results in a decreased incidence of mammary tumors and hyperplastic alveolar nodules. Although there are strain differences in magnitude, the suppressive effect of thymectomy seems to be general. Some data indicate that thymectomy acts on the transforming activity of MTV, whereas the growth and autonomy of induced tumors is favoured in such hosts. No effect of thymectomy could be detected on the

presence of either blood associated or of milk-borne MTV activity. Splenectomy also decreased both the incidence and age onset of mammary tumors. However, in females of the same strain, previously thymectomized, splenectomy suppressed the depressive effect of thymectomy on mammary-tumorigenesis and restored to a large extent the pattern of intact controls. Decreased capacity to produce enhancing antibody, or direct action of the thymus (hormonal) on development of ovary, uterus and mammary glands were suggested as possible explanations (72; 412; 780; 829; 830).

B. Humoral immunity

Studies on both animal and human neoplasms revealed that antigenic tumors do elicit antibody formation as a rule. Indirect evidence suggests that in fact all hosts with progressing tumors would have an enhancing serum factor, which involves antibody, while in other cases antibodies contribute to host resistance (397; 399).

The high sensitivity of viruses to antibodies in general is a well known phenomenon, which applies to oncogenic viruses as well. Seroprotection has been demonstrated against oncogenesis with a number of viruses including polyoma virus (707), SV40 (20), and feline sarcoma virus (270). Spontaneous regression of Friend virus induced mouse leukemias was accompanied by the appearance of C' fixing antibodies against viral antigens (749). Removal of mammary carcinomas from mice caused an increase of serum resistant

activity which was ten times higher than before (868). A rat antiserum to wild type Gross virus was able to protect mice against Gross-positive transplanted leukemias but not against Gross-negative leukemias. Protection was directly related to in vitro cytotoxic tests. Complete protection could be achieved in some mice by serum given as late as 3 days after i.v. inoculation of leukemia cells. Protected mice were not immune to rechallenge with minimal numbers of Gross-positive cells after the transferred antibody had been cleared (663).

Female mice infected prenatally or neonatally with MTV were shown to produce antibodies to a soluble viral envelope antigen both in tumor bearing and tumor free state. These findings indicate that true tolerance does not exist to a viral antigen which is obviously common to several MTV strains harbored by the serum donor mice (634).

Some experimental studies on the function of different antibody classes in tumor immunity tend to indicate that IgM is responsible primarily for host resistance in the mouse and IgG₂ is involved in the blocking of cell-mediated immunity against solid tumors (25; 860; 862). Thus, IgG₂ was eluted from chemically induced mouse sarcomas by a glycine-HCl buffer at pH 2.4. Tumor cells incubated and injected with autologous eluates gave in some experiments a higher yield of tumors in syngeneic mice than did cells not subjected to this treatment. Some isologous eluates originating from several primary tumors, or from syngeneic tumors

belonging to a different line from the one tested, were also capable of enhancing tumor growth (727; 728; 946; 947). Moreover, blocking material in the form of sera of rats carrying progressively growing polyoma tumors and low pH eluates of polyoma tumor tissue inoculated into polyoma isografted rats, caused an enhanced tumor growth. The corresponding serum from animals carrying an MC-induced sarcoma and eluates from such tumor tissue had no significant enhancing effect. The administered blocking activity was detectable in the sera of recipients 3 days after inoculation. However, no delay or decrease in the level of cell-mediated anti-tumor immunity could be detected by in vitro tests for lymphocyte cytotoxicity against polyoma tumor cells (55).

An "unblocking" serum factor was also found in sera of tumor bearing rats which was able to neutralize serum factors that block cell-mediated tumor cell destruction in vitro. This effect seemed to be correlated with the therapeutic action of the sera against syngeneic polyoma virus-induced rat tumors. The class of antibody involved, and the mechanism of action remain unknown (56).

Antibody dependent lymphocyte mediated killing of tumor cells was recently demonstrated. Target cells obtained from MC-sarcomas, MTV-induced tumors and MSV (Moloney)-induced sarcomas were killed in an immunologically specific way by non-sensitized lymphoid cells in the presence of heat inactivated sera from mice bearing the respective neoplasms or whose tumor had been removed.

Neoplastic target cell death was not characteristically mediated by such sera alone or by normal lymphoid cells in the presence of serum from animals bearing some other tumor but appeared to be a specific synergistically produced cytotoxicity (705).

Humoral response to a number of human tumors has clearly been demonstrated. Burkitt lymphoma (BL) and nasopharyngeal carcinoma are distinguished by outstanding high EB-virus antibody titers, eight times higher than in various control groups including other lymphoproliferative disease, and other carcinomas of the head and neck. Low anti EB-virus titers were very rare among BL patients (moribund cases) and there are no histologically confirmed cases with negative titers. The highest titers were found in sera of patients whose tumors have undergone long term regression. In the individual patient the titers may change considerably in the course of the disease but, as a rule, they remain within the same order of magnitude; the patients can therefore be classified into groups of low, medium and high reactivity. Antibody levels probably fall when rapid and extensive tumor growth takes place. When the patient receives chemotherapy and the tumor regresses, there is often an increase in titer. (This increase was particularly apparent in a patient who received local radiotherapy). If tumor persists, the lymphoma cells become coated with IgG as a rule (491; 918). Since both the intracellular and membrane-antigen systems consist of more than one antigenic component, some positive sera contain antibodies against a larger number of components than others (857).

Each of twenty commercially prepared lots of human globulins, produced specific immunofluorescence reactions with EB3 cells, which is a continuous cell line derived from Burkitt's lymphoma. Titers varied between 1:60 and 1:640. The preparations also exerted growth inhibitory effect on EB3 cells, for which C' was not required, and it was related to the immunofluorescence titers (960). EB virus antibodies were also found in blood specimens from three different chimpanzee groups which had not been in contact with humans, indicating that wild chimpanzee were also infected by EB-virus or a very similar herpes-like virus (540).

Another intensively studied group of human tumors is osteosarcomas and various soft tissue sarcomas. Immunofluorescence studies have revealed a high incidence of antitumor antibodies in sera of patients (13/13) with these diseases and of their close relatives (34/40) and associates (11 of 12 positive), which react with common antigen(s) in osteosarcomas. The incidence of antibodies in normal blood bank donors was much lower (29%). Similar antibodies were found in patients with liposarcomas and fibrosarcomas. Cytotoxic antibody was found in serums from 70% of sarcoma patients, 58% of their family members, and 8% of serums from normal donors. Antibodies which fix only human complement were demonstrated in sarcoma patients. A rising antisarcoma antibody titer was consistently observed following surgical removal of the tumor mass in sarcoma patients who remained free of the disease, whereas patients with recurrent disease were found to have a progressive decline in

their titers of antisarcoma antibody (236; 259; 260; 261; 625; 627; 628; 953).

Malignant melanoma patients exhibit also a high frequency of anti-tumor antibodies. Antimelanoma antibodies were revealed by complement fixation in 67% of melanoma patients and in only 22% of sera from normal blood bank donors. All patients with spontaneous regression had cytotoxic antibodies, 89% of patients with localized melanoma, but only 26% of patients with advanced metastatic disease exhibited cytotoxic antibody activity. A study of serial serum specimens on melanoma patients revealed a drop in antibody titer to an undetectable level with advancing metastatic disease; both IgG and IgM antibodies were present in some cases as revealed by immunofluorescence (624; 625).

Two seronegative and one weakly positive patients were immunized with autologous irradiated melanoma cells. Membrane immunofluorescence obtained with the autologous (post-immunized) sera was greatly reduced when it was first mixed with negative (preimmunized) sera. The mixing reduced the fluorescence titers from 1:64 to 1:2 in two cases and from 1:32 to 1:4 in the third case. None of the control sera, pooled human sera and heterologous negative melanoma sera altered the titers appreciably. Blocking effect was largely in the IgG fractions. Using double diffusion a precipitin line was seen between the positive serum up to a titer of 1:16 with the negative serum, but neither reacted with the controls. Significant lines of reaction occurred only between the IgG fraction

of the negative serum and the gammaglobulin fraction positive for melanoma antibodies. Formalinized sheep erythrocytes coated with gammaglobulin of the positive sera were agglutinated by addition of the gammaglobulin fraction of negative sera. In one case a positive serum produced a complement dependent cytotoxic effect on the autologous melanoma cells, which was considerably reduced by adding an equal volume of negative serum before testing. It was suggested that a specific antibody against the original tumor specific antibody, rather than an antigen-antibody complex is the blocking agent (546).

Patients with colon carcinomas were found to produce tumor specific antibodies (344; 345; 420; 640); however, LoGerfo et al. (554) could not detect antibody activity against CEA in sera from 265 patients.

Antibodies to genital herpesvirus type -2 were found in sera of women with cervical atypia, carcinoma in situ and invasive carcinoma in a significantly higher frequency than in sera of control women ($P < 0.0005$) (36; 142).

When 437 sera were tested by mixed hemadsorption against 102 different specimens of leukemic tissues, approximately 30% of leukemia and lymphoma cells contained antigens which reacted with 30% of sera of leukemic patients. Normal cells reacted with only 10% of sera of leukemic patients but not with sera of normal donors. Not all positive results could be attributed to isoantibodies. The detected antibodies were shown to be IgG heat stable and C' independent (579).

Seven of 8 patients' sera with carcinoma of the pancreas showed positive reactions by electroimmunodiffusion. Positive reactions were also noted in sera of some patients with pancreatitis and with unrelated malignant lesions. Antibodies from these latter sera were completely removed by absorbing the sera with extracts of normal or inflamed pancreas. However, absorption of sera from patients with carcinoma of the pancreas with these extracts still left a component that reacted only with malignant extracts (932).

Five of 8 sera from patients with primary lung cancer of oat cell type gave positive membrane fluorescence with anti IgM reagent, but not with anti IgG with cell line OAT, established from lung cancer of oat cell type. 27 sera from patients with other types of carcinoma, (including 9 cases of primary adenocarcinoma of the lung) and sera of healthy individuals gave no positive reaction (657).

Sera or peripheral blood lymphocytes of patients with squamous cell carcinoma of skin exhibited immunological reactivity against their own tumor cells as demonstrated by membrane and cytoplasmic immunofluorescence, complement-dependent cytotoxicity and lymphocyte attack, respectively (641).

Sera of patients with urinary bladder carcinomas contained C' dependent antibodies which were cytotoxic for autochthonous and allogeneic bladder tumors; the degree of cytotoxicity of serum from the same patient varied over a period of 8 months and some samples were completely devoid of cytotoxicity. Sera of some patients exhibited a blocking activity on the cell-mediated cytotoxicity

of peripheral leukocytes against tumor cells. Opsonizing antibodies were also detected which appeared when cytotoxicity of the leukocytes decreased (118).

Sera from all the seven tumor patients tested (one with melanoma, one with colonic carcinoma and four with breast carcinoma), who had become clinically tumor free could "unblock" (abrogate) the blocking effect of sera from patients bearing tumors of the respective types, thus making it possible for immune lymphocytes to kill cultivated neoplastic cells in the combined presence of the "unblocking" and blocking sera (403). Furthermore, blocking activity could also be eluted at pH 3.1 from human tumor tissues, obtained at surgery (seminomas and osteogenic sarcomas) and from tumors growing in ascites and pleural effusions (816).

C. Immunological inertia

An impaired immunological reactivity in humans and animals with lymphoproliferative diseases seems to be a direct consequence of a diseased immune system (385; 413; 451; 673; 762; 818; 961).

Southam et al. demonstrated (827) that cancer patients showed twenty-one out of twenty-three takes from inoculated allogeneic cancer cell lines and six out of twelve from "normal" allogeneic cell cultures. Normal individuals showed proliferation only in four out of fifteen cases which regressed by 14 days. A similar experiment was performed on a patient with disseminated squamous cancer of the breast, who accepted subcutaneous homotransplants of

human cancer tissue culture lines (HEp2, HEp3). The primary homotransplant persisted until the patient died of her cancer 6 months later, in spite of evidence from two subsequent homotransplants that the first homotransplant had elicited homograft immunity (824).

The immune response of patients with malignant disease, not originating from the lymphoreticular tissue, was studied with a variety of antigens (tuberculin, trichophyton, candida, streptokinase, mumps antigen, DNCB, etc.) and there is general agreement that delayed skin reactivity is greatly impaired in cancer patients (18; 562; 647; 841). Similarly, the lymphocyte response to PHA was impaired in patients with a large tumor (841).

Involution of the thymus during growth of Walker carcinoma in the rat (268) and a degeneration of the thymus in inbred mice (AB) bearing NK lymphosarcoma in ascites form (496) were described. Moreover, thymus cells of tumor bearing mice were shown to lose their sensitivity to anti- θ antibodies and the number of θ -positive cells in the lymph nodes of such mice was decreased (560). Hence it may be inferred that a deteriorating effect of neoplasia on thymic function may be the reason for immunological inertia.

D. Autoimmunity and cancer

Sensory carcinomatous neuropathy develops in a small proportion of patients with oat cell bronchial carcinoma and, more rarely, with other neoplasms. It is characterized by destruction of neurons, particularly in the dorsal root ganglia together with some lymphocyte infiltration. The disease is unrelated to metastasis. The serum of these patients contained an antibody against neurons which were detected by C' fixation and immunofluorescence (940).

Smooth muscle antibody at titers of 1:10 or more was found in a large fraction (67.5%) of patients with malignant disease and in 20% of 46 controls, i.e. the incidence of smooth muscle antibody was 70% in malignant melanoma and 83% in carcinoma of the ovary (948).

A patient with a nephrotic syndrome associated with bronchial carcinoma was shown by renal biopsy to have a diffuse membranous glomerulonephritis, and on immunofluorescence, immunoglobulins were demonstrated in the glomeruli. The patient died 3 and a half years after diagnosis, and immunoglobulins eluted from the glomeruli reacted specifically with the surface plasma membranes of the bronchial tumor cells. Immunodiffusion revealed a reaction of identity between the glomerular eluate and an extract of the tumor when tested with the patient's serum. It is suggested that deposition of tumor-specific antibody or antibody complexes in the glomeruli is one mechanism for the development of a nephrotic syndrome in patients with malignant disease (545).

Normal C58/wm mice older than 6 months rapidly lost their capacity to be immunized to a syngeneic transplantable line of I_p leukemic cells. An age dependent central nervous system (CNS) disease occurred in immunized mice characterized by a monocytic inflammatory response, some demyelination, destruction of neurons in the cord and paralysis or death. Lymphocytes from diseased mice injected i.p. into 10-14 months old mice induced CNS-disease, but not in mice of 2-6 months of age. No virus involvement could be detected in CNS-disease, which appeared to result from a serum mediated immunological response directed against θ -type iso-

antigens presumed to be present in leukemic cells and CNS tissue (637).

Evidence for local immune reactions has been described in the preleukemic thymus of AKR mice, e.g. appearance of lymphoid follicles, patchy loss of small lymphocytes and increased number of plasma cells. Although young AKR mice are tolerant to the Gross-antigen, in the preleukemic period spleen and lymph node cells of these mice exhibit an immune cytolytic reactivity to syngeneic AKR embryo cells; this suggests a partial or complete breakdown of tolerance to the Gross-antigen in the preleukemic period. It is proposed that secondary, immunologically mediated damage of virus-infected Gross-positive thymic lymphoid cells may be the ultimate process precipitating leukemia development in the thymus of AKR mice (585).

Escape Mechanisms of Antigenic Tumors

One would expect that antigenic tumors recognized as not-self by the host's immune system should be rejected. Although there is indirect evidence that malignantly transformed antigenic cells are rejected in a number of cases before the manifestation of neoplasia, yet some of them avoid rejection and initiate antigenic tumors. It is, therefore, plausible to assume that antigenic tumors interfere somehow with the defence mechanisms of the host in order to escape immunological surveillance by one of the possible mechanisms discussed below.

A. Immunodeficiency states of the host

The incidence of malignancy in patients with primary immunodeficiencies is roughly 10,000 times that of the general, age-matched population. Each type of immunodeficiency has a distinctive constellation of malignancies associated with it. In general malignancies of hematopoietic and lymphoreticular system are predominant (317; 468; 684). Furthermore, a significant risk of de novo malignancy must be accepted as part of the price for transplantation of kidney and other organs with immunosuppression therapy; thus, in renal allotransplant recipients undergoing immunosuppression the incidence of reticulum cell sarcomas was more than 100 times greater than that in the general population and far greater than that of other types of malignant tumors in transplant recipients. A greatly enhanced occurrence of squamous cell carcinomas was also reported in such patients. When malignant tumors were accidentally transplanted along with renal homografts, they developed into autonomous and invasive tumors and in two instances these tumors were rejected after stopping immunosuppression. Nevertheless, the benefits of renal transplantation with current methods make it the treatment of choice for end stage renal disease (594; 684; 695; 833; 921).

There is abundant evidence also in experimental animals that immunodeficiency states and immunosuppression favours the induction and/or progression of tumors. Female, aged, randombred Swiss mice were less capable of rejecting skin homografts and

tumor homo- and hetero-grafts than young adult mice. They showed decreased phagocytic activity and produced isoantibody of relatively low titers. In addition, the spontaneous tumor incidence increased with age (878). Similarly, susceptibility of hamsters to viral oncogenesis with SV40 was directly related to the age of the animal at infection; thus, more than 95% of the animals inoculated within 3 days after birth developed tumors after 90-150 days and the incidence of tumors fell to 20-25% in animals infected at 1 or 2 weeks of age and to zero in animals infected at 3 weeks (or more) of age. The investigation of immunocompetence in terms of the response to SRBC showed that very few PFC were generated if the animals were immunized at 1 week of age, and there was a rapid increase by the 3rd-4th week of age (310).

The importance of immunodeficiency states for the manifestation of certain type of tumors is well illustrated by viral oncogenesis with the radiation leukemia virus (RLV) (547). The interaction of this virus, isolated from irradiated tissues of C57Bl mice, with thymocytes in vivo yields a high leukemia incidence if the mice are further exposed to an immunosuppressive agent (irradiation or ALS). It seems that transformed leukemic cells can proliferate only when the host is immunologically impaired. By delaying immunosuppression after inoculation the leukemogenic virus into the thymus, the leukemia incidence was markedly reduced; this decrease may be due to triggering of the immune response. Inoculation of RLV into newborn C57Bl/6 mice decreased the takes of syngeneic leukemic cell grafts, which were inoculated s.c.

30 days later (382; 383).

The Moloney type virus induces spontaneously regressing sarcomas in mice and tumor incidence increases in thymectomized mice. Progressively growing tumors could be established, however, in adult thymectomized and x-irradiated recipients (521).

Antilymphocyte serum (ALS), a frequently used immunosuppressive agent, was reported to promote in mice the occurrence of spontaneous reticular neoplasms (132), to facilitate the induction of lung adenomas by urethan (893) and enhance carcinogenesis by oncogenic viruses (390; 519; 520; 733). Similarly, growth of plasmacytomas and sarcoma I could be accelerated in mice by ALS treatment (565; 861).

ALS treatment was found to be much more efficient in enhancing the development of primary polyoma tumors in rats than surgical thymectomy. When ALG (antilymphocyte globulin) treatment was started as late as 28 days after the polyoma virus inoculation, there was still a marked increase in tumor incidence, but this effects could not be observed if ALG treatment was started 7 or more weeks later (908; 909).

ALS treatment of Syrian golden hamsters increased the incidence of tumor occurrence as well as the total number of tumors induced by painting the cheek pouch with dimethylbenzanthracene (956). The metastatic growth of an antigenic hamster lymphoma was favoured by ALS treatment and ALS was shown to not act directly on lymphoma cells (325). Human choriocarcinoma in the cheek pouch

of ALS treated hamsters grew in a higher incidence, had an increased volume and persisted longer. Methotrexate, an anti-cancer drug, had less oncolytic effect on choriocarcinoma in ALS-treated than in untreated hamsters (543).

It is of interest that L-asparaginase, a commonly used drug in cancer therapy possessing immunosuppressive potency, increased the frequency of metastases of sarcoma 180 in mice (232).

Immunosuppression by certain chemical carcinogens and oncogenic viruses may play an important role in the success of induced antigenic tumors. A single exposure of mice to carcinogenic hydrocarbons rapidly depressed the number of plaque-forming spleen cells to SRBC during a long period of time, corresponding to the latency period of tumor development. Benz (a) pyrene (BaP), 7,12-dimethylbenz(a)anthracene (DMBA) or 3-methylcholanthrene (MCA) carcinogens decreased PFC per total spleen or nucleated cell population. However, the noncarcinogenic hydrocarbons, such as anthracene and benz(e)pyrene, had no immunodepressive effect and 7-methyldibenzanthracene had a slightly depressive effect on PFC. It was suggested that a correlation may exist between the ability of carcinogen to induce antigenic tumors and its inhibitory effect on the host's immune response. A significantly higher incidence of takes and/or earlier tumor appearance was seen in the MCA-exposed hosts after small doses of syngeneic MCA induced sarcomas (840). An extensive study of immunosuppression by polycyclic hydrocarbons MCA, BaP, DMBA, dibenz(a,h)anthracene, benzo(c)phenanthrene showed that the capacity of chemical carcinogens to inhibit

rejection of homografts is best correlated with the strength of their carcinogenic potency. Rejection of 6C3HED lymphosarcoma in DBA/2 mice was used as an assay system. The homograft tolerance assay proved a useful, sensitive, and rapid technique for detecting potential carcinogens as well as anticarcinogens. Immunological stimulants, carcinogens and adjuvants increased tumorigenesis as well as homograft tolerance in the presence of active carcinogens. It was postulated that carcinogens inhibit the cellular rejection system (768). Normal marrow and thymus cells were injected to groups of x-irradiated recipients respectively with spleen cells from DMBA treated donors and SRBC were administered as an antigen. Immune reactivity could not be restored with T-cells, but bone marrow cells fully restored the capacity of spleen cells to respond to SRBC. It was, therefore, concluded that bone marrow cells are immunologically deficient in animals suppressed by DMBA (767).

The immune response to SRBC of young adult C3Hf/Bi mice are sensitive to the oncogenic effect of MC was suppressed by the drug but it proved without effect in I strain, which is relatively resistant to MC carcinogenesis (852). Furthermore, injection of DMBA to neonatal mice permanently suppressed the humoral antibody response and resulted in a high tumor incidence. Mice injected at birth with BaP and MCA did not show any detectable immunosuppression, which is contrary to the finding that neonatal immunosuppression with DMBA correlates with a high yield of tumors of the lymphatic tissue; such a correlation was not found with BaP and MCA (53).

Immunosuppression during mineral oil carcinogenesis in mice was reported as measured by antibody production against T2 phage, PFC and agglutinin titers against SRBC, allogeneic tumor graft rejection and graft-versus-host reactivity (506).

Depression of homograft rejection and GVH-reactivity following DMBA exposure was observed in rats. Similarly, the purine derivative 3-hydroxyxanthine, which is oncogenic in rats, was moderately active in depressing hemagglutinin antibody formation and homograft response (235; 879).

The effect of murine leukemia virus (Friend) (MLV-F) was investigated on the immune response to SRBC of various mouse strains with different sensitivity to the virus. There was almost a direct relationship between the susceptibility and the degree of immunosuppression (144). Hemolysin production by spleen organ cultures derived from immunized mice and hemolytic plaque formation by peritoneal cells of nonimmunized mice were not influenced by in vitro infection of cultures with MLV-F. There was no evidence of virus replication in the cultures. Spleen organ cultures derived from MLV-F infected mice showed impairment of antibody formation only when culture was done after virus replication had taken place. A significant reduction in the number of peritoneal cells, which can be stimulated in vitro to produce antibody, occurs only in the terminal stages of MLV-F disease, 3 weeks after injection, and is due to a fall in the proportion of lymphocytes in the peritoneal population (75).

The mechanism of murine leukemia virus (Rauscher) (MLV-R) induced immunosuppression was investigated in mice. Adult mice received i.p. injections of an MLV-R preparation. The spleen, thymus, mesenteric lymph node, liver and bone marrow were studied histologically and electron microscopically at intervals between 6 hours and 20 days after injection. The experimental animals were also tested for their ability to respond to rat erythrocyte antigen during this interval. Numerous C-type viruses were observed localized extracellularly in plasma membrane infoldings of dendritic cells of spleen and mesenteric lymph node germinal centers. The number of virus particles in these germinal centers increased markedly during the first 10 days of injection. Immunoblasts were participating in the replication of C-type particles. Budding of C-type particles from the parenchymal immunoblasts of the germinal centers occurred as early as 24 hours, after injection. By 7 days the normal antigen stimulated emigration of immunoblasts of the germinal centers to the spleen red pulp was observed. Since these cells participate in viral replication, it is considered that they may provide a source of C-type particles to proliferating cells of the spleen red pulp, such as megakaryocytes and erythrocytic precursor cells. It is concluded, that morphologically the response to MLV-R starts as an immune response but it results in virus replication (380).

A virus of minimal pathogenicity for mice, which was derived from Friend-virus infected animals, and designated as "hypertrophic spleen virus" (HSV), was investigated for immunosuppressive effect in mice. Immune response to SRBC was strongly

suppressed if HSV was given 4 days before SRBC, as measured by the Jerne plaque assay. Similarly, mice infected with the murine parasite *Pl. berghei yoelii*, which in itself produces a severe but self-limiting disease in BALB/C mice, die rapidly if infected with HSV with $\pm$ 4 days of parasite inoculation (781).

B. Immunological tolerance

As already discussed various type-C RNA oncogenic viruses may be transmitted vertically by ovum, sperm or intrauterine infections. Some strains of the mammary tumor viruses of mice are transmitted by milk to the immunologically immature offspring (76), and according to the classical rules of immunological tolerance, immune responsiveness is therefore not expected to be present to these oncogenic agents under these conditions. Blair (91) concludes in a recent review of MTV-host relationship in the mouse, that evidence indicates that immunological reactivity is established to the antigens of MTV, both to those of the virion itself and to those expressed on the cells infected by the virus. However, the neonatally infected mouse is essentially tolerant to these viral antigens. Specific antibody formation and cell-mediated immune reactions can sometimes be induced in MTV-infected mice by immunization with MTV antigens, but the presence of any immunologic reactivity to MTV in the normal neonatally infected mouse has been difficult to demonstrate.

It was observed that MTV-induced hyperplastic alveolar nodules grew poorly or not at all in adult C3H/2 hosts, which are free of MTV and conferred a strong protection against subsequent

challenge with syngeneic MTV-induced mammary tumor. No such protection was conferred upon immature (3 to 4 week old) C3H/2 hosts by MTV-infected tissues. Similarly, MTV-infected C3H/2 mice did not respond to such immunization (516). When (C3Hf/Gs x CBA - T6T6)F₁ mice were injected with Graffi leukemia virus within the first days of life they failed to produce transplantation resistance or cytotoxic antibodies against tumor antigens following subsequent immunizing procedures. Such mice were also susceptible to the leukemogenic action of the virus. The observed lack of immune response was shown not to be related to a general immune depression but dependent on a specific tolerance to the injected virus. The tolerant state could be abolished by adoptive immunization with syngeneic spleen cells from donors immunized as adults. Furthermore, nursing by preimmunized mothers was also capable of preventing the development of tolerance in the offspring injected with the virus at birth. Thus, antibodies passively transferred via the maternal milk are also able to abolish tolerance (158).

It was found, however, that after hyperimmunization with MTV several inbred mouse strains produce precipitating antibodies to the MTV virion. Even mice which are neonatally infected with the virus can make such antibodies. One inoculation with MTV emulsified in complete Freund's adjuvant into BALB/cf-C3H mice, which are neonatally infected with the virus, leads to a significant enhancement of tumor development. Adjuvant or MTV alone had no such effect. These results indicate that an "underground" cellular

immunity exists in MTV-induced mice. GR mice, which are hereditarily infected with the plaque forming variant of MTV, to which they are tolerant, produce antibodies to MTV virions after hyperimmunization with the standard virus. One single injection of standard virus or H_2O_2 treated plaque forming variant into GR mice leads to a significant delay in tumor-development and spleen cells can transfer this resistance (76).

The absence of true immunological tolerance was also repeatedly observed to members of the murine leukemia-sarcoma virus complex. Gross virus is the etiologic agent of spontaneous leukemias of AKR mice. Despite the vertical transmission and the persistence of Gross virus throughout their life, these mice are not immunologically tolerant to the virus. Specific antibodies to Gross antigen can be detected in the kidney where they have been deposited in the glomeruli, apparently in the form of antigen-antibody complexes (664). It was also observed that BALB/c mice infected neonatally or by vertical transmission from the mother with Moloney leukemogenic virus or mouse sarcoma virus develop chronic, proliferative glomerulonephritis. The abnormalities were more severe when leukemia became manifest. Only a few virus particles were occasionally detected in some glomeruli. Immunoglobulins and viral antigens, however, were demonstrated in practically all glomeruli by immunofluorescence and antibody capable of neutralizing MLV was eluted from the kidney of the infected mice. The findings appear to be consistent with an immune complex etiology of this glomerular disease (13;110).

Members of C57Bl mouse strain have been presumed to carry the radiation leukemia virus through vertical transmission during post natal life; yet, newborn mice injected with the virus show resistance to a further iso-transplantation of leukemic cells induced by the same virus (381).

Experiments on polyoma virus oncogenesis in mice revealed that syngeneic spleen cells from sensitized adults can prevent tumor formation when transferred early enough (one week) but they are ineffective if the transfer is delayed (by one month) after the infection of thymectomized C57Bl mice with polyoma virus. Newborn spleen cells are ineffective. Further investigations showed that in adult animals cell-mediated immunity develops more rapidly than humoral immunity after inoculation of polyoma virus i.p., while in the newborn the situation is the reverse (13).

A phenomenon designated as "sneaking through" the immune surveillance by antigenic tumors has been observed by several investigators. Leukemia L1210 and an ascitic subline M46G, carried in DBA/2J mice, and the antifolic drug resistant C82R strain, carried in (BALB/cAn x DBA/2J) F_1 hybrids, were transplanted into homologous BALB/c mice. These transplanted leukemias grew and regressed (homograft response) when inocula of 0.1 ml containing approximately 1% leukemic spleen were used. With further dilution of the inoculum, the proportion of tumor takes terminating in death of the animals was increased markedly (440). MCA-induced tumor cells were given in a wide dose range to syngeneic recipients.

Two of three mice in each group receiving 40 and 200 cells developed tumors, while only one out of three died with inocula of 1,250 and 12,500 cells. All three mice succumbed to neoplasia in groups given 62,500, 125,000 and 625,000 cells, respectively (103; 661).

It is noticeable, that this dose-dependent tumor take strikingly resembles to the low and high zone tolerance observed with a variety of antigens by many investigators and discussed in the section on general immunology.

C. Immunological enhancement

Immunological enhancement seems to be a major escape mechanism of antigenic tumors. With the aid of in vitro growth inhibition or tumor cell destruction assays it was demonstrated that most human and animal tumor bearing hosts have detectable cellular immunity against their antigenic tumor. This cell-mediated immunity, however was consistently abrogated by serum factors of hosts with progressing tumors. Such factors were not detectable in regressor sera or in sera of immunized animals. The blocking effect was immunologically specific (397; 399; 596).

In allogeneic systems immunization leads to an initial heightened resistance to the tumor graft, which is transient if immunization is carried out with killed tissue and of longer duration if living tissue is used. This period of resistance is followed by enhancement. Antibody is involved in enhancement. Accelerated graft rejection of a new inoculum, however, may be demonstrated in the same animal with the original tumor enhanced. Passive enhancement

can be induced with microliter, or even smaller quantities of antisera, and hyperimmune sera are usually more effective. In this case IgG is responsible for enhancement. Serum-versus-cell dose is extremely important; the same serum could enhance or inhibit tumor growth at different ratios. The nature of the tumor, the strain of the host, and system (passive, active, etc.) are all important factors. Some animals will continue to reject successive grafts of a tumor, though they are producing enhancing sera.

In histocompatible tumor system IgG was found to be responsible most frequently for enhancement, while IgM may enhance or inhibit as well, and this effect may be dose dependent. In this system cell-mediated immunity develops also first, but there is no complete killing of tumor cells because of "weak" tumor antigens. Antibody response follows which may result in enhancement. Factors like shifting the immunological balance in preference of the cellular response, changing the molecular composition of humoral antibodies, relative vulnerability of the tumor to C' mediated cytotoxicity are important (464).

It was observed long ago that the spleen of tumor bearing animals is enlarged and exhibits histological changes characteristic of antigenic stimulation (955). Other experiments revealed that certain mouse strains are more resistant to the homotransplantable sarcoma 180 if splenectomized (287; 288; 289; 662; 773) and to some other sarcoma homografts (615; 711). Tumors induced in BALB/c mice by the Moloney sarcoma virus were rejected more frequently in splenectomized mice. The effect of splenectomy was more pronounced

in females than in males (704). Splenectomized hamsters responded to an allotransplantable lymphoma with increased immunity in the first 2 weeks, but resistance was decreased in the following 2 weeks when compared with sham operated controls. If splenectomy was performed 7 days after tumor inoculation, there was no demonstrable effect on the subsequent immune response (324; 329).

As revealed by the colony inhibition test, splenectomized animals had less blocking activity in their serum to a transplantable MCA induced tumor, than did their sham operated controls; cellular immunity was of about the same magnitude in both groups of animals (406).

Tumor enhancing alloantibody (IgG_2) directed against a spontaneous mouse fibrosarcoma was cleaved with papain. Fab fragments induced greater enhancement than Fc. It was suggested that the peripheral concept of enhancement is more significant than the central one, although both mechanisms appear operative (204). In further studies half molecules were prepared also by reduction of the critical inter-H-chain disulfide bond using 2-mercaptoethanol and cysteine. Preparation of half molecules, Fab, and Fc fragments were assayed separately for tumor-enhancing ability. Results of this investigation proved that the Fc region of the tumor enhancing IgG_2 molecule contributed significantly to the mediation of this type of antibody-induced specific immun-suppression (205).

Blocking activity was eluted at pH 3.1 from human tumors. A high (above 100,000) and a low molecular weight (below 100,000)

fraction could be separated from the eluates by ultrafiltration. Neither of these fractions could block lymphocyte-mediated cytotoxicity in vitro when tested by itself, while a 1:1 mixture of them was effective. Blocking was also obtained when the tumor cells were first exposed to the high and then to the low molecular weight fractions, but not when the sequence was reversed. Blocking factors, therefore, can be eluted from human tumors and the blocking effect may be due to antigen-antibody complexes (404; 816).

An MLV (Rauscher) induced lymphoma of Swiss mice was immunosensitive in diploid form from which immuno-resistant tetraploid cells emerged. These immuno-resistant cells produced both leukemia virus particles and virus neutralizing globulins. On the basis of these facts a unique theory of enhancement of tetraploid lymphoid malignancies was proposed (812) suggesting that the globulins act as "self-enhancing" antibodies by coating antigenic sites of the neoplastic cells and that the neoplastic cells thus escape lymphocyte mediated rejection. According to this hypothesis, which is illustrated in Fig. 4, tetraploid immuno-resistant cells emerge when diploid lymphoma cells and antibody-producing immune spleen cells are brought together, in tissue culture. It is proposed that tetraploid immuno-resistant cells are formed by the fusion of diploid lymphoma cells and antibody-producing plasma cells (812; 804). An analogous situation has been described for Burkitt lymphoma of human, where recurrent tetraploid forms are immuno-resistant (491; 812).

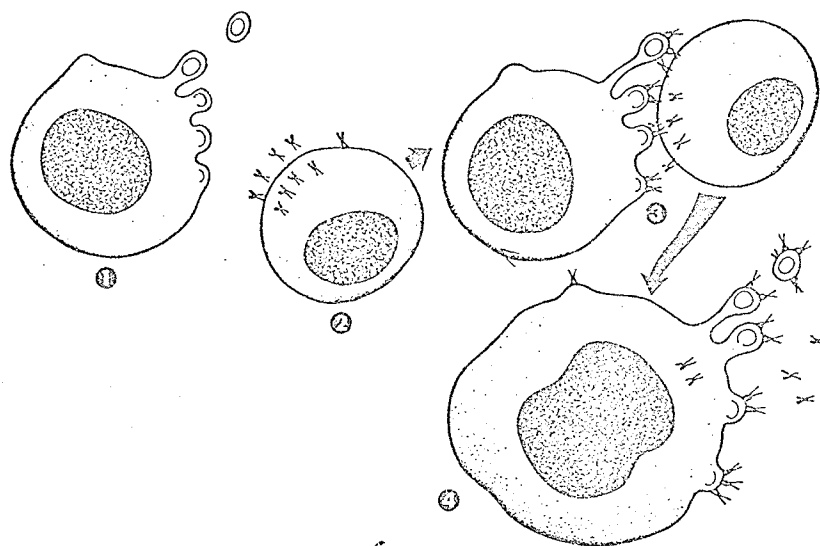


Fig.4. Hypothesis: Formation of immunoresistant tetraploid neoplastic lymphoid cells by fusion of the primary diploid neoplastic cell with plasma cell-producing virus-neutralizing antibody. The resulting tetraploid cell continues both genetically determined commitments: synthesis of leukemia virus and of virus-specific globulins; the globulin functions as "self-enhancing antibody." 1, Virus-producing diploid immunosensitive lymphoma cell; 2, Antibody (virus-neutralizing)-producing plasma cell; 3, Juxtaposition of 1 and 2; 4, Fusion of 1 and 2.

Sinkovics et al., In "Leukemia-lymphoma" Year
Book Med. Publ. 1970, p. 53.

D. Changes in antigenicity

As discussed earlier, antigenic modulation of tumors may be caused by various agents such as antibodies or drugs. There is experimental evidence that immuno-resistant tumor cell lines have a lower density of antigens on their surface than immunosensitive lines of the same tumor. Three malignant and one normal malignant cell lines were derived by cloning from the same fibrosarcoma induced by polyoma virus in H3B/Bi mice. The sensitive line induced a humoral response which could be demonstrated by in vitro colony inhibition, and conferred immunity against the malignant cell lines by preimmunization of the host; moreover, this immunosensitive line adsorbed more C' when reacted with the antiserum than did the malignant lines (645). In a similar study an immunosensitive variant cell line induced by polyoma virus was shown to have 8.8 times more tumor specific antigen by isotopic antiglobulin technique than the malignant line (886).

Ordinarily adult syngeneic rats reject radiation leukemia virus induced tumors unless irradiated. The rejection involves an immunological mechanism directed against a high concentration of virus cell surface antigen. In a few tumors that were able to grow in nonirradiated adult hosts the concentration of this antigen was lower. Some sublines lost completely the viral antigen. Electron microscopic examination of these immuno-resistant lines showed that virus particles were either absent or radically decreased in concentration as compared to the original lines (290).

A theory proposed by Cohn (184) for the escape mechanism of antigenic tumors in the presence of C' fixing cytotoxic antibodies is related to the low density of antigenic determinants on the surface of the tumor cells as illustrated in Figure 5A and B. As mentioned on page 315, it is visualized that at least two appropriately spaced (within 200 Å) antigenic determinants are necessary for activating the complement components fixed to appropriate antibodies on the cell surface and for subsequent lysis. If the density of tumor antigens is lower than that, the cell escapes lysis despite the fact that complement fixing antibodies may be produced to it.

E. Escape mediated by non-antigenic sialomucin coat

In the vast majority of cases the fetus inherits transplantation antigens from the father which are incompatible with those of the mother; yet, this antigenic graft of Nature survives. To explain this apparent immunological paradox, it was proposed that in species (including man) with a hemochorial placenta a specialized layer of fetal cells with a non-antigenic electronegative sialomucin coat constitutes a continuous layer in direct contact with the maternal blood and thus segregates the two incompatible tissues (85; 208). Although the immunobiology of maternal-fetal relationship is probably very complex, which is influenced by hormonal and other factors, this non-antigenic coat still appears to be of major importance. It was convincingly shown that nonantigenicity of early mouse trophoblast was associated with surface mucoproteins. The normal antigenicity of the fetal tissue could be restored by the removal of the sialic acid component with neuraminidase (210).

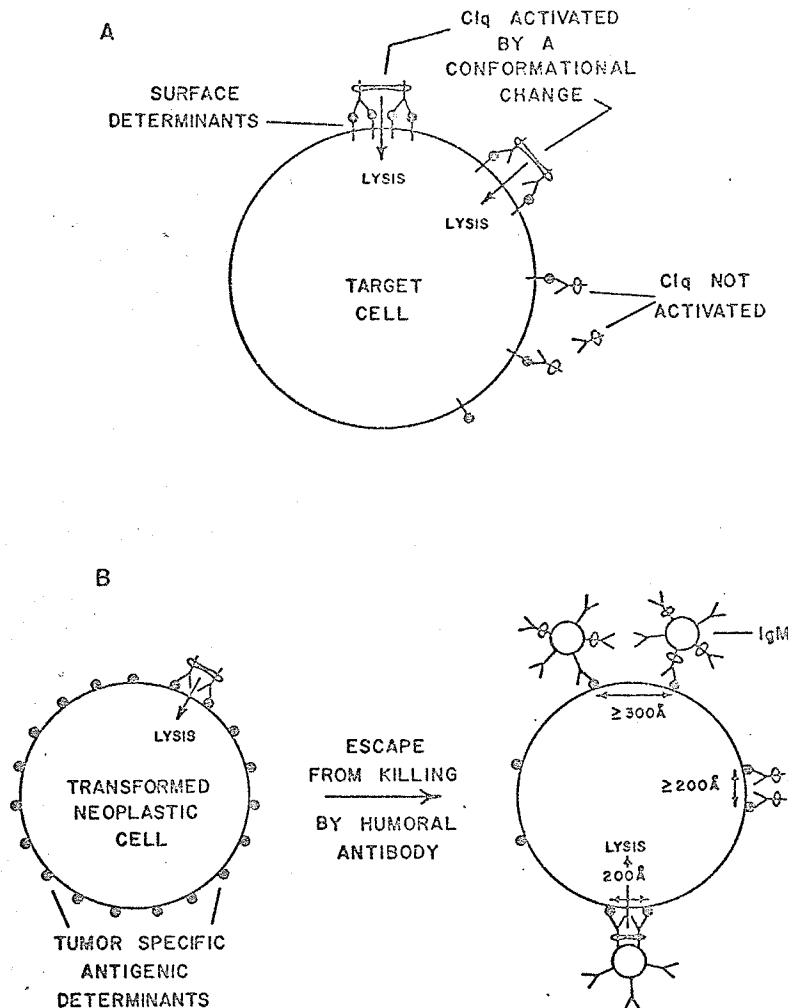


Fig.5. Models for activation of complement lysis: (a) The effect of spacing of surface determinants on the activation of complement lysis via IgG. (b) The escape from complement killing by a tumor cell.

Cohn, M., Ann.N.Y.Acad.Sci. 190: 529, 1971.

It follows that tumors arising from embryonic cells (choriocarcinoma, trophoblastic tumors) are usually just as incompatible to the host as normal embryonic tissues, yet some of them are very invasive and metastasise rapidly. This behaviour is due to the same absence of antigenic activity that is evident in normal trophoblast (373). Studies on histocompatibility between mother and child in cases of choriocarcinoma revealed that fatal cases of neoplasia occur in the presence of major histocompatibility differences, despite the fact that the patient is immunocompetent (523; 771; 772). For some unknown reasons women of blood group A married to males of group O appear to have the highest risk, whereas women of group A with group A husbands apparently have the lowest risk, the relative risk for these two groups being 10.4:1(522). Choriocarcinoma patients are able to reject the husband's skin graft, although the mean time of rejection may be as long as 18.9 days compared with a mean time of 9.5 days for grafts from unrelated donors. It was suggested that histocompatibility antigens are recognized onchoriocarcinoma, but produce tolerance rather than sensitization (46; 759).

Furthermore many tumors may express embryonic antigens and/or release embryonic proteins. Although the physiological role of these substances is hardly known, it is quite conceivable, that at least some of them are capable of inducing specific tolerance as soluble (deaggregated) antigens or favour enhancement by other mechanisms. Carcinoembryonic antigen may represent such an example,

which is located in the glycocalyx of the colonic cancer cell surface (347) and is also released into the circulation (345). So far only humoral response could be detected to this antigen, and no significant response was obtained in vitro to CEA with lymphocytes from patients with gastrointestinal cancer, pregnant women, or normal individuals (345; 530). As already mentioned, humoral antibodies appear to be inefficient against solid tumors and are frequently involved in enhancement. Therefore, CEA might be chiefly responsible for the escape of gastrointestinal carcinomas by eliciting a tumor enhancing immune response.

Evidence is rapidly accumulating that cells of a variety of tumors might have sialomucin coats. Ehrlich ascites carcinoma and L1210 leukemia cells were shown histochemically and by electron microscopy to be completely coated with a layer of acid mucosubstances; the coat contained sialic acid components (765).

Recently it was shown by several investigators that cells of various tumors treated with Vibrio cholerae neuraminidase become more immunogenic. There are few successful "takes", and tumors that do appear develop slowly and are prone to regress. Early challenge of tumor bearing mice with neuraminidase treated tumor cells either prevents or impairs growth of initial tumor graft. Late challenge of hosts with established tumors results in the arrest of tumor growth and about 30% regression occurs. Neuraminidase treated tumor cells grow like untreated cells when injected

into immunosuppressed animals. The results were specific for neuraminidase and also for the tumor, no crossreaction being obtained with a second antigenically unrelated tumor (255). Although the mechanism of action of neuraminidase on the immunogenicity of tumors is not clear, the available evidence convincingly demonstrates that a sialomucin coat is involved in some way in the protection of tumor cells against host defence mechanisms.

F. Escape by immunosuppression

Glucocorticoids are physiological materials capable of immunosuppression. Certain serum factors such as α -globulin and maternal immunosuppressive factor influence immune reactions similarly (137; 342; 472; 957). Monkeys (*M. mulatta*, *M. speciosa* and *C. aethiops*) treated with progesterone became susceptible to oncogenesis by the Carr-Zibbler strain of Rous sarcoma virus which is oncogenic in chickens. Eight juveniles and two adults were conditioned with daily i.m. injection of 5mg/kg progesterone. All developed fibrosarcomas at the site of virus inoculation in 8-15 days. Virus was demonstrable in all these tumors by back passage to chickens. Similar results were obtained in two females of *M. mulatta* in the third trimester of pregnancy. The tumors regressed after parturition or after excision. Progesterone is known to be a precursor of adrenocorticosteroids, but it is not certain whether it acts as an immunodepressant indirectly through the stimulation of adrenocorticoids, or directly on some phase of antibody formation (636).

It is obvious that antigenic tumors producing any of these physiological immunosuppressive agents in large quantities are at great advantage regarding their escape from immunological surveillance.

G. Escape by growing in immunologically privileged sites

The ultimately fatal course of Burkitt's lymphoma, a tumor in which host defenses are playing a relatively important role, is frequently caused by the involvement of the central nervous system - an immunologically privileged site (169). It follows that antigenic tumors originating from immunologically privileged tissues, such as the central nervous system, eye, testis and possibly a number of endocrine organs, will not have to face rejection, at least in the early stage of development. Later on, the tumor mass could be too voluminous for a successful immunological attack.

Experimental Immunotherapy of
Cancer

Specific destruction of cancer by immunotherapy has been a wishful dream of many investigators and physicians for decades. However, in spite of painstaking efforts in many laboratories and some encouraging results, experimental evidence to date has not provided convincing evidence for the feasibility of such therapy on a long term basis and for killing a large number of malignant cells (385; 825). Therefore, the primary role reserved now for possible immunotherapy is for a final "cleaning" operation consisting of killing off the last malignant cells remaining after

radiotherapy, chemotherapy and/or surgical removal of the tumor. Nevertheless this role is an important one. Radiotherapy and chemotherapy act by single-hit kinetics and the same therapeutic dose, is required for each tenfold decrease of the tumor cell population, irrespect of the size of the population. Obviously, the toxicity and side effects of these agents increases with increasing doses given in order to decrease further the population of neoplastic cells. Hence, with these therapeutic agents the killing of all malignant cells in the body is practically impossible. It has been shown, that animals apparently cured for lifetime by treatment with anticancer drugs relapse if immunosuppressive agents (x-ray, cortisone) were applied during therapy (312; 598). Furthermore, it was observed that when Burkitt lymphoma patients received chemotherapy and the tumor regressed, there was often an increase in serum antibody titer. An increase in antibody titers was particularly apparent in Burkitt lymphoma and nasopharyngeal carcinoma patients who received local radiotherapy (491).

The major significance of a potential immunotherapeutic approach lies, in the protection against recurrence and metastasis, in combination with other forms of therapy. The high specificity of the immune reaction, its ubiquitous action in the organism (except in certain "privileged" sites) and with certain exceptions, its nontoxic nature are all advantageous properties for the fulfilment of this role in cancer therapy.

Immunotherapy of several animal tumors has been clearly demonstrated; long term remission of human leukemias and regression

of cutaneous tumors by immunotherapeutic procedures has also been achieved with results comparable or even better than the therapeutic effect of best drugs available for the treatment of such tumors (576; 577). The biology of human oncogenic viruses is not sufficiently well understood for developing an immunopreventive strategy against certain "familial" cancers of possible viral origin such as leukemias, sarcomas etc., where a break of tolerance to the vertically transmitted virus by immunization should prove effective, as has been shown in animal experiments. Prevention of virus induced tumors in experimental animals was successful by vaccination with the causative virus during the latent period (172; 230; 233; 446), and even immunization of parents protected the offspring in the case of Friend leukemia virus in mice and the herpes virus-induced Marek's disease of chickens (257; 919). Protection against transplantable tumors in syngeneic animals may be achieved with a variety of methods and agents acting through immunological pathways. Some of the ideas which have potential importance in future immunotherapy of cancer will be surveyed in this section.

A. Active immunotherapy

As discussed earlier the host's inability to destroy a progressing antigenic tumor is considered to indicate some inadequacy or failure of the host's defensive immune response. Many strategies for immunization against cancer are based on the assumption that autochthonous tumors are weakly antigenic. Cinader

recommended in 1962 the chemical modification of autochthonous tumor antigens as an approach to immunotherapy of cancer (163). Even with non-modified tumor cells a stronger antigenic stimulus is always intended by some other means, e.g. increasing the antigen dose by nonproliferating x-irradiated or chemically restricted cells, changing the route, or by using adjuvants in addition.

Takeda and coworkers (866) investigated various parameters of immunotherapy with MCA-induced primary tumors of inbred rats. The tumors were classified as of high, medium and low antigenicity. The effect of ligation for 24 hours and release of the primary tumor on artificial metastases established by s.c. transplantation of 10^8 cells from the same tumor to a distant site was investigated. Immunotherapy of naturally metastasising tumors or prolongation of the survival of tumor bearing hosts having two tumors of the same size (20mm diameter) by ligating one of them was also attempted. The following general conclusions were drawn: (a) Low antigenic tumors develop often infiltrative growth with frequent recurrence and metastasis and survival time of the host is short. It is hard to alter the growth characteristics of the tumor by immunization and, therefore, these tumors represent "absolute malignancy". (b) Medium antigenic tumors develop infiltrative growth with recurrence and metastasis, but less frequently. Marked suppression of tumor growth, recurrence and metastasis was noted with strong immunization. (c) Highly antigenic tumors develop only rarely infiltrative growth with recurrence and metastasis. With strong immunization the growth

rate decreases markedly and the survival time is also significantly prolonged. The rationale behind the ligation and release of tumors was to induce necrosis and liberation of tumor antigens and thus expose the host's immune system to a stronger antigenic stimulus. It is to be noted that animals with large tumors were treated, i.e., when the general competence to mount an immune response is impaired, and yet regressions occurred.

The route of inoculation may play an important role in the outcome of immunization against cancer. It was found that mice inoculated with Ehrlich ascites carcinoma both via the s.c. and i.p. routes survived longer than control animals receiving the tumor only i.p. A marked regression of the s.c. transplants could be observed in the mice with two inocula (389). When the transplantability of MCA-induced sarcoma and B16 melanoma tumor cells was tested in syngeneic CBA and C57Bl/6J mice a much higher number of tumor cells was required for progressive tumor growth after i.v. transplantation than after i.p. or s.c. transplantation (97).

Successful treatment of a diethylnitrosamine induced solid tumor (hepatoma) was accomplished in inbred guinea pigs by repeated intradermal injection of living tumor cells. Immunization was started 5, 12 and 19 days after the intra-muscular inoculation of a lethal dose of tumor cells. Immunization with cells of the ascites variant of the same tumor cell line resulted in protection of 27% of the animals, and immunization with cells grown in tissue culture resulted in protection of 50% of animals. Success or failure of immunotherapy in this system depends mainly on the number of

cells in the challenge inoculum. With too high a cell inoculum host-dependent immunotherapy was unsuccessful. Intradermal immunization with ascites tumor cells, subjected to freezing and thawing, or to x-irradiation as well as immunization with similarly treated, spleen cells and with tumor cells antigenically unrelated to the challenge inoculum did not prevent growth of the intramuscular tumor (508; 938). However, intradermal immunization of syngeneic guinea pigs with a MCA-induced liposarcoma effectively prevented the growth of an intramuscular tumor challenge when immunization was initiated at the same time as, or up to 10 days after, the challenge. The most effective immunogens for intradermal immunization were viable cells from grafts of the transplanted tumor, but irradiated tumor cells and tumor cells grown in tissue culture were also effective. No protection against tumor growth was observed if the challenge dose was increased from 10^5 to 10^6 cells (258; 624).

Subcutaneous inoculation of I2C leukemia cell suspension into strain 2 or F₁ hybrid guinea pigs produced subcutaneous leukemic infiltrations at the site of inoculation leading in most of the animals to a generalized leukemia. On the other hand, when strain 2 and F₁ hybrid guinea pigs were inoculated intradermally, about two thirds of the animals developed intradermal tumors and half of these tumors regressed spontaneously. Subcutaneous reinoculation of animals in which the intradermal tumors regressed did not lead to tumors in a large fraction of them and immunity was more pronounced in females, than in males. The acquired immunity could not be

transmitted to other guinea pigs by serum collected from immunized animals (368; 369). Immunization with large doses of irradiated I2C cells intradermally or emulsified in complete Freund's adjuvant and injected into the foot pads protected almost all strain 2 guinea pigs against a subsequent leukemic challenge. However, animals similarly immunized with normal strain 2 lymphocytes or chemically induced syngeneic hepatoma were not protected. Adoptive transfer of lymph node and spleen cells from immunized animals into normal recipients afforded complete protection against leukemic challenge. Serum antibodies to I2C cells in immunized animals could not be detected by a variety of in vitro assays. Intradermal immunization of guinea pigs 2 or 4 days after the injection of a lethal inoculum was able to protect some animals against fatal leukemia (264).

In two separate experiments with C3H/Crgl mice bearing autochthonous MTV-induced mammary tumors in situ, or as heterotopic autografts, it was found that injection of an irradiated or freeze-thawed preparation of biopsy sample of the liver from the tumor bearing host interfered with the growth of the autochthonous tumor. Immunization of normal C3H/Crgl mice with liver preparations from animals bearing isografts of immunogenic mammary tumors was as effective as immunization with living tumor implants in preventing the development of tumor isografts upon subsequent challenge. However, livers from normal mice or from mice bearing isografts of nonimmunogenic mammary tumors were not effective. The immunological character of this phenomenon was

attributed to "activated" tumor antigens supposedly carried by the livers of tumor bearing mice (906).

One of the most successful modification of tumor cells used for immunization is treatment with neuraminidase. Cells from 4 of 5 MCA-induced sarcomas failed to induce tumor in vivo after incubation with *Vibrio cholerae* neuraminidase (VCN). When subsequently challenged with tumor fragments the surviving mice showed substantial anti-tumor immunity. The fifth tumor continued to grow and killed the host despite neuraminidase treatment. Neuraminidase did not kill the cells, it did not cross-react with tumor antigens, nor did it induce crossreaction with syngeneic mouse spleen cell isoantigens. Neuraminidase treated cells from an antigenic tumor grew readily in mice irradiated with 600r (209). The MCA-induced MC-42 fibrosarcoma of C3H/HeJ mice never regressed spontaneously, but VCN treated cells grew more slowly than normal and frequently regressed. The growth rate of VCN treated cells was more rapid in immunosuppressed recipients. Animals inoculated with VCN-treated MC-42 were immune to subsequent challenge with untreated tumor cells. When animals already supporting a 10-30 day growth of MC-42 (0.4-1.0cm) were challenged with an inoculum of living VCN-treated MC-42 cells, half of the original tumors completely regressed, and the growth of the other tumors was slowed down. Treatment with heat inactivated VCN was ineffective. Regression was not influenced by treating the immunizing inoculum by mitomycin-C in addition to VCN. Direct injection of VCN into 10-20 day tumors stop their growth (806; 807). The use of neuraminidase treated tumor vaccines was far more efficient

than the use of *Mycobacterium bovis* in inducing the regression of MCA-induced fibrosarcomas in mice. Multiple injections of living tumor cells treated in vitro with VCN plus mitomycin-C are more effective than a single inoculation (757). Modification of Ll210 tumor cells with VCN resulted in loss of oncogenicity; 10^7 treated cells failed to induce leukemia in DBA/2 mice. However, the immunogenicity of the treated tumor cells was increased, since DBA/2 mice were specifically resistant to a lethal (10^5) virulent Ll210 cells after single immunization. Immunogenicity was directly correlated with release of neuraminidase susceptible N-acetylneuraminic acid. However, prolonged incubation with neuraminidase, beyond 90 minutes for this particular tumor, gradually destroys the immunogenicity of the tumor cells (69). It is clear that the immunogenicity of a variety of tumors is greatly increased after incubation with neuraminidase. Sialic (N-acetyl-neuraminic) acid, linked to amino-sugars by 2-6 ketosidic bonds appears to inhibit the immunogenicity of underlying molecular sites, possibly by steric hindrance and neuraminidase is considered to "unmask" the antigens. VCN-treated cells regenerate over a period of 18 hours at 37°C and, therefore, one may infer that the mechanism responsible for tumor inhibition must occur rapidly in vivo. A considerable number of treated cells (37%) are phagocytosed by macrophages in comparison with untreated cells (6%), which suggests that enzymatic removal of sialic acid affects the primary confrontation of tumor and host. *Vibrio cholerae* neuraminidase is effective in removing sialic acid; however, similar enzymes from *Clostridium perfringens* and influenza virus are not. Trypsin treatment abolishes the antigenicity

of VCN-treated tumor cells (207). Attempts to unmask H-2 antigens in mouse trophoblast and lymphoid cells, however, have been unsuccessful. Weak antigens, such as the θ antigen may be uncovered by VCN. It is possible that VCN increases immunogenicity nonspecifically; reduction of negative charge by removing sialic acid from the membrane may increase deformability or alter the transport of amino acids. Such cells are rendered more susceptible to phagocytosis and complement mediated lysis (255).

The increase of antigenicity by modifying the tumor cell surface with chemicals or by coupling to proteins was also attempted by several investigators. Appfel et al. (31) could induce a substantial immunity against Ehrlich ascites carcinoma in A/J mice, an MCA-induced sarcoma and EL4 tumor in C57Bl mice, and against Krebs-2 tumor in Swiss mice by preimmunization with tumor cells treated with 10^{-3} - 10^{-4} M Na-iodoacetate, a sulfhydryl alkylating agent. Certain other reagents reacting with sulfhydryl groups, such as iodoacetamide, N-ethylmaleimide were also effective, but p-chloromercuribenzoate was not effective (31). In a similar study the weakly antigenic 6C3HED cells were modified with: (a) diazotized p-aminobenzoic acid, (b) fluorodinitrobenzene, (c) iodoacetamide, (d) iodoacetate, (e) N-ethylmaleimide, and (f) p-hydroximercuribenzoate. Cells modified with the strong antigenic determinants (a) and (b) failed to protect C3H mice against 10^6 6C3HED cells; (c), (d) and (e) protected fully; (f) gave partial protection. Alterations producing potent immunity involved blocking of sulfhydryl groups with reagents not generally considered good haptens. EPF-1 lymphoma cells treated with iodoacetate

provided protection against an early transplant generation of EPF-1. However, cytotoxic antibody to 6C3HED or EPF-1 in the serum of immune animals was demonstrable, but lymphoid cells from an immune C3H mouse protected a susceptible animals, indicating cell-mediated immunity (709).

Human gamma globulin was coupled to tumor cell suspensions of C3H mammary carcinomas utilizing bis-diazobenzidine as a coupling agent. The altered cells were then reinjected into the animals from which they were taken with complete Freund's adjuvant. Tumor growth was significantly retarded ($p < 0.05$) in the immunized group, and increased amounts of necrosis, lymphocytic infiltration, hydropic degeneration and fibrosis was observed on histologic section. Moreover, antibodies to tumor cells were detected in the immunized animals by microprecipitin Ouchterlony and immunofluorescent techniques. On the other hand, antibodies were not detectable in sera of control animals immunized with untreated tumor cells in complete Freund's adjuvant (211). Similarly some degree of protective immunity against subsequent challenge with isologous tumor cells was conferred on mice by immunization with tumor cells complexed electrostatically to methylated bovine γ -globulin (BGG) (base-acid interaction) or conjugated covalently to BGG with bis-diazobenzidine and immunization following tumor excision tended to decrease rather than increase resistance to further challenge with viable malignant cells (111).

Immunization of mice could be achieved against Ehrlich ascites tumor using a hybrid of Ehrlich ascites tumor cells and polyoma transformed hamster cells; the hybrid cell is analogous to an attenuated tumor cell (927).

Tissue culture line of 6C3HED ascites tumor cells, after long term propagation in vitro, failed completely to produce tumors when reinoculated into isogeneic C3H mice. However, these nontumorigenic 6C3HED tissue culture cells induced strong immunity in mice against virulent 6C3HED cells freshly drawn from mouse tumors and the sera or spleen cells of these immunized animals neutralized tumor cells in vitro. Sonically disrupted nontumorigenic cells also induced immunity, but to a much lesser degree than the intact viable cells. Inoculation of mice with the nontumorigenic 6C3HED tissue culture line also partially protected them against virulent TA3, Ehrlich, or Ehrlich-Lettre tumor cells when tested in Swiss mice (267).

Treatment of cancer with various viruses may have a beneficial effect. Possible mechanisms of action include direct cytolysis by virus multiplication; immunological cytolysis by both cell-mediated and humoral responses to "budding" viruses; and, the production of interferon by viruses. It should be remembered, however, that a variety of virus infections have a negative effect because of their marked immunodepressive action (928).

Treatment of tumors, in the ascites or solid forms, (e.g. Sarcoma-1, Ehrlich ascites carcinoma) in mice by inoculation with bovine enterovirus-1 resulted in regression of the tumors

without any pathological effect on the animals. Death of mice with lymphatic leukemia was delayed after such treatment. The oncolytic specificity of the virus does not appear to involve the production of interferon, but requires specific adsorption to the tumor cells. The susceptibility to killing was observed also in culture, since viral-transformed cells and oncogenic cells were susceptible to the virus, in contrast to cells of untransformed lines and cells primary cultures, which were resistant (876; 877).

The WSA strain of influenza A₀ virus was adapted to grow in Ehrlich ascites tumors carried in ICR mice. The strain was passaged in suckling BALB/c mice by intracerebral inoculation, then adapted to the SV3T3-T4 and BALB3T3 cell lines. The virus produced marked cytopathic effects after three passages and high hemagglutinin titers after seven passages. BALB/c mice could be made immune to challenge with syngeneic SV40-transformed fibrosarcoma cells by vaccinating them with cell-free homogenates of tumor cells which had been infected with influenza virus. No immunity was induced by vaccinating the mice with homogenates of influenza virus infected normal cells, or with homogenates of tumor cells not infected with influenza virus. It should be noted, however, that about 10% of mice died, and about 50% developed paralysis of the lower extremities because of virus infection. Influenza virus modifies the entire plasma membrane, not just the portion associated with the budding virus, because of the insertion of virus-coded

antigenic proteins (V antigen) into the membrane structure; the V antigen serves as a "helper" during immunization (100). Swiss mice receiving leukemia cells infected with Newcastle disease virus (NDV) plus mouse anti NDV serum succumbed to tumors faster than those receiving either noninfected cells or infected cells only. Newborn mice injected with infected tumor cells lived longer but some of them died because of viral encephalitis. Hence both delayed and accelerated tumor growth ("enhancement"?) could be induced by NDV-infected tumor cells. Vesicular stomatitis virus exerted good oncolytic effect (811).

The action of Friend virus was investigated on 2 spontaneous undifferentiated sarcoma of Wistar/Mk, noninbred Gifu, and Donryu rats; on four nitroquinoline-induced fibrosarcoma of Wistar KA/Mk rats; and on diaminoazobenzene-induced hepatoma of Donryu rats. Any line of rat tumor cells infected with Friend virus either failed to grow in susceptible hosts, or grew and soon regressed. Friend virus specific membrane antigens were detected in infected tumor cells by immunofluorescence and complement cytotoxic tests. After the rejection of Friend virus infected tumor cells rats showed resistance to the growth of noninfected tumors of the same type. In some cases immune effects were recognized even when the tumor cells were injected before immunization (498).

B. Adoptive immunotherapy

Transfer of 1.2×10^8 peritoneal exudate cells intracardially from strain 2 guinea pigs immunized with a diethylnitrosamine-induced

hepatoma to tumor bearing animals on days 5, 12 and 19 protected 40% of the animals which had received a relatively small tumor-inoculum (1.1×10^4 cells/animal i.m.) (938). Immuno-therapy against I2C leukemia in strain 2 guinea pigs was also achieved when initiated early in the course of the disease, i.e. tumor bearing animals injected with 3×10^5 I2C cells intradermally were protected if given immune spleen and lymph node cells ($3.4 - 9 \times 10^6$ per animal) intravenously 2 days (6 out of 8 protected), 4 days (3 out of 8 protected) and 7 days (1 out of 4 protected) later (264).

The effects of sensitized syngeneic, allogeneic, and xenogeneic lymphocytes on small (5mm) transplanted MCA-induced and spontaneous mammary tumors in syngeneic mice and rats was investigated. Sensitized lymphocytes were obtained from blood, lymph, lymph nodes, spleen and thymus. In a number of studies, zymosan was employed alone and with lymphocytes. Despite a variety of therapeutic regimens, these studies failed to demonstrate convincingly that employment of such cells in the model systems utilized could influence tumor growth, when the tumor was applied in the form of an inoculum of cells or of a small growing tumor. Of the many experiments carried out, only the system consisting of sensitized syngeneic circulating lymphocytes in animals harboring MCA-induced tumor cell inocula was effective in this regard. Allogeneic and xenogeneic cells were uniformly ineffectual (292).

Pilch and coworkers demonstrated recently that resistance to chemically induced mouse, rat and guinea pig tumors may be transferred by "immune" RNA prepared from lymph nodes and spleens of syngeneic, allogeneic or xenogeneic animals immunized with the same tumor. Normal syngeneic lymphoid cells treated with this immune RNA in vitro could confer protection on adoptive transfer and similarly this immune RNA protected animals against a subsequent tumor graft on injection into animals in the presence of dextran sulfate, a potent RNase inhibitor. Furthermore lymphoid cells treated with immune RNA exerted target cell lysis in vitro. However, nonspecific RNA was ineffective in all such experiments and the immune RNA preparations were inactivated by treatment with RNase (227; 228; 697; 698; 699; 724; 725; 726; 755).

C. Passive immunotherapy

Although attempts to apply passive immunotherapy for the control of malignant disease have proven disappointing, rather than encouraging, it would be imprudent at this stage of our incomplete knowledge of the host-tumor relationship to categorically dismiss this approach. Thus one may visualize that antibodies coupled to any cytotoxic agent may be highly effective in concentrating the cytopathic factor in the tumor foci. In principle, this method would be promising, if one could link to specific antitumor antibodies products which are highly toxic when administered as conventional drugs (629). Clearly, this system is essentially similar to the strategy devised in the first part of the present study, except that in the author's system the function of antitumor antibodies is

to attract killer cells into the foci of malignant cells, on the assumption that the immune cells would become activated for the release of their cytotoxic agents on interaction with the antibodies coating the tumor.

It was already discussed that viral oncogenesis may be prevented by antibodies, and that an "unblocking" antibody is probably able to exert therapeutic effect in vivo. The increasing level of cytotoxic and other antibodies in malignant melanoma and sarcoma patients showing resistance to their disease, and the decrease of such antibodies in cases of progressing disseminated neoplasia was also presented. It is not completely clear, however, whether in these human malignancies the change in antibody levels is causatively related to resistance or if it is a consequence of the reduction in tumor size.

As a general rule complement dependent cytotoxic antibodies are active against dispersed malignant cells such as leukemia or against malignant cells circulating in the blood. If the density of antigenic determinants on the surface of tumor cells is too low, complement fixation is inefficient or may not take place at all. In the extravascular space, where the cells of solid tumors are located, IgM antibodies which are most efficient in C' fixation, are present only in low concentration or not at all, which may be one of the reasons why solid tumors resist so well attack by antibodies.

Various leukemias of mice could be prevented or even cured by antisera produced in rabbits (418; 537; 735; 736). Takes of Walker 256 rat carcinoma could be prevented by rabbit antiserum in more than half of the rats inoculated with 10^4 cells. Moreover, administration of the globulin antibody fraction led to a marked decrease in size of the tumor, but the tumor masses did not completely disappear (589).

D. Interferon as an anti-tumor agent

Interferons are small proteins able to inhibit viral multiplication in uninfected cells of the species of origin by interfering with the combination of viral messenger RNA with cellular ribosomes to form functional polysomes. Interferons are produced by cells infected by almost any virus, or exposed to interferon inducing compounds, and also produced during cell-mediated immune reactions by sensitized lymphocytes. Mouse interferon preparations enhanced the specific cytotoxic action of sensitized lymphocytes on allogeneic tumor cells indicating that, beyond its well known anti-viral effect, interferon might play an important role in immune reactions as well (please see "interferon" in the section on Basic Immunology).

Interferon produced in mouse L cells by the Newcastle disease virus activated normal allogeneic or syngeneic lymphocytes for target cell destruction ($P < 0.05$). When cells from DMBA-induced primary sarcomas of C57Bl mice were mixed with normal syngeneic

spleen cells and interferon was added prior to injection to C57Bl recipients, a reduction in tumor incidence and delayed growth occurred ($P \leq 0.002$ by χ^2 test). Interferon or syngeneic lymphocytes alone did not affect tumor growth (157).

The lethal dose of Ehrlich ascites tumor could be increased by treatment of inoculated mice with interferon prepared from brain tissue. Intraperitoneal application gave better results than s.c. and no effect was obtained if interferon was given before tumor inoculation. Phagocytosis of tumor cells by macrophages was observed on smears obtained from the peritoneal cavities of mice treated with interferon, but not from untreated or mice treated with normal brain extract. It was proposed that in addition to the direct inhibition of tumor growth, interferon stimulated phagocytosis (360).

Lewis lung tumor inoculated s.c. to C57Bl mice was inhibited by daily i.v. treatment with a crude interferon preparation (256,000 NIH International Units) derived from the brains of mice inoculated with West Nile virus. Treatment was continued for 3 weeks. Only interferon treatment was associated with a significant inhibition of the growth of primary tumor; treatment with control preparation was ineffective. A decreased number of pulmonary metastases was observed in interferon treated mice in comparison with controls. Interferon injected i.v. daily (128,000 NIH International Units) was effective even when the treatment was started 6 days after tumor inoculation, when palpable tumors had already developed in all mice. It was not possible to determine

if the decreased number of pulmonary metastases in this case was the consequence of an inhibition of the primary tumor, or if it represented an independent effect of interferon on the development of metastatic foci (361).

A comparison of the antitumor effect of interferon and interferon inducers, e.g. Newcastle disease virus (NDV), statolon, poly I:C, poly A:U, was made on EAC₃H (Ehrlich ascites) and RHT-I (Rauscher leukemia virus-transformed hamster embryo cells) tumors in vitro and in vivo. In mice NDV, statolon and poly I:C were equally effective as antitumor agents. However, interferon and poly A:U at the dose level used were not effective. In hamsters, the antitumor effects of the inducers were much less pronounced than they were in mice. In vitro the three interferon inducers and interferon itself were highly effective in inhibition of focus formation by murine sarcoma virus. It was concluded that in vivo the antitumor effects of interferon inducers may bear a relationship to the amount of interferon induced, since NDV, statolon and poly I:C are strong inducers in mice. Antitumor effect could, however, also be mediated by enhancing the cell-mediated immune response or by a direct chemotherapeutic effect of inducers (poly I:C) (745).

Prolonged interferon treatment of AKR mice in the form of concentrated mouse brain interferon, started at 3 months of age, significantly increased the survival time of male mice and diminished the incidence of spontaneous lymphoid leukemia. No difference was detectable between treated and untreated female mice. In a second experiment interferon treatment was continued for one year. The

survival time of both male and female mice was prolonged and the incidence of leukemia was reduced in both sexes. A similar interferon treatment also increased the survival time of male and female AKR mice inoculated at birth with extract of leukemic tissue (362).

The oncogenicity of murine sarcoma virus (Harvey) and Friend virus leukemogenesis in mice could also be inhibited by daily interferon treatment (82; 363).

E. Nonspecific stimulation of host resistance to syngeneic tumors

A variety of immunological adjuvants and other materials have been tested for the stimulation of host resistance against tumors. Living, killed or fractionated *Bacillus Calmette-Guerin* (BCG), *Corynebacterium parvum*, *Bordetella pertussis*, endotoxin, digests of DNA and synthetic polynucleotides have been used most frequently. However, occasionally, experiments with nonspecific stimulants, e.g. BCG, a methanol extraction residue of BCG and with *B. pertussis*, have resulted in increased tumor growth rather than in resistance. Immunization with tumor tissue or oncogenic virus in Freund's adjuvant can also lead to enhancement of tumor growth; this may be due to the administration of killed tissue rather than to the use of adjuvant. Most of the materials tested for the stimulation of host resistance against cancer have been shown, however, to elevate levels of antibody and antibody-producing cells when given some time before or simultaneously with antigen (958).

The effect of living BCG, intact phenol killed organisms and acetone extracted BCG, several methanolic extracts and their residues obtained from the phenolized and acetone-treated bacilli were tested on a variety of mouse tumors, e.g. syngeneic uterine sarcoma of BALB/c mice, two mammary carcinomas, a hepatoma and an osteosarcoma of C3H mice. Animals given single i.p. injections of the various materials in saline were challenged with s.c. trocar injections of approximately 0.5mm^3 tumor tissue. Prophylactic effects of several types and of varying degree were exhibited by all the preparations tested, at certain dose levels. Among the protective effects one may cite: initial retardation of the tumor followed by a normal growth, total prevention of development, regression and disappearance, an inhibition of metastatic spread, and normally a prolongation of life. The dose of the materials was critical, the optimum quantities varying somewhat from one preparation to the next and with the tumor. The protection elicited by some of the preparations was as great, or greater, three months after treatment as it was when only 2-4 weeks elapsed between vaccination and transplantation (934).

Three mouse tumor lines, i.e. two MCA-induced and one spontaneous, normally growing progressively when grafted intradermally into syngeneic C3H mice, failed to grow when injected as a mixture with BCG. Experiments to investigate the process involved in this tumor suppression at the site of BCG injection led to the following conclusions: (a) the effect of BCG is local, not systemic; (b) lack

of tumor growth cannot be accounted for the death of tumor cells in the BCG mixture before injection; (c) evidence was obtained that tumor growth is slightly retarded in animals unable to respond immunologically to either BCG or tumor antigens; however, this apparently nonimmunological process is not sufficient to account for the complete suppression of tumor growth observed; (d) host immune reactivity is necessary to BCG to prevent growth of viable tumor cells injected with BCG; (e) tumor growth at the site of BCG injection may be suppressed in the absence of tumor-specific immunity; (f) established, growing tumors may regress if infiltrated with BCG; (g) combination of tumor infiltration with BCG and of subsequent surgery is more likely to be curative than either modality alone; (h) with the tumor most thoroughly studied, injection of a mixture of tumor and BCG does not induce tumor immunity, as does temporary growth of the tumor alone (62).

Administration of BCG i.v. had a very weak effect, if any, on the subsequent growth of Ehrlich ascites cells in the peritoneal cavity of mice. However, the growth of the tumor cells was strongly suppressed at the same site after a previous i.p. infection with BCG. Trehalose-6,6-dimycolate (cord factor) extracted from tubercle bacilli or living BCG suppressed the induction of lung adenomas in mice by urethan (70).

Treatment with BCG increased the resistance of mice to oncogenesis by adenovirus type 12 (815) and murine leukemia virus (Friend) (515).

In strain 2 guinea pigs complete inhibition of syngeneic diethylnitrosamine induced hepatoma was observed when infection with BCG was induced at the site of tumor inoculation. This inhibition was not attributable to a direct cytotoxic effect of living BCG on tumor cells, but it was mediated by the host during a delayed hypersensitivity-type immunologic response to the infecting organism. Animals that rejected tumor cells at the site of an infection with BCG showed suppressed tumor growth and delayed hypersensitivity reactions to a subsequent challenge of tumor cells. The minimum number of living BCG capable of eliciting a host response that would completely kill tumor cells was 6×10^6 organism in immunized and 6×10^5 organisms in unimmunized animals. Optimal suppression of tumor required living BCG, close contact between tumor and BCG, and a cell-mediated immune response by the host. This was the only method of inducing specific immunity to line-10 hepatoma in strain 2 guinea pigs (963; 964). Further experiments with the same tumor-host system revealed that the growth of intradermal tumor transplants was suppressed if the tumor cells were inoculated together with BCG cell walls attached to oil droplets. Tumor growth was not inhibited if the tumor cell were given together with BCG cell walls, oil droplets or oil droplets plus BCG walls prepared so as to prevent attachment of the walls to oil droplets. Animals in which tumor growth was suppressed acquired systemic tumor immunity (965).

Mammary tumors were induced by the oral administration of DMBA in Sprague-Dawley female rats. When one mammary tumor was

present the rats were paired according to the surface area of this primary tumor. One member of each pair received a single dose of BCG s.c., containing 1mg of living suspension in 0.5ml culture medium. Controls received culture medium only. The animals were observed weekly for a total of eight weeks. The total tumor surface per rat, the number of rats developing new tumors and the number of tumors per animal were significantly higher in the BCG treated group. The delay between the administration of BCG and the appearance of a second palpable tumor was very significantly shortened in the BCG treated group. It should be stressed that BCG can enhance tumor growth in certain conditions and should, therefore, not to be used indiscriminately in the treatment of cancer and related conditions (696).

The effect of polyadenylic-polyuridylic acid (poly A:U), a known nontoxic stimulator of antibody formation, on chemically induced and viral mouse tumors were less recurrence after excision or a reduced rate of tumor growth when given at the time of implantation. The effects of poly A:U, a poor interferon inducer, were as good as those with a good interferon inducer, namely toxic and pyrogenic polyinosinic-polycytidylic (poly I:C) acid. Polynucleotides stimulate classical immune responses against tumors rather than act through interferon induction (111).

Studies on Moloney virus oncogenesis in mice revealed that there is a critical dilution of virus against which poly I:C

is effective. Marked differences in response to the anti-tumor effect of poly I:C were observed in different strains of mice. In 4 week old BALB/cCR and N:NIH(SW) animals there was a significant decrease in the incidence of tumors after treatment. Four week old C3H/HeN and NZB/HeN and NZB/BLN mice, however, were not protected by the compound. There was no difference in serum interferon level at 0, 3, 6, 8, 12, 18 and 24 hours after a single s.c. injection of 200 µg poly I:C between NZB/BLN and N:NIH(SW) strains, and in C3H/HeN mice it was higher than in BALB/c. Interferon failed to protect BALB/cCR mice against Moloney virus induced tumors. Pretreatment with a single dose of poly I:C, or simultaneous administration with the virus resulted in enhancement of tumor induction in AL/N mice at all virus dilutions tested. The data illustrate that the relationship between host, drug, and virus is dependent on multiple factors and is more complex than previously envisaged. Strain of mouse, dose and time of administration, number of injections, site of injections, dose of virus all influence the result. Adjuvant effect of poly I:C on immunological reactions and direct cytotoxicity on tumor cells by inhibiting RNA and protein synthesis could be involved in the mechanism of action. Interferon production by poly I:C is not related to its suppression of tumors induced by murine sarcoma virus Moloney in mice (319; 933).

It was repeatedly observed by several investigators that allogeneic or xenogeneic "immune" lymphoid cells exhibited an

anti-cancer effect when given to tumor bearing hosts (9; 11; 37; 456; 613; 895). The mechanism of this well demonstrable effect, however, remained vague. Alexander (9) suggested that RNA could be the acting agent. It was recently demonstrated that the passive transfer of immunocompetent lymphoid cells from allogeneic strain 13 guinea pigs to normal adult strain 2 recipients which were subsequently challenged with a lethal inoculum of I2C leukemia significantly altered the development of leukemia. Prolongation of survival even occurred at a dose 30-fold higher than the lethal dose. The temporal relationship between cell transfer and leukemic challenge was shown to be crucial, and the antileukemic effect was observed even at a time when all donor cells had presumably been rejected. Allogeneic cells given 1-13 days before leukemia challenge were effective. Fifty per cent of allogeneic cell recipients subjected to a second leukemic challenge were able to reject this inoculum. Transfer of parental lymphoid cells from strain 2 or strain 13 guinea pigs to (2x13) F_1 recipients were equally effective in suppression of I2C leukemia. This indicates that the rejection of allogeneic lymphoid cells by a host response is not required. The possibility that protection results from direct rejection, by donor cells, of I2C leukemia on the basis of an immune response directed against strain 2 histocompatibility antigens present on I2C cells was also excluded, since strain 2 parental cells were equivalent to strain 13 cells in affording protection to F_1 recipients. It was suggested that immunocompetent

lymphoid cells of the host may exert the primary effector mechanism in this phenomenon although a role of cells of the reticuloendothelial system (RES) has not been excluded. A model for immunotherapy of guinea pigs with already existent leukemia utilizing repeated allogeneic cell transfers was shown to be effective in significantly prolonging the survival of such recipients ($P < 0.001$). All of the treated guinea pigs eventually died of leukemia (265; 474).

F. Combination therapy

Several studies illustrate that the number of cures in tumor bearing animals may greatly be improved if various forms of immunotherapy are combined with one another and/or with conventional therapy by drugs, surgery or x-irradiation.

Spontaneous mammary tumors of mice treated by surgery, chemotherapy (streptonigrin, thioguanine, endoxan, cyclophosphamide, mitomycin-C) and zymosan (a RES stimulating agent) were cured in 54% of cases, but absolute cure resulted in only 24% of the cases if zymosan was not given (569). In C3H/H1 mice bearing spontaneous mammary adenocarcinoma, treatment with double-stranded poly A:U was a potent adjunct to surgery. Similarly, in hamsters bearing a highly metastasising transplantable melanoma (T1196), poly A:U reduced the appearance of early metastases by 50% as compared with untreated animals or animals treated by surgery alone (512).

DBA2 mice injected with 10^3 L5178Y lymphoma cells i.p. (lethal dose is 10 cells) could be cured up to 80% if irradiated

tumor cells were injected i.p. 24 hours after tumor challenge and BCG was given separately at different time (678).

Studies on combination therapy was carried out with two MCA-induced antigenically different fibrosarcomas (MC-42, MC-43) of C3H/HeJ mice. A single injection of BCG, or MC-42 cells exposed to heat inactivated VCN plus mitomycin-C, or any of these treatments did not produce total regression of firmly established MC-42 tumors (0.5-10cm). A single injection of active VCN treated MC-42 cells induced the regression of 2 out of 7 tumors. If BCG was injected directly into the tumor mass and VCN-treated tumor vaccine was injected elsewhere, only 1 of 14 regressed. If BCG was injected at a separate location from the injection of VCN tumor vaccine, or mixed, 15 of 42 tumors totally regressed (806).

When Ehrlich ascites carcinoma (EAC) cells were exposed to 200 rads and injected to BALB/c mice there was no increase in the survival time (control 17.8 ± 0.5 , exposed 16.0 ± 1.8). If, in addition, the tumor cells were exposed to heterologous rabbit antibodies the survival time increased to 36 ± 9.4 days. Without previous irradiation anti-EAC antibody did not change the survival time. This protection was increased by increasing the radiation dose and was completed at 600 and 800 rads (332).

A variety of rat tumors could be inhibited or cured by vaccination with Friend virus infected tumor cells. This immunotherapeutic effect could be strengthened if mitomycin-C (1.5 mg/kg)

was also given to the immunized animals (498).

The response of primary benzpyrene induced fibrosarcomas of rats to local irradiation (2000r) was greatly improved in 50% of the animals by implanting autochthonous biopsy material exposed to 10,000 r in vitro. Irradiated autografts without local x-ray treatment had no effect on the growth of these tumors (374).

G. Immunotherapy of human tumors

It may be stated that practically all the basic ideas for cancer immunotherapy tested in animal systems have also been tried on cancer patients. Southam (825) concludes that although in a few patients objective documentation of tumor regression was reported, rarely if ever was it possible to conclude that the responses were due primarily to immunologic mechanisms. This statement is also valid for most of the recent clinical experiments done on cancer patients (385).

A complete remission of choriocarcinoma was observed after active and passive sensitization of the patient against her spouse's tissue (162).

Fourteen volunteers with far-advanced malignant tumors were treated with autologous tumor coupled to rabbit gamma-globulin with bis-diazotized benzidine. Of the 14 treated 2 recovered and had been tumor-free for about 4 years. Three patients have shown stabilization or slow progression of tumor; nine have died. Antibodies in high titers directed toward the tumors, were demonstrated by various means in thirteen patients (212). This study was repeated

by others on 42 patients with advanced cancer. Only one of the cases provided objective evidence for tumor regression. A partial immunologic survey showed no evidence of delayed hypersensitivity and minimal evidence of humoral antibody to tumor extracts. There was no evidence of tumor growth enhancement (206). An additional study on cervical carcinoma in situ with identical method of immunization was performed on seven volunteers. All patients exhibited histologically regressive changes during treatment. In two cases there was gradual but total regression a year after therapy. In a third case, regression was marked from carcinoma in situ to minimal dysplasia. Two were treated and cured surgically and final tissue findings confirmed moderate regression to a stage of residual dysplasia. Two others were still being observed at the time of publication of the original article. During immunization polymorphs attacked cancer cells, they penetrated intracellularly and caused degeneration (39).

An experimental and clinical study of the effect of alterations of tumor cell surface topography on surface charge, cell viability and immunogenicity was performed. It was shown that a large number of positively and negatively charged molecules, ranging from a molecular weight of 500 to 1,000,000 could be attached chemically to tumor cell surfaces without damaging their capacity to multiply and form tumors. Tumor cells thus conjugated, with even the most immunogenic surface antigens, did not increase protective immunity to cancer cells but in a number of instances tumor enhancement was observed (35).

Twenty patients with advanced cancer have been treated by a vigorous course of immunotherapy with tumor extract. No striking clinical response were observed (436). However, long remissions occurred in some patients with carcinoma in which electrocoagulation of the tumor was the treatment of choice (848). Cryotherapy of primary and metastatic tumors of breast, skin, colon, prostate and pharynx has been reported to result in elevated antibody levels directed toward subcellular components of the destroyed tumor tissues (623).

Daily infusions of brinase (protease-1 of *Aspergillus oryzae*) for 10 to 30 days in six patients (3 children and 3 adults) with acute leukemia resulted in the production of complement-dependent autocytotoxicity against leukemic cells and lymphocytes and, in some instances, against platelets. Remission was obtained in three of five patients who were given combination therapy and in the one patient with acute myeloblastic leukemia who was treated with brinase alone (883; 884).

Cross immunization with non-viable tumor suspensions was carried out between 14 pairs of patients with the same tumor type and same major blood group. Seventeen patients underwent exchange of plasma white blood cells without complications. Three patients with disseminated malignant melanoma were alive and well 10, 7 and 5 month after treatment. Two patients with disseminated carcinoma of the colon responded to treatment, one gained weight, returned to full time employment for 3 months and then died, the other

was alive without evidence of tumor 8 months after treatment (437). Twenty one patients with acute leukemia, resistant to the available forms of chemotherapy or unable to be treated by drugs mainly due to a significant neutropenia, were given transfusions of leukocytes from donors with chronic myeloid leukemia. There were 9 remissions, 6 complete with normal blood and bone marrow, and 3 incomplete; however, the remissions were of a short duration (801). The use of pig mesenteric lymph node cells, from animals immunized by implantation of multiple fragments of the appropriate tumor in the mesentery, for the treatment of five patients with advanced neoplasia was attempted; regression was observed in a case of hepatic metastases of colon carcinoma with 150 day survival (854).

In two patients with metastatic melanoma, reciprocal tumor grafts were followed by injections of buffy-coat extracts (transfer factor) from the recipient to the donor in each case; tumor regression was observed in one case (108).

Large amounts of either autochthonous or allogeneic cultured lymphocytes were infused i.v. into 31 patients with advanced malignancy. Of three patients infused with over 300 grams of autochthonous lymphocytes, two had significant remissions without exhibiting any severe side effects (622).

Of four patients from one family, all with clear-cell carcinoma of the kidney, two patients (nos. 2 and 3) have been symptom-free since nephrectomy. Patient no. 3 was found to have symptomatic pulmonary metastases approximately a year after nephrectomy and was given plasma transfusions for over 15 months from patient no. 2, who has been plasmapheresed first weekly

and then biweekly. This procedure proved successful providing sustained remission in the recipient no.3 and no untoward effect on the donor patient no.2 (427).

One of the most convincing clinical experiments was done by Mathe for the immunotherapy of acute lymphoblastic leukemia (574; 576; 577). In the first step the number of leukemic cells were reduced by chemotherapy as much as possible, from approximately 10^{12} to 10^8 cells. Then a further reduction was attempted by sequential administration of all available active drugs (cyclophosphamide, cytosine arabinose, dipropylxyethyl-imino-benzoquinone, 6-mercaptopurine, methotrexate, methyl-glyoxal-bis, prednisolone, rubidomycine, vinblastin, vincristine, vinleurosine). Some patients also underwent meningeal radiotherapy, which was subsequently abandoned erroneously, and disappointing results occurred mainly because of several meningeal relapses. Splenectomy was carried out in patients with splenomegaly, and any enlarged lymph nodes were treated by radiotherapy. The patients undergoing immunotherapy were 3-50 years old and have been highly selected, that is, they all received prolonged chemotherapy with little effect. Groups with the following treatment have been established: (i) chemotherapy only (10 patients); (ii) BCG on every 4th day, then every 8th day applied by scarification; scarification consisted of application on each occasion of 2 ml BCG with 75 mg living bacteria per ml on twenty skin scratches of 5cm each (10 patients); (iii) immunized with pooled leukemic cells from allogeneic donors s.c. or intradermally. The cells were stored in dimethylsulfoxide at

-70°C until used. The first six injections (4×10^7 leukemic cells each) were pretreated with 4% formalin in order to inactivate any possible leukemogenic virus; later cells irradiated by 4,000 rads in vitro were injected (5 patients); (iv) this group received a combination therapy consisting of the combined therapies given to (ii) and (iii) (7 patients). The 10 control patients of the first group all relapsed quickly with an average time of 66 days. Out of the 22 patients treated by immunotherapy, 9 had relapsed by the 130th day ($\chi^2 = 6.18$, $P = 0.02$). Later, three other patients relapsed. Most relapses occurred early: 5 before day 30, 9 before day 100 (including the 5); four of these were late in onset at day 210, 324 and 950. Seven patients were still in remission more than one year after chemotherapy was terminated, a total of six were well for more than two years; two of them for more than three years, and one for more than four years. There were no significant differences among the groups given BCG, leukemic cells alone, or the association of these two forms of immunotherapy. Meningeal radiotherapy is important and more intensive chemotherapy is needed. The complementary application of an antiviral agent (amantadine) is being used in one group, and intermittent chemotherapy (vincristine) in another. No difference has yet appeared. A similar program was carried out by the Medical Research Council of Great Britain, in which BCG was applied to leukemic patients without the intensive chemotherapy and radiotherapy of the nervous system. The duration of remissions was not greater than in the controls (576).

As referred to in the Foreword, immunotherapy of primary and secondary cutaneous tumors in patients has been achieved by inducing local delayed hypersensitivity reactions with a variety of agents. Klein (486) observed first that a chemotherapeutic agent, trenimon (2,3,5-ethylene-imino-(1,4)-benzoquinone), which is an inducer of allergic contact dermatitis, when applied topically to tumors, was followed by complete regression of all epitheliomas, a total of 110 in one patient. Later DNCB, a skin sensitizing agent without direct chemotherapeutic action was found to be equally effective in the treatment of cutaneous and mucosal tumors. A synergistic effect between immunotherapy and chemotherapy with 5-fluorouracil was also noted. Deeper lesions responded less favourably; but became smaller, and regressed to a second course of immunotherapy or combined treatment. Lesions which had recurred following surgery or x-irradiation gave unsatisfactory results, and after 2-5 successive courses of immunotherapy less than 60% regressed. More importantly, sensitivity to the compound used to elicit the immune reaction could be transferred to non-reactive patients with white blood cells (486). In a further study local chemotherapy with 5-fluorouracil and immunotherapy were compared. Resolution of neoplasms of 700 patients with chemotherapy occurred in more than 95% and lasted for observation periods of up to 6 years. Local side effects were minor and no systemic toxicity was observed. More than 50 patients have been studied by this immunotherapeutic approach and the results were essentially similar to those obtained by topical chemotherapy. Steroid administration did not interfere with resolution of the neoplasms, although the inflammatory component was suppressed (941).

Other investigators using DNCB for the induction of delayed hypersensitivity reactions over skin tumors noted that not only primary skin cancers but also carcinomas and lymphomas metastatic to the skin regressed. Twenty of 21 patients could be sensitized to DNCB and, in 13 patients, regression of treated nodules compared to untreated nodules was noted. Even when the control nodule was 1 to 2cm from the treated nodule there was no effect visible in the control nodule (844). Furthermore regression of melanomas were observed in 5 of 8 cases after injection with BCG, noninjected nodules regressed in 2, and one patient recovered completely. Two patients with osteosarcoma, one with fibrosarcoma and one with rhabdomyosarcoma, all with unresectable multiple bilateral pulmonary metastatic lesions were injected with BCG into the metastatic nodules and with irradiated tumor cells mixed with BCG intradermally. A temporary decrease in growth rate of pulmonary metastatic lesions was observed in 2 patients and rising antibody titers were detected in all patients, but all of them died subsequently (624). A skin lesion that recurred after total excision, was resistant to irradiation and resembling either mycosis fungoides or a plasmocytoma on histologic examination, was induced to regress by a local delayed hypersensitivity reaction. Nontoxic extracts of the hemolytic streptococcus, streptokinase and streptodornase were used for sensitization (383).

Of 19 patients with proved metastases from malignant melanoma treated by inoculation of smallpox vaccine, intradermal deposits disappeared completely in six of ten cases. Five of these remained well 2 to 22 months after initial treatment. The

response was limited strictly to the site of inoculation (441).

Immunological surveillance of cancer

Thomas (881) suggested during a discussion at a conference that "it is a universal requirement of multicellular organisms to preserve uniformity of cell type... the phenomenon of homograft rejection will turn out to represent a primary mechanism for natural defence against neoplasia". Over the years Burnet (127; 128; 129; 130; 131) developed further this idea into a theory known as immunological surveillance of cancer, according to which mechanisms ought to exist in large, long lived animals for eliminating or inactivating potentially dangerous mutant cells. These aberrant cells are visualized as carrying new antigenic determinants, which are recognized by the thymus dependent immunocytes and eliminated by processes identically to those responsible for homograft rejection. Moreover, he suggested that a major reason for the development of the adaptive immunity system in vertebrates is their susceptibility to neoplasia.

Abundant evidence cited by Burnet, and discussed also in this survey, from both experimental and clinical studies supports the existence of tumor antigens and an effective host defence mechanism of immunological nature against such neoplastic cells. It is worth noting, however, that lower animals also have neoplasms and ways of controlling cancer, some of which resemble immune reactions (225). In addition, non-immunological control mechanisms, recently acknowledged also by Burnet (131), do exist in higher animals.

Allogenic inhibition may represent an important defence mechanism against neoplasia (489). Lymphomas, sarcomas and carcinomas originating in homozygous hosts grow better when transplanted into syngeneic mice than in F_1 hybrids of the syngeneic strain and an allogeneic strain. This growth inhibition in F_1 hybrids has been termed allogeneic inhibition (398). The phenomenon was subsequently observed by a number of other investigators in various tumor systems (113; 293; 764), and it would appear that allogeneic inhibition could efficiently inhibit or even destroy small clones of antigenic tumor cells.

It is well known that surface interactions between stromal connective tissue and parenchymal cells are essential for the differentiation and the normal function of cells in higher organisms (871). A breakdown of such control mechanism is evidenced in neoplasia by the absence of "contact inhibition" in vitro (846) and might be also witnessed in vivo (279; 869; 870). Denervation of the ear of rabbits was found to increase its sensitivity to skin carcinogenesis by DMBA and MCA as compared to that of the innervated ear (680), suggesting the possibility of a neural control. The susceptibility of mammary carcinoma and some other cancers to hormonal control is also well known (366; 435; 476; 936; 937). Other factors such as interferon or chalcones (122; 156; 777) were also shown to play a role in the control of neoplasia.

Finally various mechanisms of DNA repair exist, and a human disease, xeroderma pigmentosum, in which neoplasia frequently occurs, was shown to be caused by a defect of this mechanism (168; 262; 535).

Prehn and Lappe proposed recently that in-certain situations a weak immune reaction actually would facilitate neoplastic growth, which is conceivable, but more evidence is needed for the clarification of this point (712; 713).

It may be concluded, therefore, that immunological surveillance is not the only control mechanism of neoplasia in higher animals and man, but probably the ultimate one. Other mechanisms involving cell contact (allogeneic inhibition) hormones and several of the intracellular mechanisms such as DNA repair and interferon production seem to play also an important role. Nevertheless convincing evidence proves that the immune system serves as an effective control mechanism of neoplastic disease.

Chapter III

EXPERIMENTALIn vivo destruction of tumor cells by an indirect cell-mediated immune mechanism

As outlined in the first chapter this experimental model has been devised with a view to developing an immunotherapeutic procedure for the destruction of antigenic tumors. For this purpose, the first step of the system involves the establishment of artificial neo-antigens of tumor cells which consist of tumor specific antibodies capable of coating these cells with the additional condition that these antibodies should be recognizable as foreign by the tumor-bearing hosts or by animals syngeneic with such hosts. The second step involves the destruction of coated tumor cells by sensitized lymphoid cells, that are capable of initiating a cell-mediated immune reaction on contact with the foreign antigenic determinants of the tumor specific antibodies. As illustrated in Fig. 1, the antitumor antibodies in this system serve (i) as "homing" devices for concentrating the sensitized cells into the foci of malignant cells and (ii) as factors capable of activating cytotoxic potential of the sensitized cells through the release of cytopathic mediators within the tumor foci. Hence the destruction of tumor cells in this system is considered to occur through an indirect cell-mediated immune mechanism.

To test the validity of this working hypothesis, a polyoma virus (DNA oncogeneic virus belonging to Papova viruses) induced

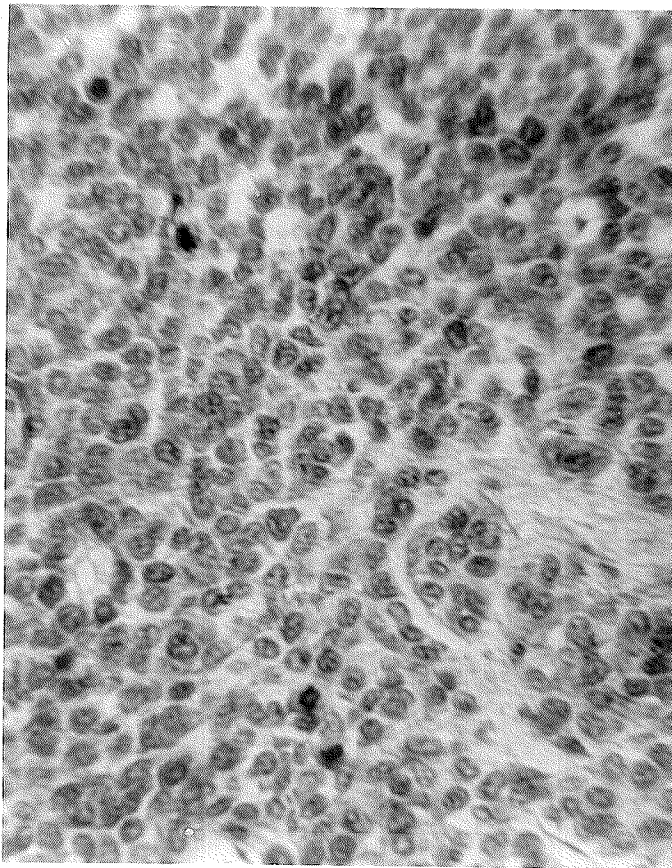
tumor of inbred C57Bl/6J mice was chosen as a model system (Fig.6). Polyoma virus-induced tumors were described very early as being antigenic. Both intracellular (T antigen) (989) and surface antigens are present on the polyoma cells. The surface antigens are non-virion antigens, but are virus dependent and, therefore, the antigens of all polyoma virus transformed cells are crossreacting regardless of the species of origin. As previously discussed, in addition to the crossreacting antigens, virus-independent surface antigens may also be present on tumor cells transformed by oncogenic viruses, which may be manifested as non-crossreacting, individually specific antigens (372). A relationship between polyoma virus induced transplantation antigen and normal embryonic antigen in hamsters was also suggested (681). Similarly, as in the case of other oncogenic DNA viruses of the Papova and Adenovirus classes, polyoma virus is not produced by malignantly transformed cells as a rule, which is an advantage for immunological work, because the released antigenic virus particles do not complicate the system. An additional reason for choosing this tumor was that syngeneic C57Bl/6J mice are readily available commercially (from Jackson Laboratories) and hence, in principle, such studies would not be limited by the number of animals available for experimentation.

A. Materials and Methods

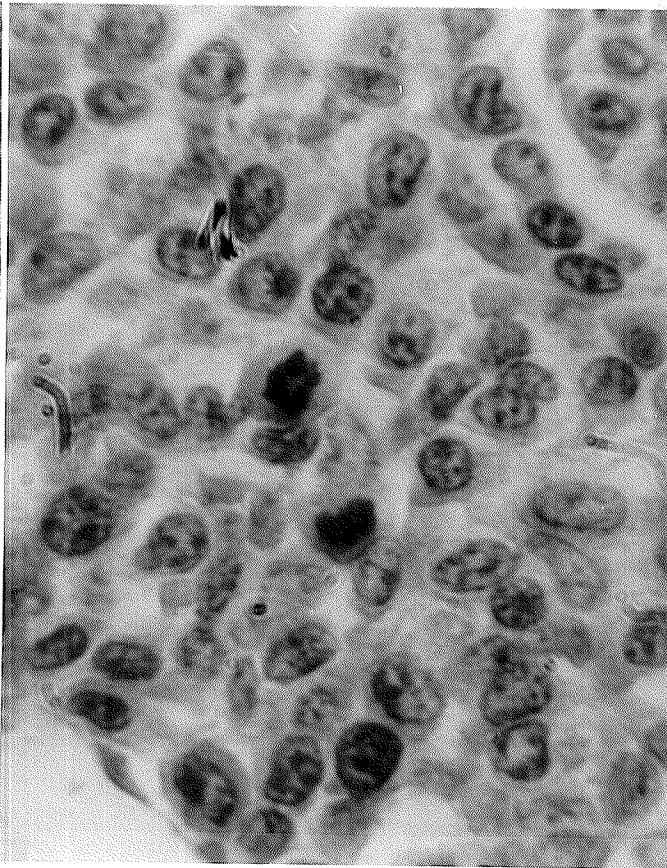
Animals: Young adult C57Bl/6J (C57) mice of both sexes, purchased from the Jackson Laboratories (Bar Harbor, Maine, USA) were used throughout the experiments. In experiments where transfer

Figure 6. HISTOLOGICAL PICTURE OF POLYOMA VIRUS INDUCED TUMOR OF C57 MICE
(Hematoxylin and eosin; approximate magnification on left: 100x,
right: 400x)

Left



Right



of lymphoid cells was necessary, the donors and recipients always belonged to the same sex. New Zealand white rabbits were obtained from local breeders.

Tumors: A tumor (Po) induced by polyoma virus in inbred C57 mice in Dr. K. Habel's laboratory (Fig. 6) and the B16 melanoma of C57 mice originating from Jackson Laboratories were used. Both tumors are readily transplantable in C57 mice and cause fatal neoplasia. Spontaneous regressions never occurred during the three year period of experimentation.

Tissue culture materials: All tissue culture materials, e.g. Eagle's minimal essential medium (MEM); MEM spinner modified; Eagle's medium L; medium HeLa; medium NCTC 109; medium 199 (ML99), medium 1066; Hanks balanced salt solution (H); Earles balanced salt solution; non essential amino acids for MEM; glutamine; penicillin and streptomycin; trypsin; and lactalbumin hydrolyzate were purchased in dry powder form from Difco Laboratories (Detroit, Michigan, USA). Tissue culture media were made with triple distilled water according to the directions supplied by the manufacturer.

Fetal calf serum: Fetal calf serum (FCS) was supplied by the Grand Island Biological Company (Grand Island, New York, USA)

Sheep red blood cells (SRBC) were obtained in Alsever's solution from National Biological Laboratory, Winnipeg.

Complete Freund's Adjuvant (CFA): CFA with 1 mg/ml *Mycobacterium butyricum* was used throughout the experiments and obtained from Difco Laboratories.

Salts for buffer and other solutions were obtained from Fisher Scientific Company, (Fair Lawn, New Jersey, USA.)

Antisera: Rabbit anti-sheep red blood cell (SRBC) sera were produced in 3 animals. SRBC were washed three times in phosphate buffered saline, pH 7.2 (PBS), and a 5 ml suspension containing 10^9 SRBC/ml was injected into the rabbits three times weekly i.v. for three weeks. The animals were bled 7 days later. The collected sera had hemagglutinating titers ranging from 1024 to 4096.

Goat anti-rabbit Ig sera were obtained from Microbiological Associates (Bethesda, Maryland, USA).

Sheep anti-rabbit IgM was kindly supplied by Dr. S. Fujimoto of this Department.

Sterilization: All materials and equipment used for handling tissues and cells were sterilized. Tissue culture media and solutions were filtered by pressure through 0.22 μ Millipore filters. Glassware and surgical instruments were autoclaved at 121°C for 15-20 minutes. Some flasks, dishes and tubes made of tissue culture quality plastic material were purchased in a sterile form, ready for use (Falcon Plastics, Division of Becton-Dickinson & Co., Los Angeles, California, USA). Alcoholic iodine solution containing approximately 1% iodine was used for disinfection of the shaved skin of animals before surgery for the removal of tissues and organs.

Preparation of tumor cell suspensions: Subcutaneous or intraperitoneal tumors were aseptically removed from C57 mice and

placed into a plastic Petri-dish containing a balanced salt solution (Hanks or Earle solution) or tissue culture medium. Non-necrotic tissue was then selected and minced into fine pieces of 1-2 mm diameters by the opposite movement of two sharp scalpels. The minced tissue was transferred into an Erlenmeyer-flask, which contained 30-50 ml of spinner-modified Eagle's Minimal Essential medium (SMEM), which is free of calcium ions. A gentle stirring by a magnetic mixer with a speed of about one revolution per second for 15 minutes followed as a washing procedure. The tissue was then allowed to settle for a few seconds, the supernatant discarded with the aid of a Pasteur pipette and replaced with SMEM containing 0.25% trypsin. Trypsinization was carried out repeatedly for 30 minute periods stirred gently at room temperature until the required quantity of tumor cells was collected. An equal amount of Eagle's Minimal Essential medium (MEM) containing 20% FCS (which protects the cells from trypsin injury) was added to the collected supernatants and the cells were sedimented in capped tubes by centrifugation (400 G, 10 minutes, 4°C). After centrifugation the supernatants were discarded and the cells resuspended in freshly made CMEM. All procedures were carried out under sterile conditions. Suspensions of kidney and testis cells have been prepared in an identical manner.

Preparation of lymphoid cells suspensions: The aseptically removed organs (lymph nodes, spleen, thymus) were placed into a

plastic Petri-dish containing medium 199 (ML99), and teased with needles or by two pieces of stainless steel mesh fixed in hemostats. In the latter case a couple of horizontal cross wires were removed from the meshes providing a multiple needle effect. The procedure was carried out gently, involving mainly the opening of capsule and stromal elements of the organs and letting the lymphoid cells float out into the medium. After removing the cell debris by passage of cells through a fine stainless steel mesh, centrifugation followed (400 G, 10 min. 4°C) and the cells were resuspended in the appropriate tissue culture medium required. The lymphoid organs as well as the resuspended cells were kept in an ice bath or refrigerator (4°C) for a few hours at the most until used in further steps of the experiment; all procedures involving cells were carried out as quickly as possible.

Determination of cell count and viability: An equal volume of PBS containing 0.2% trypan blue (Fisher) was added to small amounts (0.1-0.2 ml) of cell suspensions. After 3 minutes of incubation the cells were counted in a hemocytometer and the viability determined. Cells excluding the dye (unstained) were considered as viable. Cells prepared by trypsinization had over 95% viability. Lymphoid cells varied regarding viability between 70 and 90%.

Cell cultures: In preliminary experiments it was essential to establish the appropriate conditions for the culture of Po cells which were needed for the serological method to be used. The following tissue culture media were tested for supporting the growth

of Po cells: MEM, Eagle's medium L, medium HeLa, medium NCTC 109, M199, medium 1066. Best results were obtained with MEM in Earle's base, that has a high buffering capacity, which is essential for the culture of the highly metabolising tumor cells. MEM was supplemented with non-essential amino acids and glutamine, 100 unit/ml penicillin, 100 mcg/ml streptomycin and 15-20% heat inactivated (56°C, 30 min.) FCS (CMEM). An initial density of $5 \times 10^5 - 10^6$ Po cells/ml was required for satisfactory growth. The culture was performed at 37°C in a water saturated atmosphere containing 5% CO₂.

Hemagglutination: A micromethod described by Takatsy (858) was adapted using a "Microtiter" kit obtained from Microbiological Associates. The SRBC were washed three times in PBS, then resuspended to a concentration of 0.5% (packed cells/volume) in PBS which contained 1% FCS. For the determination of mouse antibodies to rabbit immunoglobulins, washed SRBC (4%) were incubated with a subagglutinating dilution of rabbit anti-SRBC serum for 30 min. at 37°C, washed again 3 times, and resuspended to 0.5%. The complement of test sera was always inactivated (56°C, 30 mins.) before use, and dilution was carried out in PBS containing 1% FCS. Sera tested for antiglobulin activity had previously been absorbed with an equal volume of packed SRBC at 37°C for 30 min.

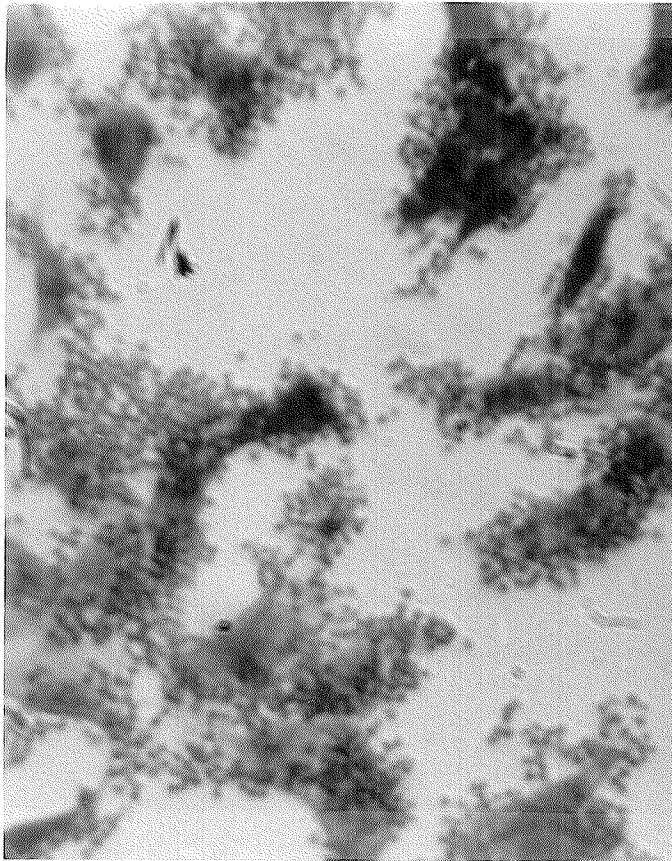
Mixed hemadsorption test: This method is basically a Coombs-type antiglobulin reaction (193) as modified by Fagraeus et al. (276a). The basic principle of the test is that different cell types having the same immunoglobulin on their surface can be

agglutinated with anti-immunoglobulin antibodies. For the purpose of these experiments SRBC acted as indicator cells and the coating antibodies to SRBC or Po were produced in rabbits, and goat anti-rabbit IgG or sheep anti-rabbit IgM were used as agglutinating reagents. Rabbit antibodies to Po in the test sera were detected by the binding (attachment) of SRBC coated with rabbit antibodies to the Po cells which had been also coated with homologous rabbit antibodies, the cross-linking reagent for the two types of cells being the antiglobulin serum (i.e. antiserum to rabbit globulins). As is evident from Figure 7, the indicator SRBC adsorbed only onto the Po cells which had rabbit antibodies on their surface.

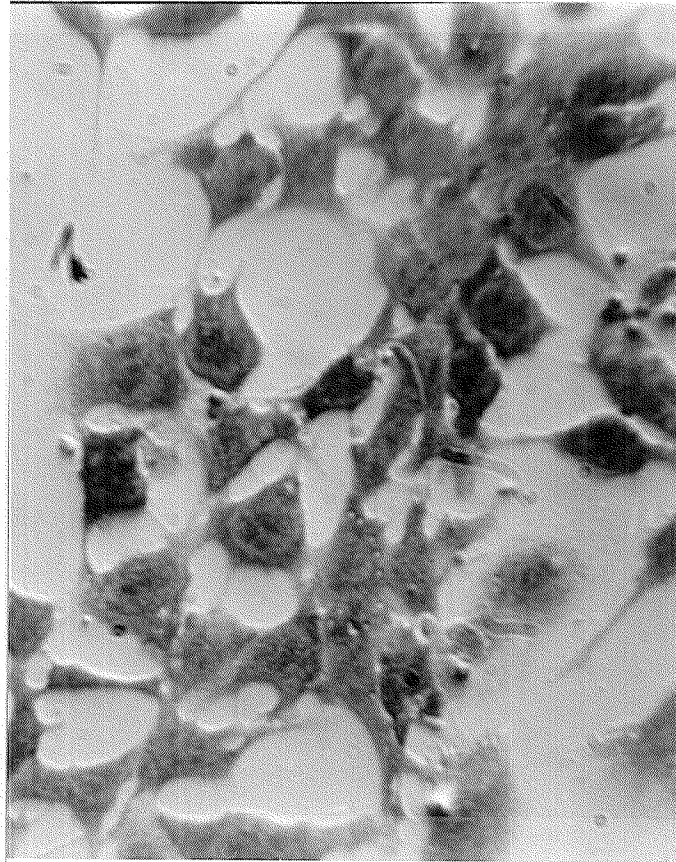
The test was carried out as follows: Po was grown in tissue culture tubes (Leighton-type or round) of 1 ml capacity for 48-72 hours as primary cultures. Similar cultures of C57 testis, kidney or B16 melanoma cells were used as controls. These cultured cells were washed 3 times with 1 ml of Hanks solution (HS), then 1 ml of the control or test serum was added, diluted in HS supplemented with 5% lactalbumin hydrolizate (HSL) (2-fold, 5-fold or 10-fold serial dilutions were used), and maintained for 2 hours at room temperature. The cells were washed again 3 times and to each tube 1 ml of a suspension (0.25%) of indicator (antibody coated) SRBC was added and the mixture of cells was kept at room temperature for 90 minutes. After gentle rinsing with HS the tubes were drained and read under an inverted microscope. The strength of the mixed agglutination reaction was graded as follows: all cells adsorbed

Figure 7. MIXED HEMADSORPTION TEST: POSITIVE REACTION ON THE LEFT,
NEGATIVE REACTION ON RIGHT
(Wright stain, approximate magnification 100x)

Left



Right



to indicator SRBC =++++; approximately 75% of the cells reacted = +++ ; around 50% of the cells reacted = ++; at least 25% of the cells reacted = +. As a general rule 25-50% positivity was taken as the end point for defining the titer of a given serum.

For the preparation of indicator SRBC a 4% suspension of washed SRBC were incubated first with rabbit anti-SRBC serum which had been diluted with PBS to a sub-agglutinating concentration and maintained for 30 min. at 37°C. The cells were washed 3 times in PBS and then incubated in a sheep or goat anti-rabbit immunoglobulin serum diluted tenfold with PBS for 45 min. at room temperature, washed again 3 times, and resuspended to 0.25% concentration in HSL; these double antibody coated SRBC were used as indicator cells for the detection of rabbit antibodies on Po cells. For the successful outcome of the mixed hemadsorption (agglutination) test, it is important to use a strong antiglobulin serum (with a minimum hemagglutination titer of 1000) which contains antibodies in excess of those required to combine with all the immunoglobulins present on the surface of SRBC; a good preparation of indicator SRBC must not agglutinate by itself, and these cells must have free antibody combining sites which are capable of binding specifically to the immunoglobulins used for coating the test (Po) cells.

B. The estimation of minimal lethal dose of polyoma tumor cells in C57 mice

The capacity of the immune system to destroy even the most antigenic tumors is very limited in syngeneic or autochthonous hosts and can be easily overwhelmed by a high cell dose. Therefore, it

is necessary to establish at least the approximate minimal lethal dose for each tumor-host system in order to be able to observe immune reactions directed against antigenic tumors. In addition to the number of cells, the method used for the preparation of cell suspensions should also be stabilized, because differences in cell viability may greatly influence the lethal dose. For all the experiments presented in this Chapter, cell-suspensions were prepared by trypsinization as described earlier.

To determine the minimal lethal dose of tumor cells, groups of 5 mice were given 10 , 10^2 , 10^3 , 10^4 , 10^5 and 10^6 Po cells i.p. in 1 ml of CMEM. Po cells grow as multiple solid tumors in the peritoneal cavity as shown in Figure 8. Three weeks after the injection, the surviving animals were killed, the tumors were counted, excised and weighed individually. At this time all the mice inoculated with 10^6 Po cells were dead, and one mouse given 10^4 cells also died. None of the mice injected with 10 Po cells had tumors; only one of 5 mice given 10^2 cells had 3 small tumors and tumors were found in four of 5 mice given 10^3 Po cells. Hundred per cent take was obtained with a dose of 10^4 Po cells or with higher doses. The average numbers and weights $\pm$ standard errors of tumors for the different groups of mice are shown in Figure 9, from which it can be concluded that the minimal lethal dose of Po cells for C57 mice is between 10^3 and 10^4 cells. After extensive experimentation, it was established that for reproducible results that it was essential to use a medium containing FCS (CMEM) for inoculation.

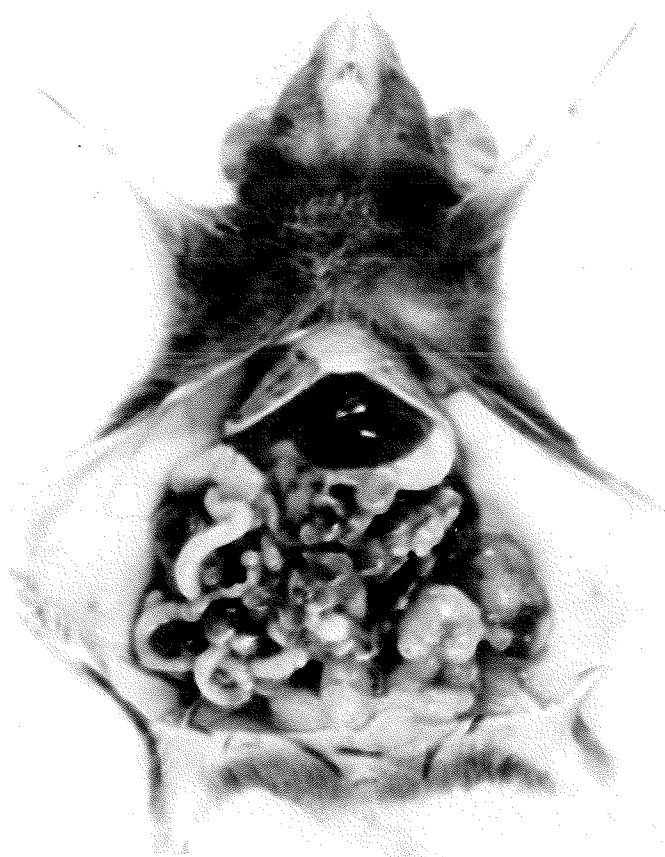
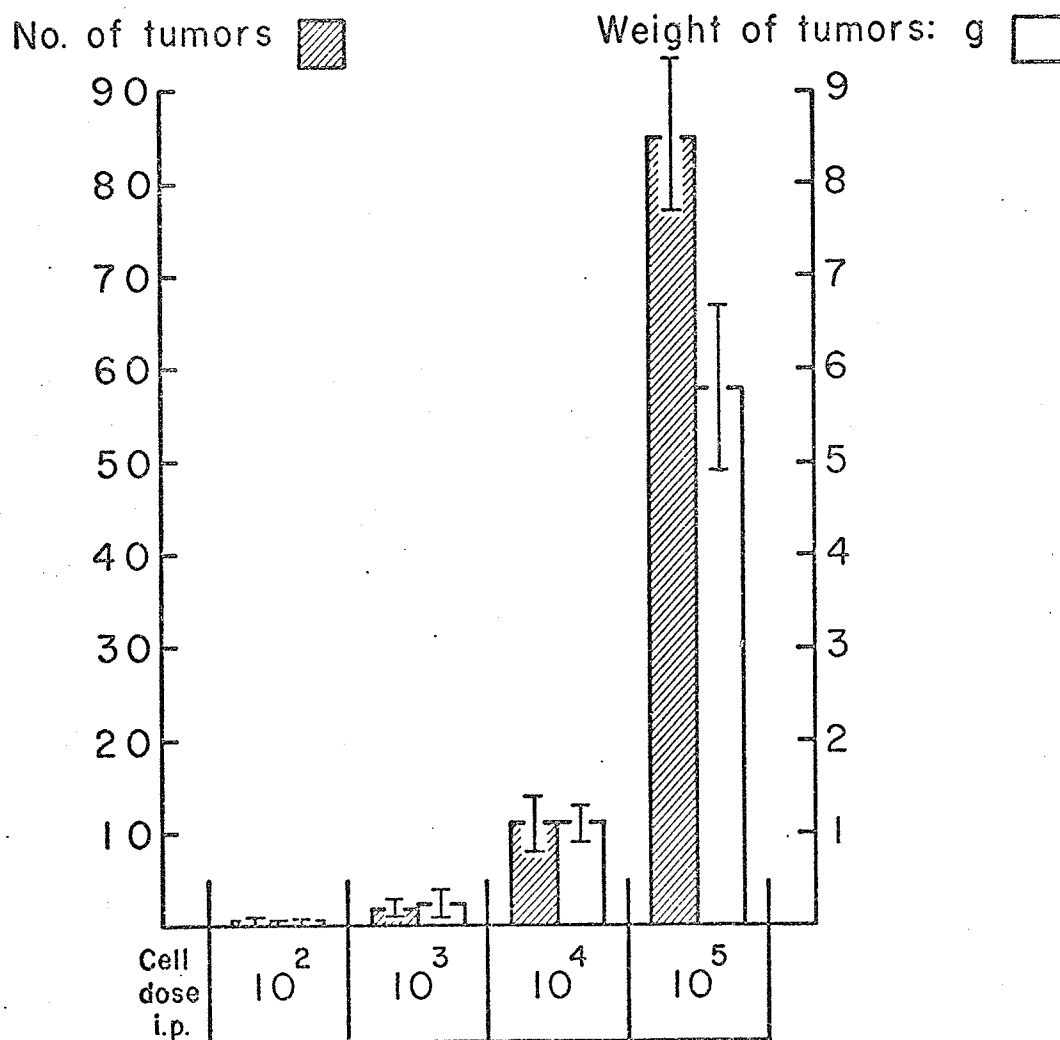


Figure 8. DEVELOPMENT OF MULTIPLE SOLID TUMORS IN THE
PERITONEAL CAVITY OF C57 MOUSE THREE WEEKS AFTER THE
INJECTION OF POLYOMA CELLS

Figure 9.

The number and total weight of intraperitoneal polyoma-tumors corresponding to different cell doses.



C. The investigation of antigenicity of Po in C57 mice

Although polyoma tumors are generally known to be highly antigenic, changes in antigenicity were also observed and variants of high and low antigenicity could be segregated in vitro (645; 886). Therefore, experiments were first performed to investigate whether or not this tumor fulfilled the requirement of antigenicity, which was basic for this model system.

Groups of 10 C57 mice were immunized as follows: with CFA only, with syngeneic testis cells in CFA and with Po cells in CFA, respectively. Suspensions of testis and Po cells were prepared by trypsinization and adjusted to 10% in M199, then emulsified with an equal volume of CFA. In the group immunized with adjuvant only, CFA was emulsified with M199. A total volume of 0.1 ml antigen was distributed into the four foot-pads of each mouse. Two weeks later a second injection of identically prepared antigen was injected s.c. into the back of the corresponding groups of mice. All immunized and a control mice were challenged 3 weeks after the second injection with 10^4 Po cells in 1 ml CMEM i.p. The experiment was terminated 19 days later by killing the surviving animals with ether. The tumors were collected, and individually counted and weighed for each mouse. The results (average $\pm$ SE) are shown in Table 1. Values of tumors for mice which died within 3 days prior to termination of the experiment are also included in this Table. As can be seen from these results, preimmunization with Po in CFA markedly reduced both the number of tumors and the mortality, but a relatively high tumor weight was obtained.

Table 1.

THE EFFECT OF PREIMMUNIZATION ON THE GROWTH OF POLYOMA TUMOR IN C57 MICE

Groups	Number of animals per group	Preimmunization	Intraperitoneal tumors number	tumors weight (g)	Mortality (%)
1	10	none	19.1±9.29	1.40±0.63	80
2	10	CFA*	25.8±4.93	2.24±0.43	70
3	10	syngeneic testis in CFA	33.1±9.22	2.03±0.52	80
4	10	polyoma in CFA	12.6±3.70	2.11±0.72	20

* Complete Freund's adjuvant

No specific resistance could be detected if immunization was carried out with a single injection of identically prepared antigens. Nevertheless, these results were considered to indicate the presence of tumor antigen(s) (TA) on Po cells, which was confirmed on further experimentation.

D. Alteration of tumor growth by normal lymphoid cells

It was found during experimentation that normal lymphoid cells, when administered i.p. conjointly with tumor cells which had been coated with rabbit antibodies, enhance tumor growth (Fig. 12) while no such effect was observed if a subcutaneous route was used for the injection of the cell mixture (Fig. 15). A number of experiments were carried out to obtain more information about the nature of this phenomenon.

Lymphoid cells were prepared from the lymph nodes, spleens and thymuses of normal C57 mice, mixed in various combinations with Po cells, and applied i.p. to normal recipients. The experiments were terminated 3 weeks later according to the standardized procedure, the tumors were collected, counted and weighed. Further details and the results (mean $\pm$ SE) of these experiments are shown in Table 2, which contains four experimental groups treated with spleen cells and another four which received spleen cells in combination with other lymphoid cells. In all these cases an enhancement of tumor growth occurred in comparison with the controls receiving Po alone, as indicated by the higher tumor growth rate (number and weight) and mortality. According to the χ^2 test this increase in the number and weight of tumors was statistically significant ($P < 0.01$). Lymph node cells, however, enhanced tumor growth in one of the four experiments and suppression of tumor growth occurred in the remaining three cases as indicated in Table 2. It should be noted, however, that a limiting factor in the experiments is the wide dose range

Table 2.

ENHANCEMENT OF TUMOR GROWTH BY NORMAL LYMPHOID CELLS UPON CONJOINT INTRAPERITONEAL
APPLICATION

Number of ex- periment	Groups	Number of animals per group	Dose of polyoma cells	Lymphoid cells source	dose	Tumors at 21 days number	weight (g)	Per cent morta- lity
I	1	5	5×10^5	-	-	14.8 ± 5.1	1.96 ± 0.76	0
	2	5	"	Ln	2.9×10^7	10.7 ± 1.9	1.38 ± 0.52	20
	3	5	"	Sp	6×10^7	21	2.58	40
II	1	10	5×10^4	-	-	19.8 ± 9.0	1.59 ± 0.46	10
	2	10	"	Ln	3.4×10^7	24.7 ± 5.6	2.50 ± 0.51	10
	3	10	"	Sp	1×10^8	29.0 ± 9.0	2.13 ± 0.52	40
III	1	10	7×10^4	-	-	26.0 ± 4.6	4.52 ± 0.89	20
	2	10	"	Ln	6.7×10^7	19.2 ± 5.4	2.57 ± 0.80	20
	3	10	"	Sp	8×10^7	48.3 ± 3.4	5.52 ± 0.41	60
	4	10	"	Ln+Sp	7.3×10^7	42.6 ± 2.9	6.05 ± 0.28	50
IV	1	5	5×10^4	-	-	22.0 ± 12	1.82 ± 0.65	40
	2	5	"	Ln	2.2×10^7	8.0 ± 3.4	1.43 ± 0.72	20
	3	5	"	Sp	3.5×10^7	27.4 ± 7.0	2.19 ± 0.50	40
	4	5	"	Tm	7×10^7	19.7 ± 6.7	3.22 ± 1.61	40
	5	5	"	Ln+Sp+Tm	as above	38.6 ± 7.0	3.25 ± 0.67	80
V	1	5	3×10^4	-	-	4.8 ± 1.4	0.44 ± 0.16	0
	2	5	"	Tm	8×10^7	2.2 ± 0.9	0.34 ± 0.18	0
	3	5	"	Tm+Ln*	3.9×10^7	6.6 ± 2.0	0.61 ± 0.20	0
	4	5	"	Tm+Sp*	3.2×10^7	16.2 ± 6.1	3.20 ± 0.84	0
	5	5	"	Tm+Ln+Sp	as above	9.0 ± 2.7	0.76 ± 0.27	0

Ln= lymph node cells; Sp = spleen cells; Tm = thymus cells.

* The dose of Ln or Sp cells are given only, for Tm cell dose see group No. 2.
As above = cell doses indicated in previous groups, are given conjointly.

of lymphoid cells applied which makes it impossible to reach a firm conclusion as to the effect of lymphoid cells from various sources on tumor growth. Despite of this difficulty it would appear that spleen cells are able to induce enhanced tumor growth. This enhancing effect of spleen cells was reproducible, whereas the effect of other lymphoid cells varied and was not consistent.

In further experiments it was investigated whether the course of tumor growth could be influenced by splenectomy. Young adult C57 mice were splenectomized as follows: a vertical incision of 3-4 mm was made behind the left costal arc on the flank. Through the incision the spleen was gently pulled out and detached from its serous ligaments, which were then replaced into the cavity and the wound was closed with one stitch.

The experiments involving the i.p. route are summarized in Table 3. As indicated in the Table a trend toward suppression of tumor growth was observed in splenectomized animals and this effect appeared reproducible. Simultaneously with experiment II of Table 3, ten splenectomized and ten normal animals were also administered 5×10^4 Po subcutaneously. The growth of tumors is indicated in Figure 10 (mean diameter $\pm$ SE). Again a reduced growth rate was observed in splenectomized animals.

It can be concluded that the conjoint intraperitoneal application of Po cells with normal spleen cells tends to enhance tumor growth, while slower tumor growth was observed in splenectomized animals in comparison with unoperated normal controls. It should be remembered that the splenectomized animals were inoculated with Po after their recovery from the operation (i.e. 1 or 2 weeks later as indicated in Table 3), therefore were considered to be comparable to normal healthy controls. Nevertheless an additional sham-operated control group could have been included for more accurate comparison. Beside this limitation both experimental approaches indicate the active role of the spleen in enhanced tumor

Table 3.

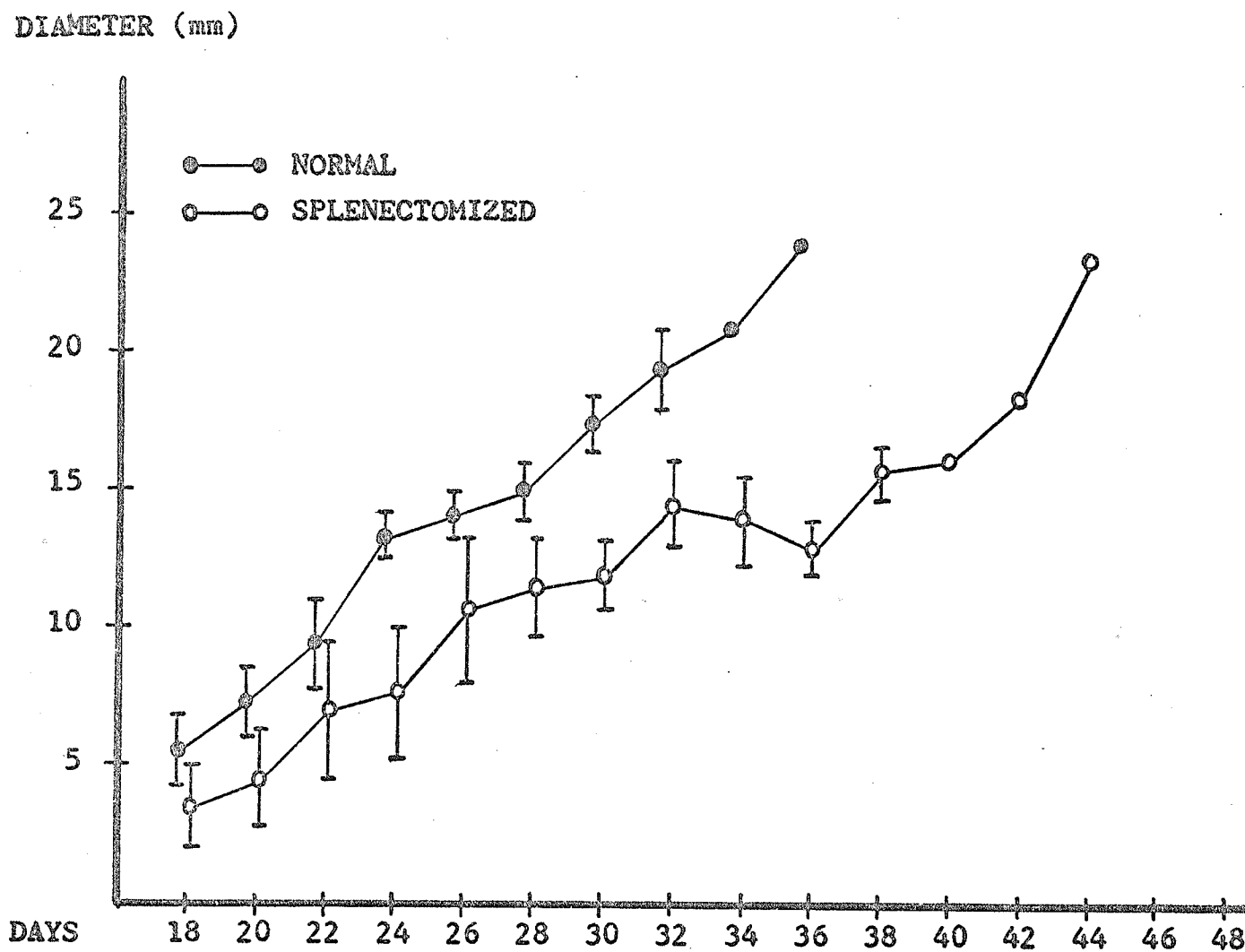
THE EFFECT OF SPLENECTOMY ON THE INTRAPERITONEAL GROWTH OF POLYOMA

Experiment	Groups	Number of animals per group	Splenectomy	Dose of polyoma cells	Dose of lymphoid cells	Tumors at day 21* number	weight (g)	Per cent mortality
I	1	5	NP	1.2×10^5	none	12.6 ± 2.0	2.2 ± 0.1	0
	2	5	- 7 days	"	none	11.8 ± 3.9	1.7 ± 0.7	20
II	1	10	NP	5×10^4	none	13.3 ± 2.3	2.7 ± 0.6	10
	2	10	-14 days	"	none	5.7 ± 1.7	1.1 ± 0.3	0
	3	10	NP	"	1×10^8	14.0 ± 1.2	2.9 ± 0.3	20
	4	10	-14 days	"	"	9.0 ± 1.5	2.4 ± 0.4	10

* Mean values $\pm$ standard error

NP = not performed

Figure 10. THE EFFECT OF SPLENECTOMY ON THE SUBCUTANEOUS GROWTH OF
POLYOMA TUMORS IN C57 MICE



growth. It is not clear presently whether this effect is mediated by enhancing antibodies or by other unknown mechanism(s).

E. Preparation of specific rabbit anti-Po serum

Preliminary experiments revealed that the Po-C57 tumor host system was suitable for testing the working hypothesis that destruction of tumor cells may be achieved by an indirect immune mechanism in vivo. It was, therefore, decided to use an appropriate rabbit antiserum as a source of tumor specific antibodies, which would be in turn recognized as foreign in mice and would thus serve the function of antigen(s) coating the tumor cells.

Six New Zealand white rabbits were immunized with fresh Po, homogenized (20% by wet weight) in PBS and emulsified with an equal volume of CFA. Seven days after six weekly s.c. injections of 0.5 ml of this antigen the animals were bled. To render the serum tumor specific, it was subjected to a series of exhaustive absorption steps as described in Table 4. It is evident from this Table that the initial hemadsorption titer of 31,250 of the serum to Po was thus reduced to a value of 40-160. However, it is worth-noting that by the end of this absorption procedure the original serum was diluted sixfold and, therefore, this final titer must be considered to be higher. Marginal reactions were obtained with testis cells with this absorbed serum and these cells gave always the strongest reactions of the three control tissues used throughout the whole absorption procedure. However, no hemadsorption was detected even with testis cells, when the absorbed antiserum was used at a dilution of 1:5 or higher. It

Table 4.

ABSORPTION AND TITRATION OF RABBIT ANTI-POLYOMA SERUM

Procedures for sequential absorption with normal C57B1/6J mouse tissues	Hemadsorption titers tested on monolayers of C57B1/6J mouse cells derived from:			
	POLYOMA	TESTIS	KIDNEY	B16-MELANOMA
Nonadsorbed serum	31,250	31,250	6,250	NT*
Ten ml serum absorbed with:				
- 4 g (wet weight) 2,000 rpm pellet of homogenized muscle at 4°C overnight				
- 0.5 g 2,000 rpm pellet of spleen and kidney at room temperature for 30 min.				
- 0.5 g testis teased at room temperature for 30 min.:	1,250	1,250	NT	1,250
- 5 g 15,000 rpm muscle pellet, 30 min. room temp.				
- 5 g 15,000 rpm liver pellet, 30 min. room temp.:	250	250	NT	125
- citrated whole blood of 14 mice, 4°C overnight:	250	50	NT	NT
- tissue culture monolayers, 75 cm ² in each flask, 5 testis and 3 kidney flasks for 45 min. at room temp. consecutively				
- citrated blood of 15 animals at 4°C overnight:	128	32	16	NT
- citrated blood of 13 mice at 4°C overnight				
- 75 cm ² monolayers, 4 testis and 5 kidney flasks for 45 min. each at room temperature consecutively				
Final titer:	40-160	10	NIL**	NIL**

* NT = not tested;

** = serum was tested at the lowest dilution of 1:5

is, therefore, considered that a reasonably specific antiserum was thus obtained. The anti-Po antibodies were identified as IgG, with the aid of specific anti-IgG and IgM sera described earlier.

F. Production of lymphoid cells sensitized to rabbit immunoglobulins

Preliminary experiments revealed that mice do not develop readily an adoptively transferable, delayed hypersensitivity reaction to proteins injected as an emulsion in CFA. Therefore, in an attempt to induce cellular immunity in C57 female mice to rabbit immunoglobulins (RIg), i.e. to immunoglobulins isotypic with antibodies proposed to coat Po cells, these proteins were attached to SRBC on the assumption that they may elicit delayed hypersensitivity if presented as an integral part of a foreign cell to the immunological system of the mouse. The following immunization procedure was used. SRBC were suspended (1% by volume) in a 100-fold diluted (heat inactivated) rabbit anti-SRBC serum (with a hemagglutination titer of 1:1024) and incubated at 37°C for 30 minutes. Coated SRBC (SRBC-RIg) were then washed 3 times in PBS and finally resuspended in PBS to 1%, emulsified with an equal volume of CFA and injected into the four foot pads of mice in a total dose of 0.05-0.06 ml per mouse. Two weeks later 0.1 ml of identically prepared antigen was injected s.c. on the back. In some experiments were used Po cells, which were coated similarly with rabbit anti-Po antibodies, and emulsified in CFA; the first series of injections were administered into the foot pads (3×10^5 Po cells in 0.05 ml) and two weeks later 0.1 ml was injected s.c. into the back of each mouse.

Two weeks after the second injection the animals were skin tested with various antigens by injecting a minute amount of antigen in 2.5-5 μ l volume, with gauge #30 needles, into the skin of the back. Extreme care was taken to inject this antigen as superficially as possible and most likely between the dermis and the muscle layer (panniculus carnosus) of the skin. A commercially obtained preparation of rabbit gamma globulins (RGG) or normal rabbit serum was used to test the sensitivity of mice to RIg. A local reaction was thus elicited in sensitized mice.

A crude Po-antigen (PoA) was also prepared for skin testing as follows: 75g wet Po tissue was homogenized in 150 ml of distilled water, centrifuged at 20,000G for 30 minutes and the supernatant was dialysed against 30 volumes of distilled water three times, for 12 hours each time. Some precipitation occurred during dialysis and was removed by centrifugation (20,000 G, 30 min.); the supernatant was lyophilized. A total of 550 mg crude antigen was prepared in this manner; it was found to be skin active as a test antigen when redissolved in PBS.

The results of four separate experiments in which SRBC-RIg and Po-RIg were used as sensitizing antigens are shown in Table 5. The sensitivity developed by the mice was detected by skin testing with the soluble antigens indicated in this Table. Skin thickness was measured at the injection sites with a caliper just before injection (Oh.) and 24 and 48 hours after the injection. The average values and standard errors are listed in the Table.

Table 5.

DELAYED SKIN REACTION IN MICE SENSITIZED TO RABBIT IMMUNOGLOBULINS

Experi- ment	Mice per group	Antigen sensitizing	test	Skin thickness (μ)			Hemagglu- tination titer to RIg
				0h.	24 h	48 h	
I	12	SRBC-RIg	RGG 50 μ g	729 $\pm$ 16	1025 $\pm$ 28*		
			PoA 50 μ g	679 $\pm$ 21	717 $\pm$ 18		
II	21	SRBC-RIg	RS 2.5 μ l	481 $\pm$ 11	776 $\pm$ 17*	743 $\pm$ 22 ⁺	256
	10	none	RS 2.5 μ l	480 $\pm$ 13	670 $\pm$ 21	670 $\pm$ 26	
III	15	Po-RIg	RGG 50 μ g	767 $\pm$ 23	1053 $\pm$ 42*	900 $\pm$ 49*	
			PoA 50 μ g	640 $\pm$ 36	910 $\pm$ 59*	791 $\pm$ 39*	
IV	21	Po-RIg	RS 5 μ l	648 $\pm$ 11	776 $\pm$ 14*	779 $\pm$ 20*	64-128
			PoA 50 μ g	643 $\pm$ 11	752 $\pm$ 16*	716 $\pm$ 12*	
	10	none	RS 5 μ l	656 $\pm$ 18	633 $\pm$ 17	644 $\pm$ 24	
			PoA 50 μ g	667 $\pm$ 17	678 $\pm$ 15	644 $\pm$ 18	

SRBC-RIg = Sheep red blood cells coated with rabbit immunoglobulins

Po-RIg = Polyoma tumor cells coated with rabbit immunoglobulins

RGG = Rabbit gamma globulins

RS = Rabbit serum

PoA = Polyoma tumor antigen

(μ) - skin thickness is given in microns (mean $\pm$ SE)

* P < 0.001, + P = 0.06 (compared to control site or control group respectively)

In all experiments a significant skin thickening occurred in sensitized mice at injection sites of the specific, but not the control antigen. However, non-sensitized animals did not give these reactions. Mice sensitized with Po-RIg exhibited sensitivity to both RIg and PoA. At this stage of sensitization some antibody activity to RIg (titers of 256) was also present in sensitized animals as shown in the Table.

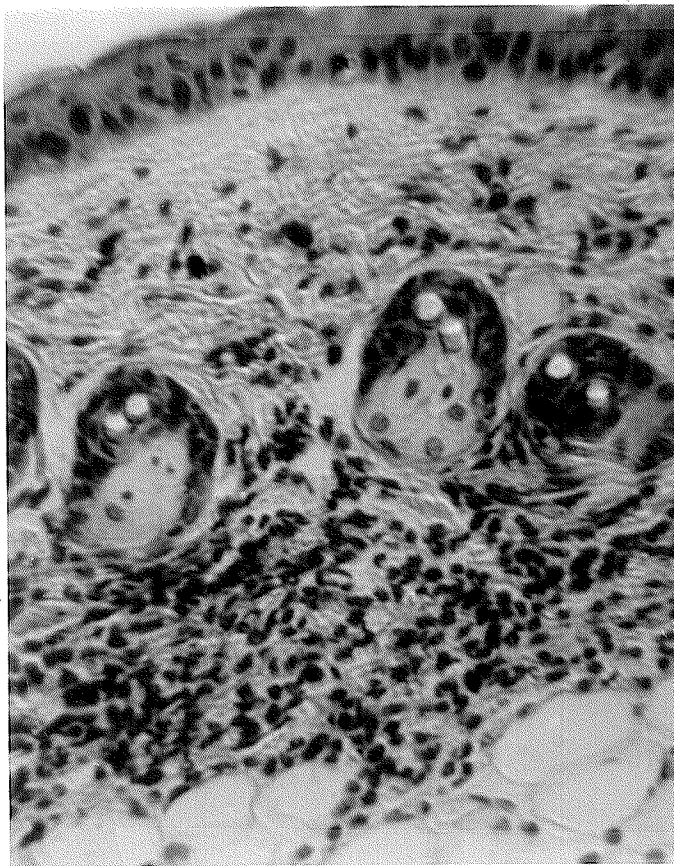
Histological examination of the reaction sites revealed the presence of both mononuclear and polymorphonuclear leukocytes. An area with predominantly mononuclear infiltration at the injection site 24 hours after administration of 50 μ g RGG (experiment I) is illustrated in Figure 11.

Spleen cells of sensitized rodents had repeatedly been described to react specifically by inhibition of migration of antigen (275; 276; 548; 874). Spleen cells of sensitized mice from experiment I were examined in a capillary tube migration system in comparison with controls. Medium 199 with 10% FCS was used with or without RGG, as specific antigen. Mean areas of migration determined at 24 hours and standard errors are listed in Table 6. It is clear from this Table that the strongest inhibition of migration occurred when spleen cells sensitized to RIg were incubated with RGG.

All the results presented indicate that cell-mediated immunity was induced in mice by this method of immunization, in addition to the weak humoral antibody response.

Figure 11. DELAYED SKIN REACTION IN C57 MICE SENSITIZED WITH SRBC-Rig AT
24 HOURS AFTER THE INJECTION OF 50 μ g RABBIT GAMMA GLOBULINS
The cellular infiltration is predominantly mononuclear.
(Hematoxylin and eosin; approximate magnification on left, 100x,
on right, 400x)

Left



Right

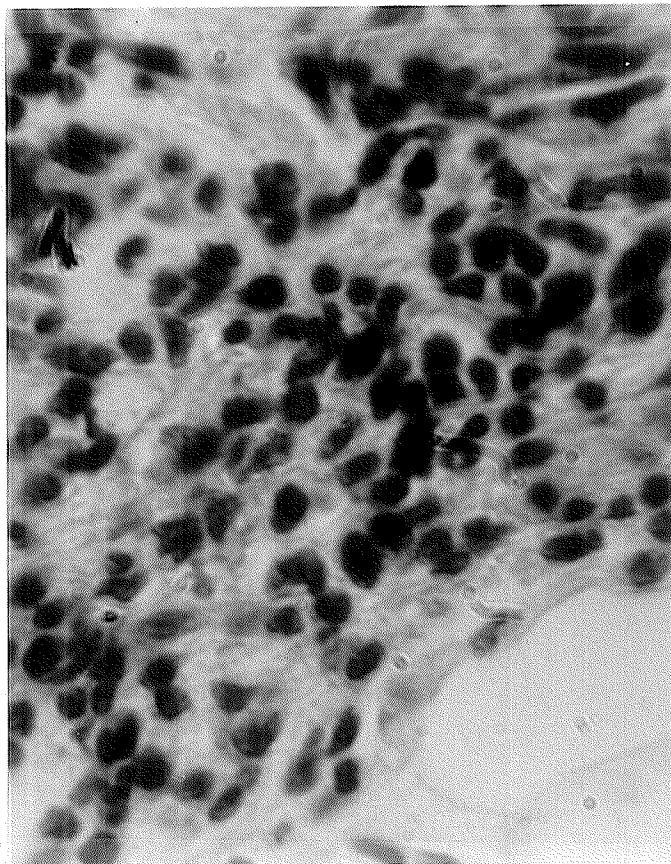


Table 6.

INHIBITION OF MIGRATION OF MOUSE SPLEEN CELLS BY RABBIT GAMMA GLOBULINS

Experi- ment	Sensitizing antigen	Mean areas of migration $\pm$ SE*		
		no RGG	on addition of antigen 2.5mg/ml RGG	5mg/ml RGG
I	none	98 $\pm$ 3.3	88 $\pm$ 2.3 (10%)	101 $\pm$ 2.7 (-3%)
II	none	82 $\pm$ 4.4	66 $\pm$ 2.1 (19%)	84 $\pm$ 6.6 (-2%)
III	SRBC-RIg	57 $\pm$ 5.0	35 $\pm$ 0.6 (39%)	44 $\pm$ 3.2 (22%)

* The corresponding per cent inhibition of migration observed on addition of antigen is indicated in brackets

RGG = Rabbit gamma globulins

SRBC-RIg= Sheep red blood cells coated with rabbit immunoglobulin

G. Adoptive transfer of immunity

In order to investigate if lymphoid cells sensitive to RIg were able to inhibit the growth of or destroy Po cells which had been coated with specific antibody (Po-Ab), these lymphoid cells were injected with Po-Ab into normal C57 recipients either intra-peritoneally or subcutaneously. Several experiments of this nature were performed.

Polyoma cells were obtained by trypsinization in all cases, washed once by centrifugation (400 G, 10 minutes, 4°C) and resuspended in ML99 at a density of 10^6 cells per ml. The tumor cell suspensions were divided into two equal parts; to one part was added the absorbed rabbit anti-Po antiserum at a final concentration of 1:100. Both samples of cells were incubated at 37°C for 30 minutes, followed by two washings in ML99 and resuspended in CMEM. With the exception of coating the cells, all other procedures including the mixing of tumor cells with lymphoid cells were carried out at 2-4°C and maintained at this temperature (ice bath) until injected into mice.

For the first experiment sensitized lymphoid cells (SLC) were prepared from the spleens, mesenteric, axillary and inguinal lymph nodes of immunized animals listed under experiment II of Table 5. Twenty days after the second antigen administration, normal lymphoid cells (NLC) were obtained from nontreated mice. Three groups of 5 mice each received 10^5 Po-Ab, 10^5 Po-Ab plus 10^8 NLC and 10^5 Po-Ab plus 10^8 SLC, respectively. In addition,

10 mice, which had been preimmunized with SRBC-RIg in CFA, were injected with 10^5 Po-Ab. All injections were given in a total of 1 ml CMEM intraperitoneally. Three weeks later the animals were killed and the intraperitoneal tumors were collected, counted and weighed. Figure 12 shows the results. The most striking feature of these results is the marked enhancement of tumor growth in the presence of NLC as well as in animals preimmunized with SRBC-RIg.

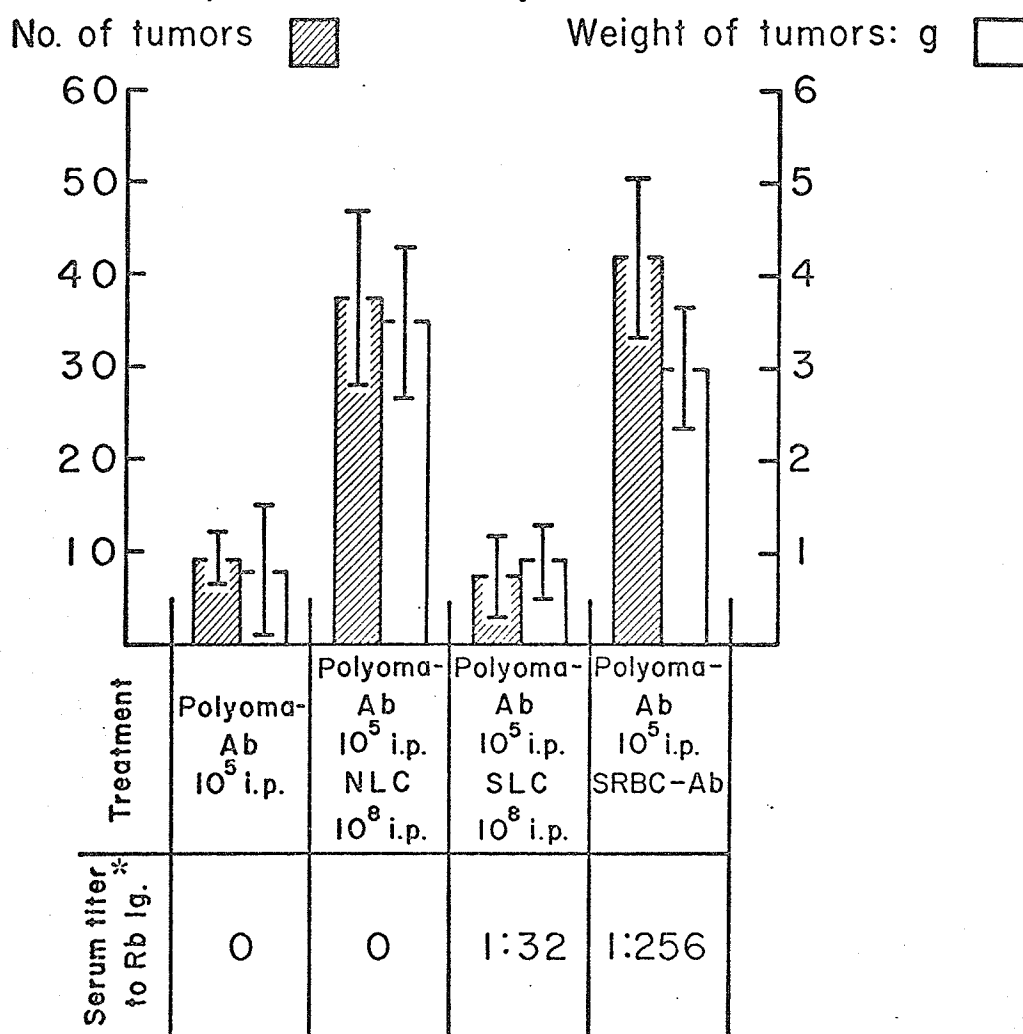
In the next experiment sensitized lymph node cells (LNC) and sensitized spleen cells (SPC) were prepared as before 20 days after the second injection of SRBC-RIg and injected with coated or noncoated Po cells to eight groups of 5 animals as listed below:

- (1) 5×10^4 Po
- (2) " + 7.2×10^7 LNC
- (3) " + 6×10^7 SPC
- (4) " + 7.2×10^7 LNC + 6×10^7 SPC
- (5) 5×10^4 Po-Ab
- (6) " + 7.2×10^7 LNC
- (7) " + 6×10^7 SPC
- (8) " + 7.2×10^7 LNC + 6×10^7 SPC

All cell doses were administered in a total volume of 1 ml CMEM i.p. The experiment was terminated as previously and the results are shown in Figure 13 for groups 1-4 in the upper graph and for groups 5-8 in the lower one. The upper graph indicates that non-coated cells (i.e. in absence of specific antigen) were enhanced by all three lymphoid cell populations, while enhancement of coated cells did not occur with LNC + SPC (lower graph), which is in good

Figure 12.

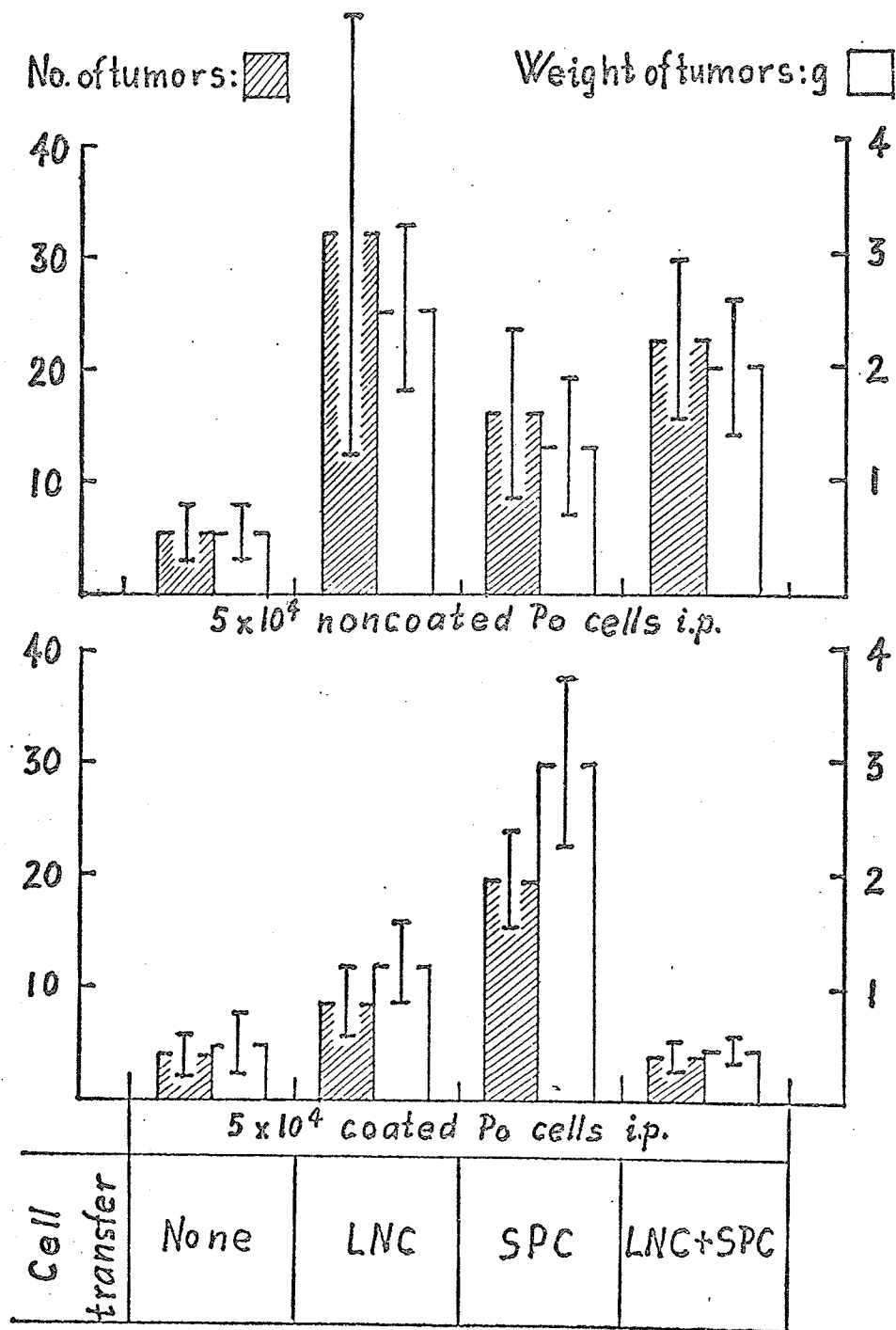
The effect of normal and sensitized lymphocytes, and circulating mouse-rabbit antiglobulins on the growth of rabbit antibody coated polyoma cells in the peritoneal cavity.



* By hemagglutination.

Figure 13.

INHIBITION OF COATED POLYOMA CELLS BY SENSITIZED LYMPHOID CELLS



correlation with the previous experiment. Nevertheless even coated cells were enhanced by spleen cells.

One more experiment was performed with cell transfer by the i.p. route. Sensitized lymphoid cells were prepared from the spleens and lymph nodes of mice immunized with Po-RIg three weeks after the second injection. These cell donors showed a delayed skin reaction to both RIg and PoA as tested one week earlier (experiment IV of Table 7). Normal lymphoid cells were obtained from nontreated animals. Groups of 5 animals each were injected with 4×10^4 Po-Ab, 4×10^4 Po-Ab + 3×10^7 NLC, and 4×10^4 Po-Ab + 4.5×10^7 SLC i.p. respectively. As shown in Table 7 normal lymphoid cells greatly enhanced the growth of Po in the peritoneal cavity, while sensitized lymphoid cells protected completely 4 of 5 animals. All the other animals had tumors at 21 days after injection as indicated in this Table. It may be inferred from these results that lymphoid cells sensitized against RIg, as well as to Po, provide a better protection than cells immune to RIg only.

A series of experiments were performed on preimmunized animals which were challenged, together with groups of normal controls, two weeks after the second injection of SRBC-RIg with either Po or Po-Ab i.p. The experiments were evaluated 3 weeks later in the usual way. The results (mean values $\pm$ SE) are summarized in Table 8. Except for the first experiment, where enhancement appears in preimmunized animals, no significant change in tumor growth was detected.

Table 7.

SUPPRESSION OF TUMOR GROWTH IN VIVO BY LYMPHOID CELLS SENSITIZED WITH RABBIT
ANTIBODY COATED POLYOMA TUMOR CELLS

Number of animals	Intraperitoneal tumors in animals injected with					
	Po-Ab number	Po-Ab weight (g)	Po-Ab+NLC number	Po-Ab+NLC weight (g)	Po-Ab+SLC number	Po-Ab+SLC weight (g)
1	5	0.38	24	2.75	0	0
2	2	0.81	81	5.30	0	0
3	3	0.43	40	3.05	0	0
4	3	0.21	32	1.92	0	0
5	died	died	10	1.30	3	0.10
Average	3.25±1.87	0.45±0.09	37.4±11.9	2.86±0.68	0.6	0.02

Po-Ab = polyoma tumor cells coated with rabbit antibodies

NLC = normal lymph node cells plus spleen cells

SLC = sensitized lymph node cells plus spleen cells

Table 8.

THE GROWTH RATE OF POLYOMA TUMOR CELLS COATED AND NON-COATED WITH RABBIT ANTIBODY IN PREIMMUNIZED
AND CONTROL ANIMALS

Experi- ment	Groups	Number of animals per group	Preimmuni- zation	Dose of tu- mor cells	Intraperitoneal growth of			
					Po cells number	Po cells weight (g)	Po-Ab cells number	Po-Ab cells weight (g)
I	1	5	none	10^5 i.p.	ND	ND	9.4 $\pm$ 7.2	0.8 $\pm$ 0.7
	2	5	SRBC-Ab	"	ND	ND	41.9 $\pm$ 8.6	3.0 $\pm$ 0.7
II	1	10	none	5×10^4 i.p.	19.4 $\pm$ 5.6	1.7 $\pm$ 0.1	13.0 $\pm$ 3.3	1.6 $\pm$ 0.1
	2	10	SRBC-Ab	"	19.6 $\pm$ 5.5	2.4 $\pm$ 0.6	10.1 $\pm$ 3.9	1.7 $\pm$ 0.5
III	1	10	none	10^5 i.p.	8.5 $\pm$ 2.1	1.4 $\pm$ 0.6	4.9 $\pm$ 1.3	0.6 $\pm$ 0.3
	2	10	SRBC-Ab	"	6.8 $\pm$ 1.4	1.6 $\pm$ 0.6	6.8 $\pm$ 0.7	0.7 $\pm$ 0.2

Po = polyoma tumor

Po-Ab = polyoma tumor cells coated with rabbit antibodies

SRBC-Ab = sheep red blood cells coated with rabbit antibodies

ND = not done

Mean values $\pm$ standard error are listed in the table

A number of experiments were also performed with lymphoid cells from animals immunized with a single dose of SRBC-RIg in CFA, given into the foot pads. The cells transferred i.p. with Po-Ab to normal recipients 5-10 days after immunization never exerted any specific effect on tumor growth.

H. Investigation of the effect of immune serum on tumor growth

Four experiments were performed to investigate if sera obtained from animals sensitized with SRBC-RIg have any effect on tumor growth. The sera used had a hemagglutination titer of 64-128 after absorption with an equal volume of packed SRBC and tested with SRBC-RIg. Table 9 gives further details of these experiments. In the first two experiments some nonspecific effects of serum treatment can be seen on the growth of both Po and Po-Ab. Heat-inactivated sera (56°C, 30 min.), stored at -20°C, were administered into mice in these experiments. In the next two experiments, fresh (i.e. not inactivated) sera were used, but no significant effect was observed, although in one of these experiments (experiment IV) both the tumor cells and the immune serum were given intraperitoneally.

I. Tumor suppression in vivo

It was obvious from the previous experiments that the intraperitoneal route favours enhancement, rather than tumor inhibition, therefore, in subsequent experiments the route of administration was changed from intraperitoneal to subcutaneous. In these experiments, the tumor diameters were measured with a caliper, from the appearance of the tumor until the death of the animal. Groups

Table 9.

THE GROWTH RATE OF POLYOMA TUMOR CELLS COATED AND NON-COATED WITH RABBIT ANTIBODY IN RECIPIENTS OF
IMMUNE SERUM AND IN CONTROLS

Experiment	Number of animals per group	Dose of immune serum	Dose of tumor cells	Intraperitoneal growth of			
				Po cells number	Po cells weight (g)	Po-Ab cells number	Po-Ab cells weight (g)
I	5	none	5×10^4 i.p.	18.6 ± 4.6	2.3 ± 0.7	18.4 ± 14.1	1.0 ± 0.6
	5	0.4ml i.v.	"	39.8 ± 18.8	2.4 ± 0.9	34.0 ± 10.2	2.5 ± 0.5
II	5	none	10^5 i.p.	25.2 ± 9.6	3.0 ± 0.4	23.8 ± 1.1	3.7 ± 0.4
	5	0.5ml i.v.	"	13.2 ± 5.6	1.5 ± 0.5	10.6 ± 0.7	1.1 ± 0.4
III	10	none	5×10^4 i.p.	19.4 ± 5.6	1.7 ± 0.1	13.0 ± 3.3	1.6 ± 0.1
	10	0.2ml*i.v.	"	18.2 ± 0.4	2.7 ± 0.5	10.7 ± 3.2	1.8 ± 0.5
IV	10	none	10^5 i.p.	8.5 ± 2.2	1.4 ± 0.5	4.0 ± 1.1	0.5 ± 0.2
	10	0.4ml*i.p.	"	7.6 ± 2.6	0.7 ± 0.4	7.2 ± 1.3	0.7 ± 0.2

* Fresh serum, i.e. not heat-inactivated

Po = polyoma tumor cells

Po-Ab = polyoma tumor cells coated with rabbit antibodies

of ten C57 mice received on their back s.c. 10^5 Po or 10^5 Po-Ab cells, with and without lymphoid cells from mice immunized with SRBC-RIg prepared 3 weeks after the second injection or from normal donors in the combinations indicated in Figures 14 and 15. As an additional control, the effect of preimmunization of mice with SRBC-RIg on the growth of Po and Po-Ab cells was also established in two groups of ten mice each (Figure 16). The points plotted in Figures 13-15 represent mean tumor-diameters in mm $\pm$ standard error. Standard errors were calculated as long as at least 4 animals remained alive in the group. The breaks in linearity of the curves are considered to be due to the death of animals bearing the largest tumors.

As is evident from the results plotted in Figure 14, coating of Po cells alone did not affect significantly the growth rate of the tumors and the addition of SLC to non-coated Po cells resulted in some suppression of tumor growth. Clearly, however, the growth of Po-Ab cells was greatly suppressed in the presence of SLC and it is to be noted that 100% mortality was observed in all groups of mice except in the last group receiving jointly Po-Ab and SLC; two animals (20%) of this group remained tumor free for over one year after treatment.

The results shown in Figure 15 demonstrate that NLC were not effective in suppressing the growth of Po-Ab cells, when substituted for SLC. It is to be stressed that in order to bring out any possible weak cytotoxic effects of NLC, the dose of Po

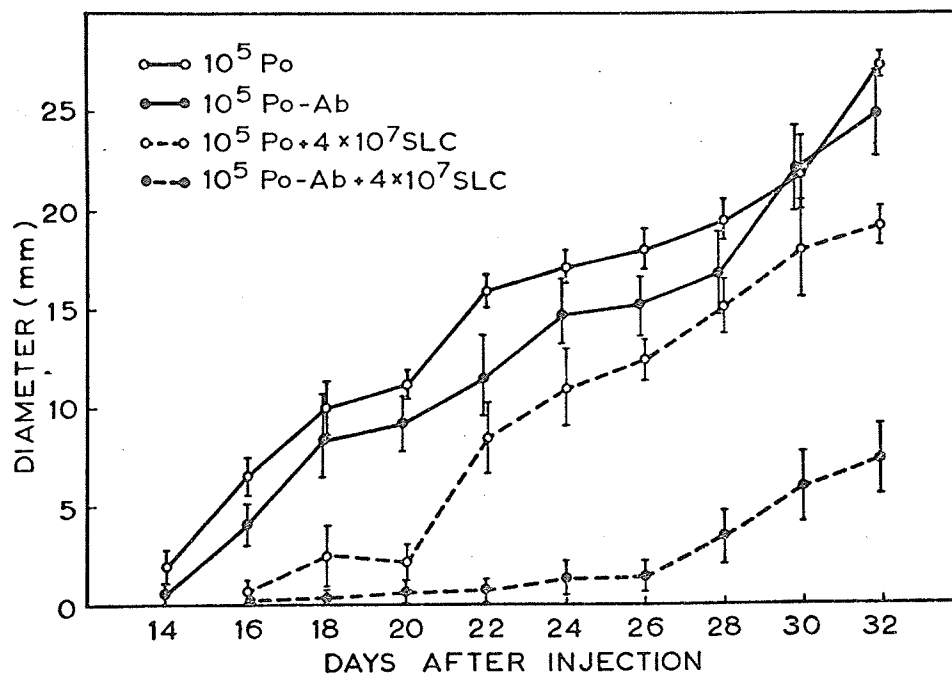


FIGURE 14. Inhibition of tumour growth by an indirect cellular immune mechanism.

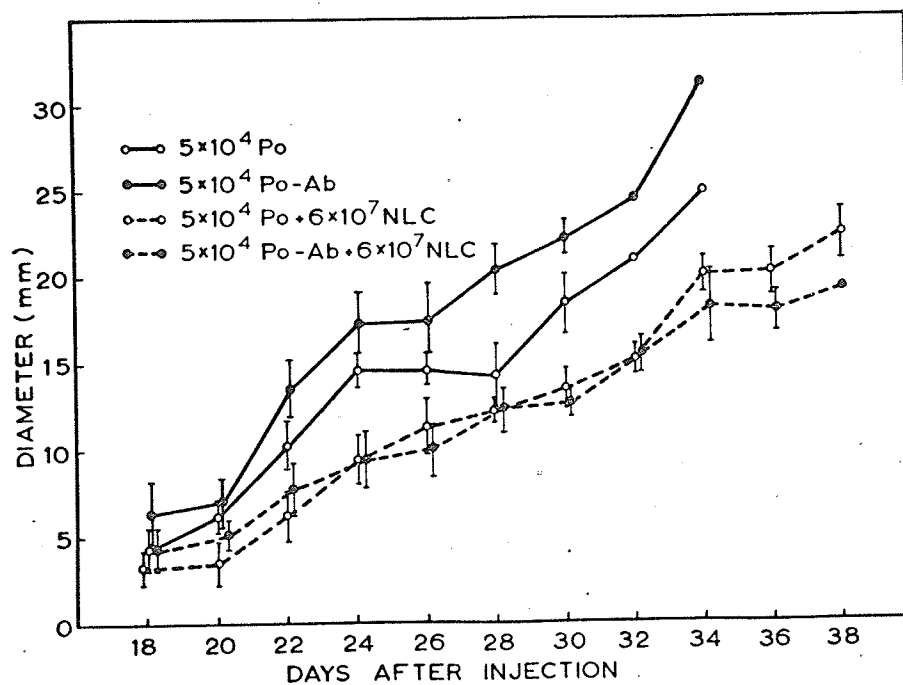


FIGURE 15. Growth rate of non-coated and coated polyoma cells in the presence of normal lymphoid cells.

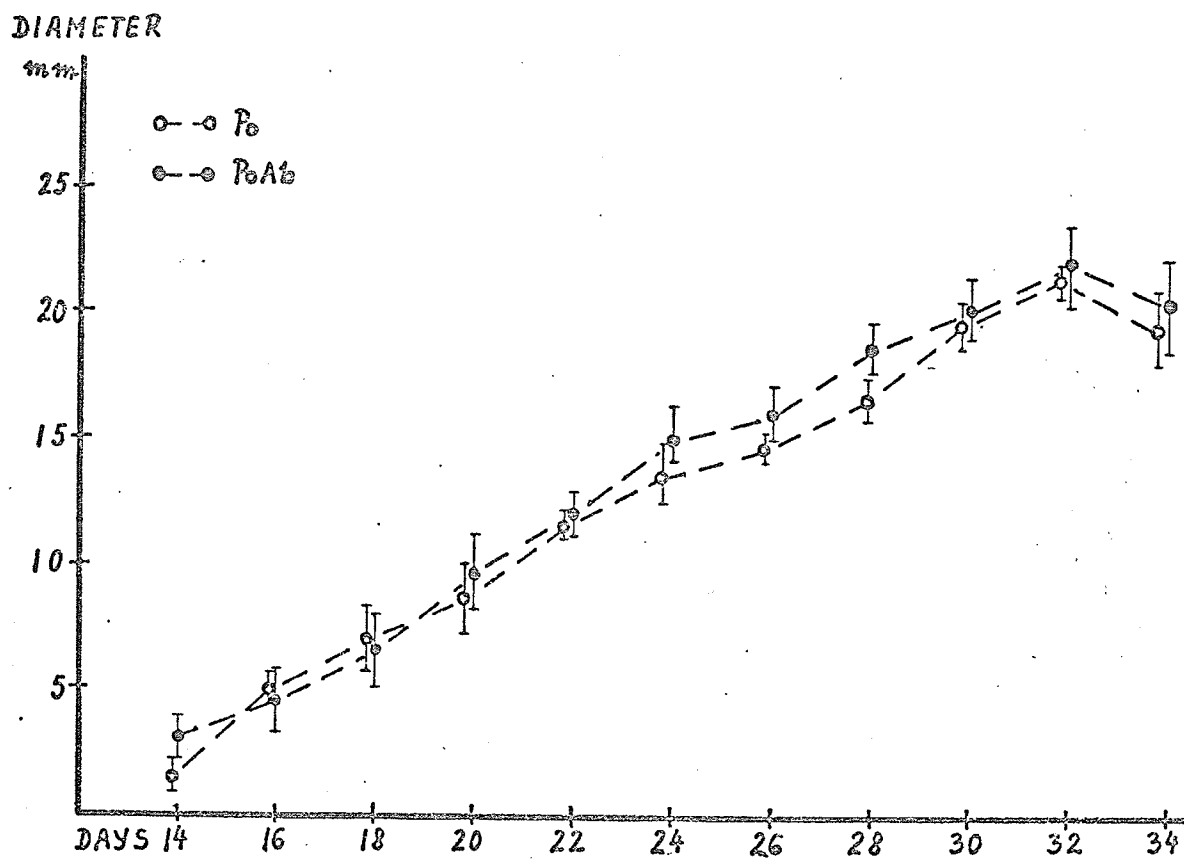


Figure 16. THE GROWTH OF RABBIT ANTIBODY COATED AND NON-COATED POLYOMA TUMOR CELLS IN SRBC-R1g IMMUNIZED C57 MICE

and Po-Ab cells was smaller (5×10^4) and the dose of NLC larger (6×10^7) than the corresponding doses in the experiments with SLC (please compare doses of cells in Figure 14-15). Moreover, in the 'preimmunized control' animals (i.e. which had been sensitized with SRBC-Ab) the growth rates of Po and Po-Ab cells were not significantly different and also did not differ from those determined in normal mice as illustrated in Figure 16. Humoral antibodies against RIg were present in these animals and it may be visualized that these antibodies were responsible for this uninhibited growth by playing a role similar to that of enhancing antibodies.

J. Discussion and conclusions

The salient feature of this model is the prerequisite of being able to coat malignant cells with the corresponding tumor specific antibody, which thus assumes the role of carrier of new antigenic determinants. In these experiments a heterologous antibody was produced in rabbits against the polyoma tumor grown in C57 mice. As demonstrated in this study with the aid of the highly sensitive mixed hemadsorption technique, it was a very difficult task to prepare an absolutely specific, heterologous antitumor serum by exhaustive absorption with normal tissues. Finally, a reasonably specific antiserum was obtained, but it still possessed a minimal residual reactivity against testis cells, which reacted always very strongly with the rabbit anti-Po serum during absorption. Although no definite proof was obtained that the Po tumor and testis cells shared a common antigenic determinant which was not present on cells of other organs, the experimental

findings suggest that testis cells and this Po tumor may possess common, perhaps embryonic-type, antigenic determinants. This tentative suggestion is also favoured by the recent report (681) that there exists a relationship between polyoma virus induced antigen and normal embryonic antigen in hamsters. In spite of this difficulty, the experimental results reported in Section I of this Chapter provide strong evidence that the absorbed rabbit antiserum contained antibodies capable of binding specifically to Po cells; the fact that the absorbed serum had no antibodies which could become attached to B16 melanoma cells (grown also in C57 mice) is considered to provide further (though indirect) evidence that the serum had been depleted of antibodies to most, if not to all, normal tissue antigens of the C57 strain.

The second condition, on which this model is predicated is that one should be able to mount a strong cell-mediated immunity against the antigenic determinants of the antibodies coating the tumor. No definite proof was obtained in this study that rabbit gamma globulins, which are highly immunogenic proteins, are actually suitable for the induction of this type of sensitization. In this connection it is worth noting that even in the guinea pig, which is the species of choice for induction of cell-mediated immune reactions ("delayed hypersensitivity") to various antigens, stimulation with protein antigens given intradermally or in incomplete Freund's adjuvant has been recently shown to result in a response involving predominantly basophilic granulocytes. This reaction was claimed to be fundamentally different from the classical

(tuberculin-type) delayed hypersensitivity reaction and termed as "cutaneous basophil hypersensitivity" (751, 752). Therefore, in retrospect, the fact that in the present study it was not possible to demonstrate a clear-cut cell-mediated immunity in mice to rabbit proteins is not totally suprising. Nevertheless, the experimental results presented in this Chapter demonstrate that the model proposed for the immunotherapy of cancer is feasible.

An immunologically specific inhibition of tumor growth was achieved by sensitized lymphoid cells in contact with tumor cells coated with the specific antigen. It is, therefore, proposed that the model underlying this study represents a promising approach for the development of immunotherapeutic procedure for the destruction of antigenic tumors. The use of rabbit antibodies as 'artificial' neo-antigens presents the advantages that (i) they can be prepared in relatively larger quantities than the corresponding tumor antigens themselves and (ii) cell-mediated immunity can be directed against antigenic determinants of normal rabbit globulins. Hence, it is anticipated that such neo-antigens may be used as a 'homing' device for localizing in tumor foci lymphoid cells specifically sensitized to the antibodies coating the tumor cells and that this model may be thus exploited for the immunotherapy of cancer on a systemic level.

Although mouse antibodies to rabbit globulins were present in sera of sensitized mice, such 'immune' sera did not

have any specific effect on the growth of rabbit antibody-coated Po cells, even when injected simultaneously. Therefore, it would appear that under the experimental conditions chosen for this investigation, tumor destruction was mediated by immune cells rather than by serum factors. However, further studies are needed for the detailed elucidation of the immunological mechanisms involved.

In additional experiments, related to the model system under investigation, it was revealed that spleen cells enhanced tumor growth upon conjoint intraperitoneal application with Po cells, but not when administered by the subcutaneous route. Conversely, splenectomy increased host resistance to this tumor. These results indicate the complexity of the factors determining tumor immunity in Po bearing hosts, and it may be suggested that immunological enhancement is an important escape mechanism for this antigenic tumor.

Chapter IV

Immune Response in Vitro against Tumor CellsRationale

During in vitro experimentation with a methylcholanthrene induced tumor in inbred strain 13 guinea pigs, the candidate discovered that when lymph node cells from outbred Hartley guinea pigs were cultured together with tumor cells in Leighton tubes, foci of round cells appeared on the monolayer by day 6. The round cells rapidly proliferated and within the next 2-3 days the monolayer disappeared completely leaving behind numerous round cells which floated freely in the medium. The floating cells obtained from such cultures morphologically resembled transformed lymphocytes (e.g. lymphoblast, plasma cell). This finding prompted the candidate to explore the possibility of developing an in vitro system for the study of the immune response against tumor cells and of the various immunological parameters determining tumor-host relationships. Obviously, underlying this study, was the expectation that this system may lead to the development of an immunotherapeutic procedure for neoplasia by immune lymphoid cells produced in vitro. Experiments done in this direction and the current phase of development of this system will be presented in this Chapter.

A. Materials and Methods

Animals: A colony of strain 13 guinea pigs (Gp 13) was founded by the author with breeders generously provided in 1969 by

Dr. Merrill W. Chase of the Rockefeller University, New York, N.Y., and since that time breeding was carried out in strict brother-sister or parent-offspring mating under his direct supervision.

A few guinea pigs of the Heston strain (GpH) were obtained from the National Institute of Medical Research, London, England and bred also in the animal facilities available to the candidate in the Department of Immunology.

Outbred guinea pigs of the Hartley (GpHa) strain were purchased from local breeders.

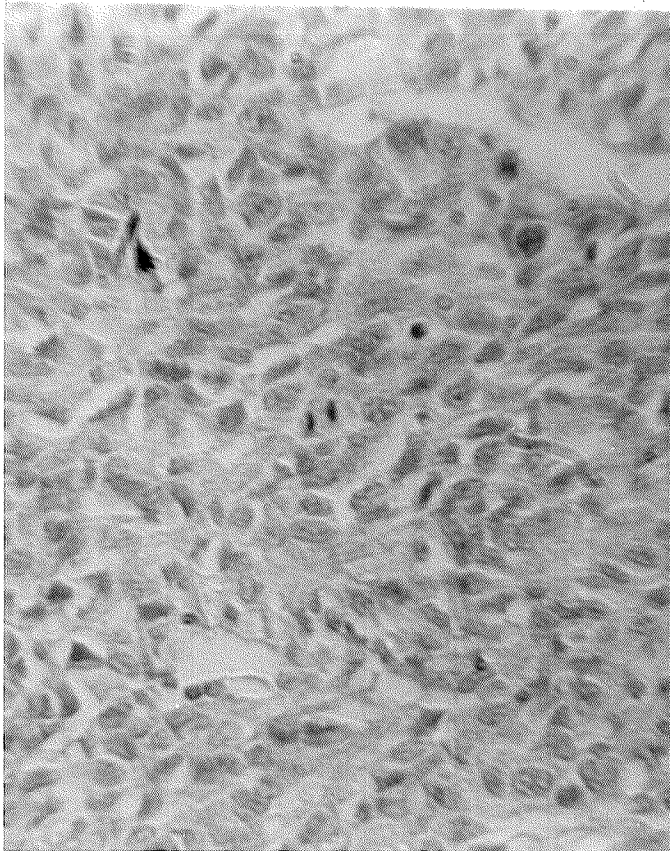
The inbred C57Bl/6J (C57)mice were obtained from Jackson Laboratories.

Tumors: A sarcoma transplantable in Gpl3, designated as MC-D, was kindly supplied by Dr. H.F. Oettgen of the Sloan-Kettering Institute for Cancer Research, New York, N.Y. This tumor had been originally induced in young (1-4 week old) female guinea pigs by injecting 5 mg of 3-methylcholanthrene (MC) subcutaneously under the shaved skin of the abdomen, and appeared after a latent period of 13-14 months (Figure 17). It had been maintained by serial passage in Gpl3 by bilateral subcutaneous implantation with a trocar piece (approximately 40 mg/animal) and was received at the 65th transplant generation in 1969. At the Sloan-Kettering Institute it had been shown that syngeneic animals which were immunized with MC-D homogenates in CFA exhibited a specific delayed hypersensitivity skin reaction at the site of

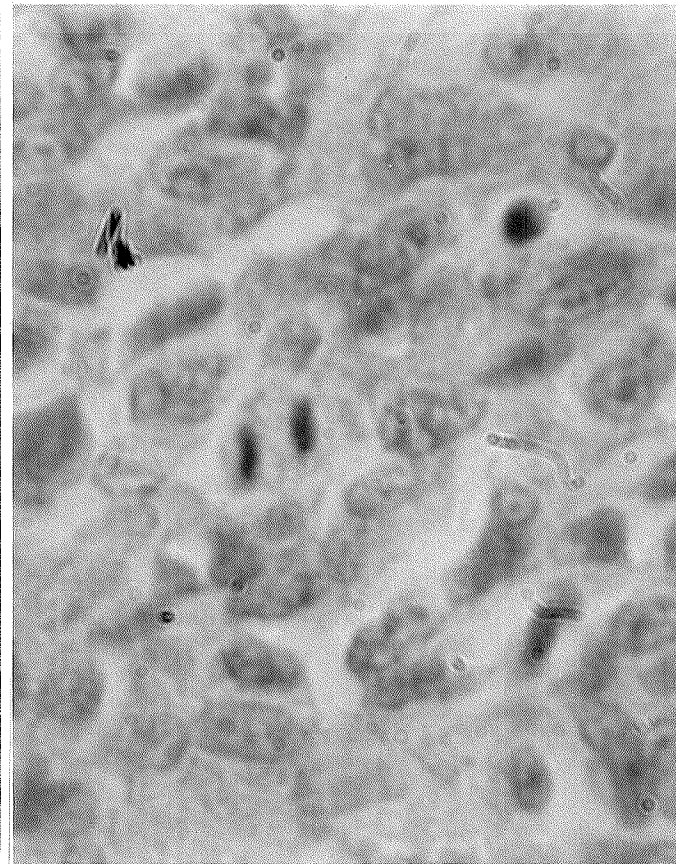
Figure 17. HISTOLOGICAL PICTURE OF MC-D SARCOMA INDUCED BY METHYLCHOLANTHRENE
IN STRAIN 13 GUINEA PIGS

Note a round cell with lymphoid morphology in the upper right corner. (Hematoxylin and eosin, approximate magnification on left 100x, right 400x).

Left



Right



injection of the tumor extract. Skin tests with extracts prepared from fetal tissues, obtained 2.5 and 5 weeks after mating, were negative, and tumor extracts did not produce skin reactions in guinea pigs immunized with homogenates of fetal tissue (658).

For details of the polyoma (Po) and B16 melanoma (B16) tumors of C57 mice, please see the previous chapter.

L929 cells representing a neoplastic cell line of C3H origin, induced by methylcholanthrene in vitro (249), were obtained from Microbiological Associates, Bethesda, Maryland, USA.

Tissue culture materials, fetal calf serum (FCS) and buffer salts were obtained from suppliers as described previously. All stains used were supplied by Fisher Scientific Company, (Fair Lawn, New Jersey, USA.) Radioactive iodine (I^{131}) was obtained from the New England Nuclear Co., (Boston, Mass., USA.) Triethyl phosphate was supplied by Eastman Kodak Co., (Rochester, USA.) Heparin-sodium was purchased from General Biochemicals, (Chagrin Falls, Ohio, USA.)

Antisera: Rabbit antiserum to guinea pig F(ab')₂ preparation and fluorescein isothiocyanate (FITC) tagged sheep anti-rabbit IgG were prepared and kindly supplied by Dr. S. Fujimoto of this Department.

I^{131} labelled rabbit antibodies to guinea pig F(ab')₂ fragments were prepared by Dr. P. Strausbauch, during his fellowship tenure in this laboratory, with whose collaboration the radioimmunoassay was carried out according to a recently described method (414).

Sheep anti guinea pig globulin was obtained from Grand Island Biological Co., (Grand Island, New York, USA.)

Syngeneic anti-MC-D sera were produced in Gpl3 animals by injecting 10^7 MC-D cells intradermally on the interscapular region. The inoculum grew progressively for 7-8 days and was then rejected. On repeated intradermal injections a local reaction occurred, which resembled macroscopically a delayed hypersensitivity skin reaction lasting for 3-4 days. As will be discussed in detail, such animals had circulating antibodies and resisted a subcutaneous dose of MC-D cells, which was much higher than the minimal lethal dose in unimmunized animals.

Outbred guinea pigs (Heston and Hartley) were also immunized by subcutaneous trocar grafts of MC-D. After an initial growth the tumors were rejected in about 10 days (allograft reaction). The injections were repeated, at monthly intervals, several times and the animals producing antibody, detectable by the hemadsorption test, were bled and their serum stored at -20°C until use.

Anti-SRBC serum was prepared also in Heston guinea pigs as follows. One ml of washed SRBC suspended in PBS at a concentration of 10% was injected i.p. 3 times weekly for 3 weeks and the animals were bled 10 days after the last injection. Hemagglutination titers of collected sera ranged from 128 to 512.

The preparation of tumor and lymphoid cell suspensions was carried out by procedures analogous to those described for mouse cells in the previous Chapter.

The separation of white blood cells (WBC) for some of the experiments was achieved by differential sedimentation in dextran. Blood was drawn into heparinized (25 units/ml) plastic syringes and placed into centrifuge tubes. To nine volumes of blood was added one volume of a 10% dextran (MW 250,000; Pharmacia, Sweden) solution in PBS, which had been autoclaved at 121°C for 20 minutes. The dextran was mixed with the blood and the tubes were incubated in a vertical position at room temperature until the red blood cells sedimented to the bottom of the tubes (40-60 minutes). The supernatants, rich in WBC, were collected, washed three times in M199 with gentle centrifugation (400 G, 4°C, 10 min.) and resuspended in CMEM.

Cell cultures: For the cultures of MC-D and other guinea pig cells, Eagle's minimal essential medium, complemented as previously described (CMEM), was used. Other media (e.g. Eagle's medium L, medium HeLa, medium NCTC 109, M199, medium 1066 and medium RPMI 1640) were also tested for supporting the growth of guinea pig cells, but they were proven to be less efficient than CMEM and were not used in later studies. Incubation was carried out at 37°C in a water saturated atmosphere containing 5% CO₂.

MC-D does not grow readily in culture. In order to initiate growth it was found necessary to start the culture with a cell number of or close to 10⁶ trypsinized cells per milliliter of medium. Some of the cells degenerate after an initial growth and eventually die, but others become adapted to the in vitro conditions in about 2 weeks and continue to grow very well with a good plating efficiency.

For the subculture of MC-D and other cell lines, the monolayers were washed 3 times with a balanced salt solution which was free of calcium and magnesium, and incubated at room temperature with 0.25% trypsin dissolved in SMEM until the monolayers could be washed off with a Pasteur pipette (10-15 min.). To facilitate the removal of the cell suspension, the tip of the pipettes were bent to a right angle over a small Bunsen flame. An equal volume of CMEM was added to the suspension of cells to protect them from further trypsin action, and the cells were sedimented in capped centrifuge tubes (400 G, 4°C 10 min.). The cells were resuspended to the desired concentrations in CMEM (usually around 10^5 cells/ml), placed into culture vessels and incubated as previously described.

When tissue culture lines of MC-D were reinjected into syngeneic animals, tumors could be induced with cells kept for 40 days in culture (6th subculture), but not with cells cultured for 130 days (25th subculture).

Staining procedures: In order to obtain good quality preparations various cells were cultured on coverslips in Leighton tubes of 1 ml capacity. The coverslips were then removed at different stages, as desired, and stained according to one of the following procedures.

Wright stain - fixation and staining with Wright solution of

the air dried preparation for 1-3 min.

- an equal amount of distilled water added to the stain and left for another 2-6 min.

- rinse with tap water, dry and mount.

Giemsa stain - fixation of the air dried preparation in absolute methanol for 3 to 5 minutes.

- stain for 30 min. in Giemsa stain (1 volume stock Giemsa stain and 2 vol. M/15 phosphate buffer pH 6.51 added to 47 vol. of distilled water).

- rinse with tap water, dry and mount.

Methyl green pyronin stain: the method recommended by Parker (676) was used, as indicated briefly below:

- fix slide preparations in Kahle's fluid and bring to water.
- stain in methyl-green-pyronin solution for 1 hr, and continue treatment by handling one slide at a time.
- plunge quickly into distilled water, renewed frequently.
- blot quickly with filter paper.
- plunge immediately into a mixture of tertiary butyl alcohol (3 parts) and absolute ethyl alcohol (1 part) $\frac{1}{2}$ min. with continuous agitation.
- rinse quickly in xylene.
- xylene, two changes, 5 min. each.
- mount in Permount.

Hemagglutination was carried out as previously discussed.

Inhibition of hemagglutination: The micromethod with volumes of 0.025 ml (858) was used for the detection of immunoglobulins

of guinea pig origin in the culture supernatants. SRBC are coated with a subagglutinating dilution of guinea pig anti-SRBC antibodies (SRBC-Ab) were reacted for agglutination with a sheep anti-guinea pig globulin serum or rabbit antiserum to guinea pig F(ab')₂ fragments. Test supernatants were diluted serially (2-fold) in PBS + 1% FCS, and to each well in the microplate was added, the agglutinating antiserum corresponding to 4 hemagglutination units (titer/4 dilution). After 30 minutes incubation at room temperature 0.025 ml of 0.5% SRBC-Ab (which was used for the titration of hemagglutination units) was added, and after a few hours the results were evaluated as discussed earlier.

Antiglobulin consumption test: This test was used to detect immunoglobulin of guinea pig origin on the surface of lymphoid cells. The rabbit anti guinea pig F(ab')₂ preparation or the sheep anti guinea pig globulin serum (diluted to four hemagglutination units) was incubated with the lymphoid cells to be tested, for 30 min. at room temperature. The cells were then removed by centrifugation and the supernatant was back titrated for its residual agglutinating capacity with reference to that of the original antibody preparation used.

Mixed hemadsorption was carried out as previously described.

Immunofluorescence test: Cells were grown in Leighton tubes which contained coverslips. The cells on coverslips were fixed in ice cold acetone for 10 min. and treated with rabbit anti guinea pig F(ab')₂ serum, or with control sera for 30 min, at room temperature, in a wet chamber (Petri-dish). The slides were washed

3 times in PBS and were reacted with FITC-tagged sheep antirabbit IgG for 30 min at room temperature, washed 3 times in PBS and finally mounted in PBS containing 10% glycerin and tested for fluorescence under a fluorescent microscope.

Radioimmunoassay: The basic principles of this procedure were identical to those described for the detection of membrane antigens associated with the Epstein-Barr virus (414). Briefly, the globulin fraction of rabbit anti guinea pig F(ab')₂ serum was precipitated with ammonium sulfate (at 50% saturation) and the IgG fraction isolated on a diethylaminoethyl-cellulose column and labelled with carrier-free ¹³¹I by a modification of the Chloramine-T method. The protein concentration of the IgG fraction was determined spectrophotometrically and adjusted to 2 mg/ml with 0.15M sodium phosphate buffer, pH 7.5. First, 50 µl of this protein solution was added to the vial containing 2 mc of carrier-free ¹³¹I and then 0.1 ml of chloramine-T (Eastman Kodak) solution was added at 1 mg/ml in the above buffer. The reaction was allowed to proceed at room temperature for 30 minutes and then stopped by addition of 0.2 ml sodium metabisulfite (1.25 mg/ml) and 0.2 ml sodium iodide solution (10 mg/ml). After 1 minute, the entire reaction mixture was transferred to the top of a small Dowex column (AG1x2, 200-400 mesh, chloride form) in a Pasteur pipette and pre-saturated with 5% rabbit serum. The radioiodinated protein was eluted from the column with 2 ml of phosphate buffer containing 5% normal rabbit serum at 4°C.

Cultures of various cell lines were initiated with 5×10^5 - 10^6 cell/ml in disposable plastic tissue culture tubes (Falcon Plastics, Cat. no. 2003) containing 1 ml CMEM each and locked in a tissue culture rack at a 15° angle. After 2-3 days of culture at 37°C the cells were washed 3 times with Hanks solution (H). The sera to be tested were diluted with CMEM, or with MEM + 10% normal rabbit serum, and 1 ml was added to each tube. Incubation followed for one hour at 37°C and the tubes were washed again 3 times with H solution. Then 50 μl of ^{131}I rabbit anti-GpIgG antibody in 1 ml of CMEM or MEM + 10% normal rabbit serum was added to each tube and incubated for 2 hours at room temperature, which was followed by washing 3 times the tubes again with H solution. Finally, the radioactivity was determined in each tube with an Automated Gamma Counting System (Model 4233, Nuclear-Chicago Corp. Des Plaines, Ill. 60018, USA).

Cytotoxicity test: All attempts to quantitate cytotoxicity by the release of isotope from ^{51}Cr -labelled MC-D cells failed, because 50-60% of the chromium was non-releasable even upon freezing and thawing 3 times. On the other hand, most of the releasable portion of the isotope was discharged spontaneously during 24-48 hours, and, therefore, only little, if any, radioactivity was available for specific release. Consequently, this procedure had to be abandoned and the surviving target cells after cytotoxicity reactions were quantitated by the method described below.

(i) Cultures of target cells were started with 10^5 cells/ml on coverslips in Leighton tubes of 1 ml capacity. The coverslips

were removed at the desired stage of the reaction, stained (Wright stain) and the survival rate of target cells was established (by comparison with controls) by counting the cells in 10 microscope fields (magnification 300x) at 3 mm distances apart along the median line of each of the 10x35 mm coverslip.

(ii) Cultures of target cells were started in plastic microtest plates, covered with a lid (Falcon Plastics, Cat. no. for plate 3040, for lid 3041); 10^3 - 10^4 cells in 0.2 ml CMEM were spaced into each well. At various intervals after adding the materials (cells or cell free supernatants) to be assayed for cytotoxicity, the plates were stained according to Giemsa. All cells in a continuous zone along the diameter of each well (5 microscope areas at magnification 300x) were counted under an inverted microscope.

Inhibition of macrophage migration: The principles of this test were discussed in a recent review (548). In order to obtain peritoneal exudate cells (PEC), normal guinea pigs received i.p. 30 ml of sterile (autoclaved at 121°C for 20 min.) light paraffin oil (Fisher). Three days later 50 ml of ML99 or SMEM was injected i.p. into each animal and, after gentle massage of the abdomen drawn back into the plastic syringe. The syringes were left in a vertical position (downward with the needle) at room temperature until the oil separated out on top of the water phase. This was followed by the collection of cells, which were located in the lower phase, into capped centrifuge tubes, and by washing them 3 times in ML99 (400G, 4°C, 10 min.). PEC were

resuspended to 10% by volume in CMEM and transferred into capillaries (1x100 mm; Kimax, Cat. no. 34502, USA) to approximately $\frac{3}{4}$ of the total length, by capillarity. The empty ($\frac{1}{4}$) ends of the capillaries were sealed over a small Bunsen flame and placed downward into capped centrifuge tubes. The cells were packed by centrifugation (400G, 4°C, 20 min.) and the capillaries were cut with a diamond pencil at the interphase between cells and supernatant. Four capillaries containing packed PEC were fixed on the bottom of each small sterile plastic Petri-dish (Falcon Plastics, Cat. no. 3001) with sterile silicon grease (High Vacuum Grease, Dow Corning Corp. Miland, Michigan, USA). The dishes were sealed with melted paraffin, perforated on the side with a hot needle and filled with test material in different dilutions. The hole on the side was closed with melted paraffin and the dishes were maintained at 37°C for 24 hours. After incubation the areas of migration was magnified 5 times with a photographic enlarger and the perimeters were drawn on paper. The magnified areas were cut out and their weight was determined with an analytical balance.

B. Estimation of the minimal lethal dose of MC-D cells for Gpl3.

For reasons already discussed, the minimal lethal dose of MC-D for Gpl3 was repeatedly investigated in order to be able to detect the onset and progress of tumor rejection in later experiments.

Initially, because of the shortage of Gpl3, single animals received six s.c. injections of MC-D cells (prepared from a solid

tumor by trypsinization) in different doses ranging from 10^3 to 10^6 cells in one ml of CMEM per site, three injections were given on the left flank (10^3) and the other three (10^4 - 10^6) on the right flank. A total of 4 animals were injected in this manner, i.e. with multiple doses of MC-D cells, using different batches of trypsinized tumor cells at different times. The results of these experiments are given in Table 10. As indicated, tumors never appeared at sites injected with 10^4 or fewer cells, and even with 10^5 cells one of four sites remained free of tumor by the time the animal was killed by the tumor growing at the site which received 10^6 cells. Therefore, on the basis of these results, the minimal lethal dose would be considered to be between 10^5 and 10^6 cells. Later, however, when the colony of Gpl3 became larger groups of 3 animals were given single doses varying from 10^2 to 10^6 of MC-D cells prepared from solid tumors in 1 ml of CMEM s.c. (please see experiments no. II and III Table 10) and surprisingly all these animals developed tumors. Therefore, it was concluded that a significant difference in dose-growth relationship existed between animals tested by the two methods of injection.

The growth rate curves of MC-D tumors in Gpl3 injected with single doses of 10^4 , 10^5 , or 10^6 cells s.c. (i.e. experiment no. II of Table 10) are plotted in Figure 18. Tumor appearance and growth rate were not significantly different in animals receiving single or multiple injections of 10^5 and 10^6 cells. Hence, it is unlikely that tumors did not develop at sites injected

Table 10.

THE MINIMAL CELL DOSE FOR TUMOR INDUCTION IN Gp13

Experiment	Number of animals injected	Dose given to each animal	Occurrence* of tumors at the injection site of					
			10	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶ cells
I	4	multiple**	0/4	0/4	0/4	0/4	3/4	4/4
II	9	single***	ND ⁺	ND	ND	3/3	3/3	3/3
III	12	single	ND	3/3	3/3	3/3	3/3	ND

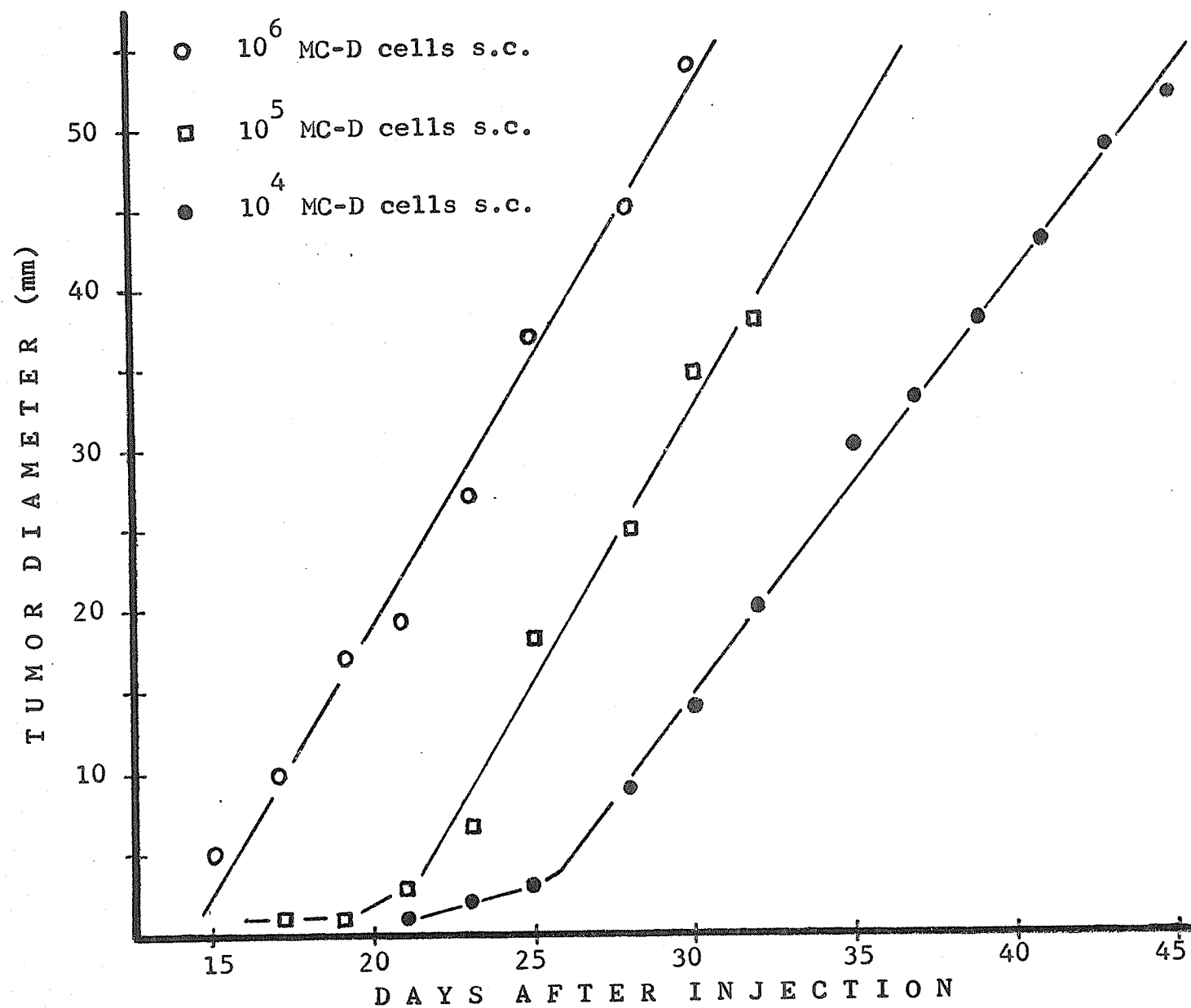
* Number of tumors per number of sites injected

** Each animal was injected with six tumor cell doses, s.c. at different sites

*** Only one dose of tumor cells was injected s.c. per animal

+ ND= not done

Figure 18. GROWTH CURVES OF MC-D TUMORS INITIATED WITH SINGLE CELL DOSES IN SYNGE-
NEIC GUINEA PIGS



with 10^4 or fewer cells in animals receiving multiple doses, because the animal succumbed to neoplasia before tumors could develop at the injection sites of lower cell doses. In actual fact, at the time of death of animals, which had received single doses of 10^6 MC-D cells, which was close to 32 days the mean tumor size was 51 mm, whereas in the group injected with 10^4 cells the tumor size was 20.5 mm (ranging from 14-24 mm). Therefore, it seems likely that immune mechanisms are responsible for the difference in the dose-growth response of animals injected with single and multiple tumor cells doses.

C. The investigation of antigenicity of MC-D in syngeneic guinea pigs

Although it had been reported that syngeneic guinea pigs immunized with homogenized MC-D in CFA gave an immunologically specific delayed hypersensitivity reaction on intradermal injection of the tumor extract (658), the candidate decided to obtain more information on the antigenicity of this tumor by a different approach.

An MC-D cell dose of 2×10^6 , prepared freshly from solid tumor, was injected intradermally into 4 animals on the interscapular region in 0.05 ml CMEM. Tumor growth reached a maximum diameter of 11 mm (average normal skin thickness was 4 mm) on day 8 after injection and the tumors regressed completely during the following week. One month later a second dose of 5×10^6 cells in 0.05 ml CMEM of a fresh cell preparation was injected intradermally into the same animals. This time the local reaction was faster,

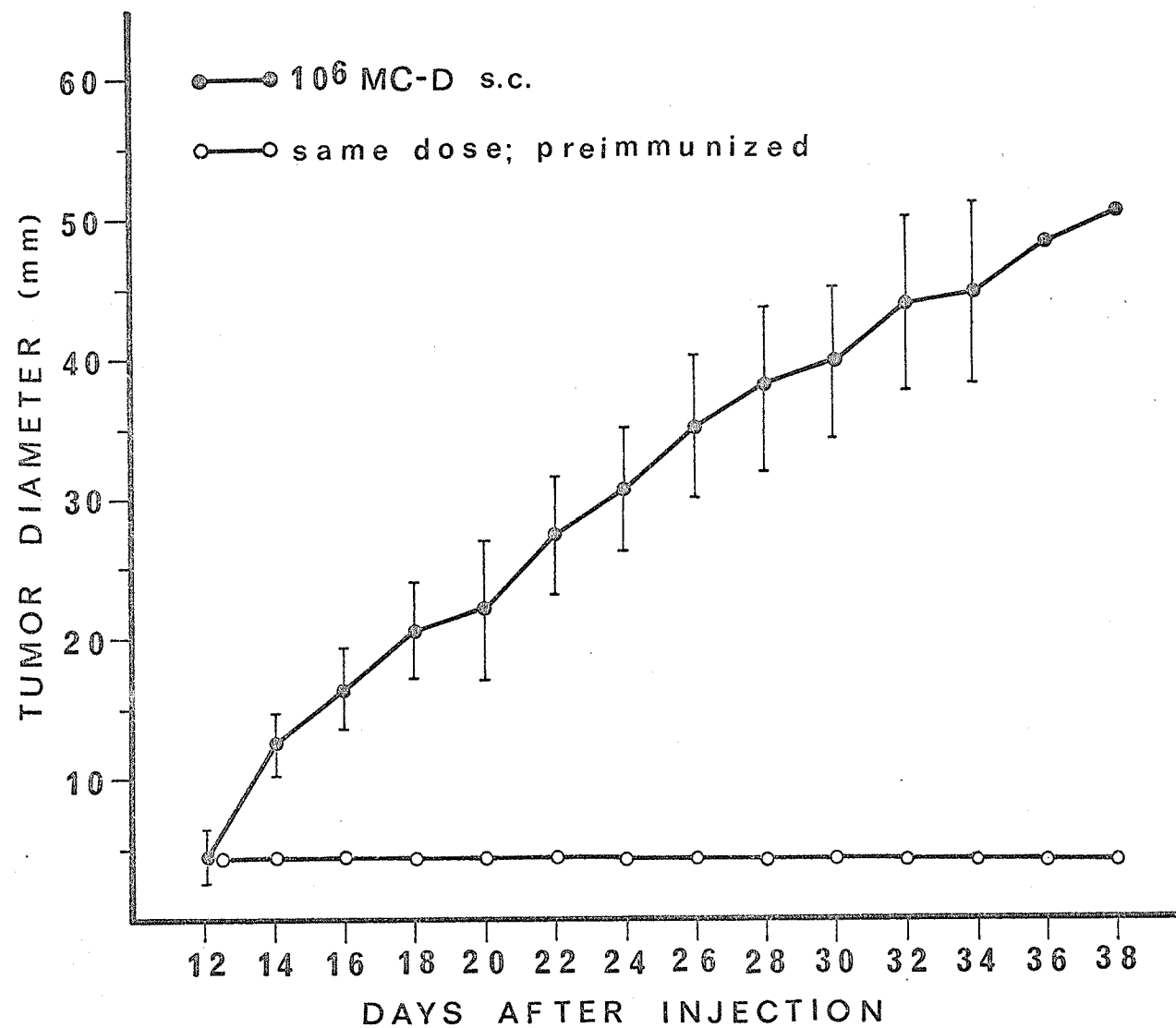
the average skin thickness reached a maximum of 5.45 mm at day 4 after injection, and the inflammation of the injection site macroscopically resembled that of cutaneous delayed hypersensitivity reactions. This reaction subsided completely by day 8. One month after the second intradermal injection all four animals were challenged with two doses of 5×10^5 MC-D cells (freshly prepared from solid tumor by trypsinization) in 1 ml CMEM s.c. on each flank; each of the four untreated control animals received two identical doses of this cell preparation. None of the preimmunized animals developed tumors, while all the controls did so as shown in Figure 19. It is interesting to note that one control animal developed tumor only at one of the injection sites and the other site was negative until death of the animal at day 42 after injection (average survival time in the control group was 40.25 days). The average skin thickness $\pm$ standard error is plotted for the immunized group.

From the available data it can be concluded that pre-immunization of syngeneic guinea pigs by intradermal injections of MC-D tumor cells rendered them resistant to at least a 10,000 fold larger dose of tumor cells than the minimal lethal dose in unprimed animals.

D. Histocompatibility studies

To eliminate the possibility that the immune phenomena observed in the previous experiments might have been due to histocompatibility differences between the colony of inbred guinea

Figure 19. RESISTANCE TO MC-D OF PREIMMUNIZED SYNGENEIC GUINEA PIGS



pigs used at the Sloan Kettering Institute for tumor induction and the colony established in Winnipeg, skin graft experiments were performed with the cooperation of Dr. E. Sabbadini of this Department.

Members of the Winnipeg colony were grafted with the skin of the two tumor bearing animals received from the Sloan Kettering and at the same time grafts were exchanged among animals of the Winnipeg breeding stock. A total of 18 grafts were transplanted. Guinea pig skin is relatively thick and it seemed that for this reason 7 grafts failed to take due to inadequate revascularization. However, all the other grafts, including 3 of 7 donated by the tumor bearing animals, survived indefinitely. Two of such surviving grafts are shown in Figure 20, which were photographed two years after transplantation.

These experiments revealed thus that no major histocompatibility difference existed within the colony of guinea pigs bred in Winnipeg and that these guinea pigs were also histocompatible with the tumor donor strain.

E. In vitro destruction of cancer cells

After having established with a reasonable confidence that MC-D is antigenic in Gpl3, the possibility of mounting an in vitro immune response against tumor cells was studied in this syngeneic tumor-host system.

Lymphoid cells were prepared from lymph nodes and peripheral blood of normal, MC-D-immune and MC-D bearing animals,

Figure 20. SKIN GRAFTS PERFORMED FOR THE ESTABLISHMENT OF HISTOCOMPATIBILITY
OF STRAIN 13 GUINEA PIGS

The pictures were taken two years after skin transplantation.

Left: a graft donated by a tumor bearing animal received from the Sloan Kettering Institute to a recipient from the Winnipeg colony.

Note the growth of hair in the center.

Right: a pigmented graft exchanged between members of the Winnipeg breeding stock was "permanently" accepted (growing black hair).

Left



Right



and cultured with established tissue culture lines of MC-D as outlined in Table 11. In the first experiment, the regional lymph nodes of syngeneic guinea pigs were removed 10 days after injection of 0.1 ml CFA into their foot pads. Adherent cells were removed by overnight incubation at 37°C under the defined conditions. The nonadherent lymphoid cells were then mixed with a trypsinized suspension of MC-D cells, distributed in 1 ml CMEM per Leighton tube, and incubated at 37°C for two days, at which time the cultures were shaken and all the floating cells were discarded by changing the medium. The cultures were inspected daily and the medium was changed at 2-3 day intervals, as required, discarding floating cells on each occasion. On the 5th or 6th day of incubation clearly discernible foci of lymphoblast-like cells (LBC) appeared on the growing monolayers of MC-D cells, which proliferated rapidly with the simultaneous destruction of tumor cells during the following two-three days. The cytotoxic LBCs were always attached to MC-D cells during their lytic activity, but floated freely in the medium when no more target cells were available.

In subsequent experiments LBC were derived from WBC of tumor bearing and normal guinea pigs, as well as from animals immune to the tumor. Cytotoxic LBC were detected, in each case, usually on day 6-7 of incubation, and continued their cytotoxic action on target cell monolayers in a manner similar to that observed in the first experiment. Furthermore, during attempts

Table 11.

THE INDUCTION OF TUMOR DESTROYING LYMPHOBLAST LIKE CELLS IN TISSUE CULTURE

Experi- ment	MC-D target subcultures	cells $\times 10^{-5}$.ml	donor	Lymphoid cells added* source	$\times 10^{-6}$.ml	Appearance of cytotoxicity (days)
I	18	5.0	CFA-immune	Ln	50.0	6-7
II	22	1.0	MC-D-immune	blood	4.4	6-7
III	2	0.6	MC-D-immune	"	0.4	7
			MC-D-bearing	"	2.8	6
IV	2	0.2	normal	"	3.6	6
			MC-D-immune	"	2.4	6
			MC-D-bearing	"	2.2	6
V	18	0.7	MC-D-immune	"	0.17	6
VI	primary	1.0, 10.0	no lymphoid cells added			10-14
VII	"	1.0, 10.0	"	"	"	10-14
VIII	"	10.0	"	"	"	20
IX	"	1.0	"	"	"	10
X	"	10.0	"	"	"	20**

MC-D = methylcholanthrene induced sarcoma (line D) of strain 13 guinea pigs;

* = Tumor and lymphoid cells were mixed prior to incubation;

Ln = lymph node;

CFA = Complete Freund's adjuvant

** = Cytotoxic reaction occurred only in two of the five culture flasks used in this experiment

to derive new tissue culture lines of MC-D, foci of LBC were detected to emerge from the tumor cell cultures around day 10 of incubation and destroyed all spindle shaped tumor cells within the following 2 days (Figure 21 A, B, C, D). This phenomenon was observed several times in primary tumor cultures, without addition of any lymphoid cells to the system as shown in Table 11. If LBC did not appear in the primary culture during the first 20 days, they did not appear later, and in such cases MC-D cell lines were successfully established. In a total of 17 primary MC-D cultures, LBC emerged in eight cases, whereas no cytotoxic cells were formed in 13 primary testis and in 8 primary kidney cultures, although some of these cultures were prepared from tissues of tumor bearing animals. It is worth mentioning that MC-D cell suspensions prepared by trypsinization of solid tumors and stained with Giemsa contained 1.5-2% small round cells with lymphoid morphology.

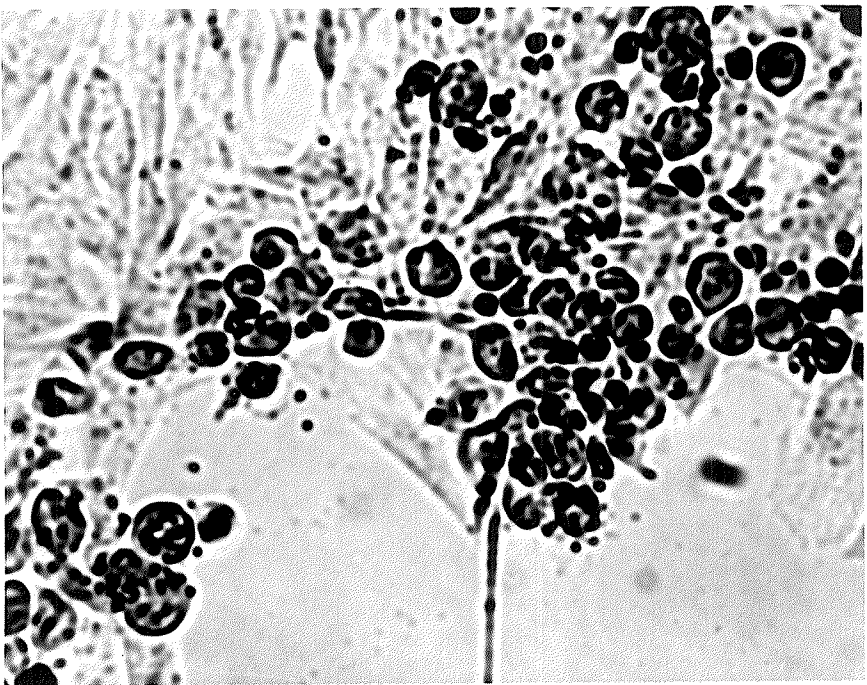
Monolayers of MC-D containing emerging LBC were removed by trypsinization at the earliest time of detection of the cytotoxic cells, i.e. when there were only a few LBC foci present, and the cell suspension was ten-fold serially diluted in Leighton tubes. In these experiments it was observed that as long as a few LBC remained in the tube the whole culture was destroyed.

It was repeatedly observed that LBC did not proliferate in the absence of MC-D cells even when fresh medium was supplied,

Figure 21. DESTRUCTION OF MC-D CELLS BY SYNGENEIC LBC

- A. Photograph of a native primary tumor cell culture showing a focus of destruction by lymphoblast like cells (LBC), which had emerged from the tumor tissue itself 10 days after initiation of the culture. LBC attached themselves to tumor cells and proliferated while lysing them. (Magnification 130x).
- B. Complete destruction of the monolayer of tumor cells two days later in the same culture. Only one spindle shaped 'target' cell is visible in the field. Clusters of LBC are floating freely in the medium. (Magnification 130x).

A.



B.

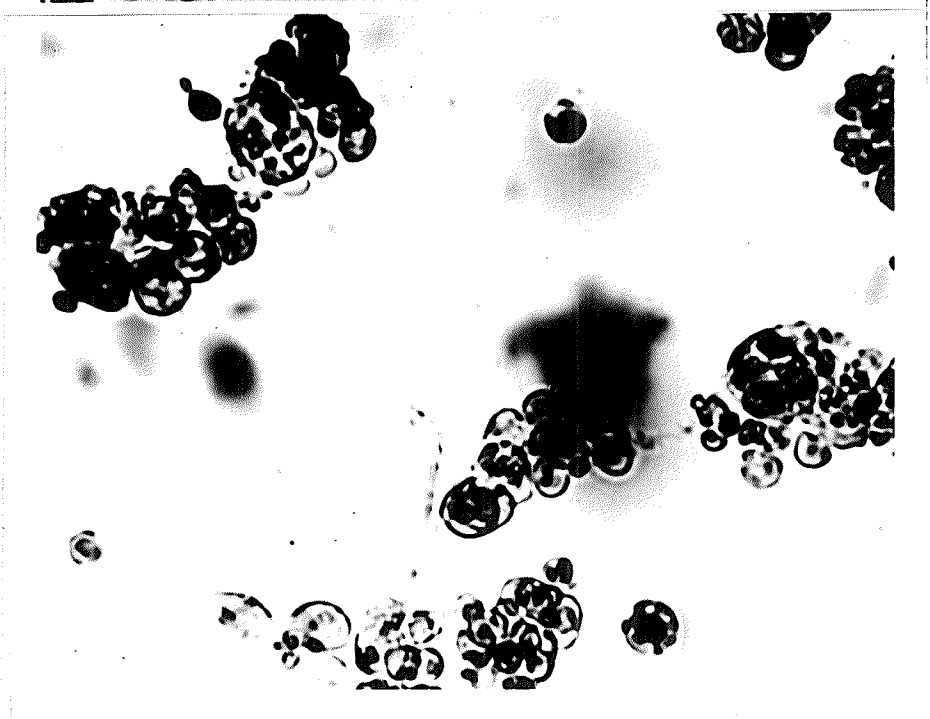
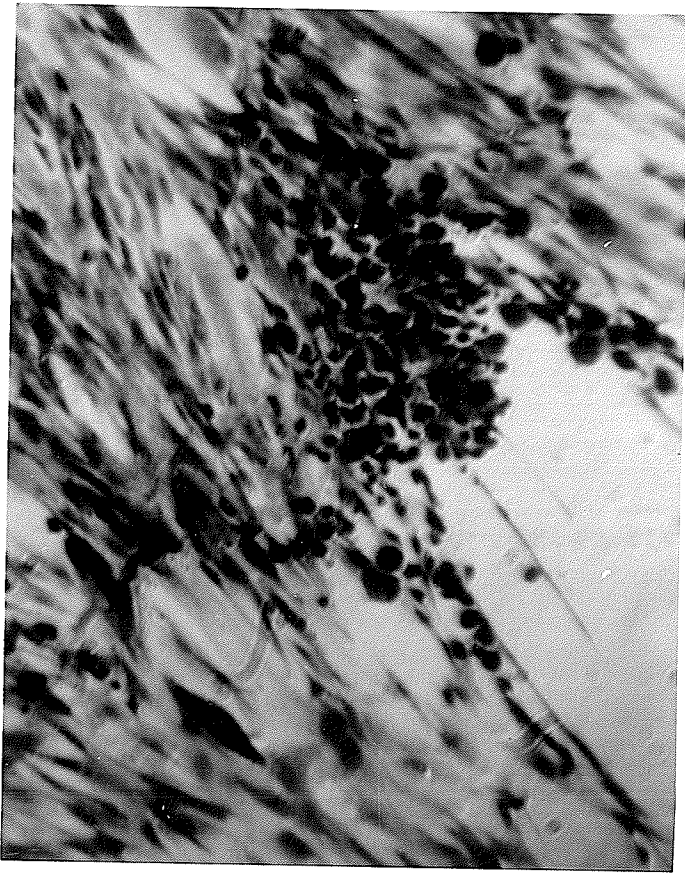


Figure 21. (continued)

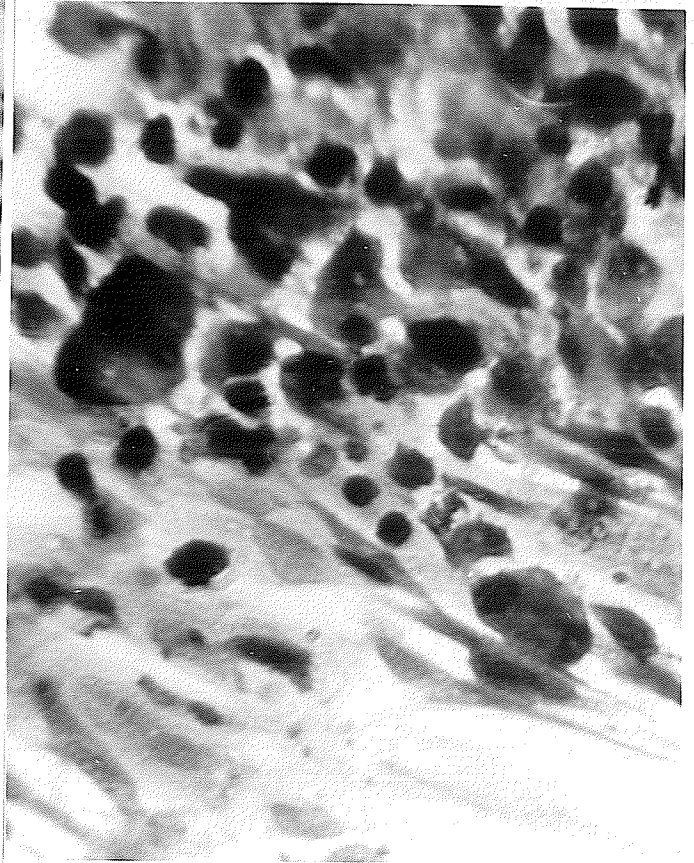
- C. Photograph of a stained preparation illustrating a focus of tumor cell destruction by LBC emerging from the primary culture. (Wright stain, approximate magnification 100x).
- D. Higher magnification of the same focus showing the cytotoxic cells which are heterogeneous in size, some of them having already prominent nucleoli. (Wright stain, approximate magnification 400x).
- E. Cytotoxic experiment with LBC. Tissue culture line of tumor cells was mixed with an identical number of cytotoxic cells (2.2×10^5 /ml, each). The picture was taken after 72 hours incubation. Only a few spindle shaped target cells survived and some of them were in the process of being lysed. (Wright stain, approximate magnification 100x).
- F. Higher magnification of the culture shown in E. Note that LBC have abundant cytoplasm around the excentric nucleus and large prominent nucleoli. (Wright stain, approximate magnification 400x).

C.



D.

230.



E.



F.



and gradually lost their viability as judged by dye exclusion (0.2% trypan blue). However, their proliferation and cytotoxicity was rapidly re-established when MC-D tumor cells were subsequently added to the culture, showing that tumor cells, supposedly the carriers of the antigenic stimulus, were in fact necessary for the maintenance of proliferation and cytotoxicity. LBC could be kept in a state of continuous proliferation and cytotoxicity by a steady supply of MC-D cells for over six months. Murine cells did not provide a similar stimulus.

The cytotoxic effect of LBC was investigated on various target cells established from Gp13, GpH or on cells of C57 mouse origin as shown in Table 12. From the results of these experiments it is obvious that LBC, although produced only in presence of MC-D cells, were cytotoxic for syngeneic, allogeneic or xenogeneic target cells. However, the destruction of MC-D cells was more effective and faster than that of other cell types; normal guinea pig cells were also destroyed completely in three to five days (Figure 21, A, B), but mouse cells were never killed out completely from the culture. For instance, when Po cells were used as targets, an initial cytotoxic effect occurred, which was followed by a slow, but steady proliferation of Po cells (Figure 22) and, finally, by day 10-14 a complete monolayer of Po resulted. On the other hand cell cultures of guinea pig origin, whether syngeneic or allogeneic, were always killed completely, that is the culture was terminated by cytotoxic LBC. In general, target

Table 12.

CYTOTOXIC EFFECT OF LBC ON VARIOUS TARGET CELLS

Experiment	Target cells	LBC added time	$\times 10^{-5}$ ml	Incubation** time	Per cent survival
I	MC-D	24 hr	1.0	72 hr	1.6*
	Gp13-Te	"	"	"	10.5
	Gp13-Ki	"	"	"	26.3
II	MC-D	24 hr	1.0	72 hr	2.9*
	Gp13-Te	"	"	"	15.5
	Gp13-Ki	"	"	"	11.6
	C57 -Te	"	"	"	35.3
III	MC-D	48 hr	4.4	24 hr	13.8
	B16	"	"	"	79.6
	Po	"	"	"	74.7
	MC-D	"	"	72 hr	0.0
	B16	"	"	"	21.0
	Po	"	"	"	56.2
IV	MC-D	48 hr	1.0	48 hr	11.0
	GpH-Te	"	"	"	25.9
	GpH-Ki	"	"	"	31.7

LBC = lymphoblast like cells; these cells emerged from mixed lymphocyte-tumor cell cultures

MC-D = methylcholanthrene induced sarcoma (line D) of strain 13 guinea pigs

Gp13 = strain 13 guinea pig; GpH = Heston strain guinea pig; C57 = C57Bl/6J mouse

Te = testis; Ki = kidney; B16 = B16 melanoma; Po = polyoma of C57 mice

* $P = 0.010$ (MC-D compared to Te); $P = 0.014$ (MC-D compared to Ki) by t test

** after addition of LBC

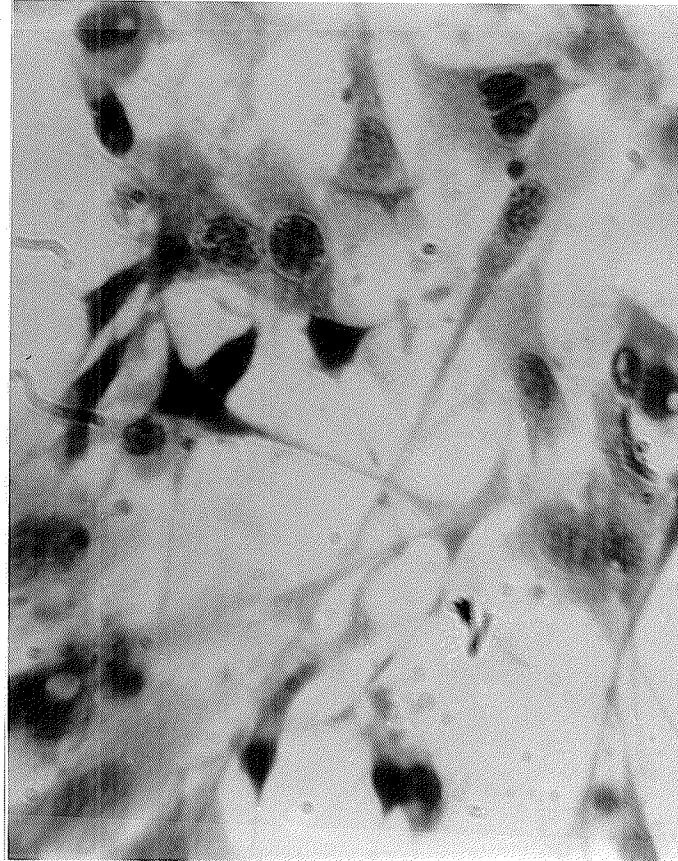
Figure 22. THE CYTOTOXIC EFFECT OF LBC ON Po TARGET CELLS.

Left: 24 hr, right 48 hr. Note the increasing density of target cells.

Left



Right



cell destruction could be accelerated by increasing the number of LBC in the system, and only a few cytotoxic cells were sufficient to initiate a reaction which followed its cytotoxic pattern to completion.

It was essential to establish if the cytotoxic effect observed in this culture system was not due to some micro-organisms (e.g. virus, bacteria) or nonspecific toxic factors, and that it was indeed a property of LBC emerging from lymphoid cells after contact of the latter in cultures with tumor cells. The effect of filtered supernatants of LBC cultures or of the supernatants collected after freezing and thawing of LBC was investigated on target cell culture in microplates. Table 13 illustrates an experiment of this nature where 10^3 MC-D cells were placed into each well of the microplate containing fresh CMEM, or dilutions of supernatants collected from MC-D and LBC cultures. All supernatants were collected after freezing and thawing of LBC except one, which had contained viable LBC. The supernatants were tested for cytotoxicity at different dilutions (in duplicates) in the presence of MC-D cells which were counted in the wells after 96 hours of incubation as previously described; the average cell numbers are indicated in the Table, from which it can be concluded that supernatants from cultures subjected to freezing and thawing had no cytotoxic effects except for a slight growth inhibition at a dilution of 2:3. However a marked cytotoxic effect with the characteristic morphological features of LBC proliferation, was

Table 13.

INVESTIGATION OF LBC SUPERNATANTS FOR CYTOTOXICITY
ON MC-D TARGET CELLS.

Supernatant after freezing and thawing	D i l u t i o n			
	2 : 3	1 : 3	1 : 6	1 : 12
MC-D	424	858	736	1065
LBC-1	367	734	632	951
LBC-2	349	634	614	1067
LBC-3	428	897	881	881
Supernatant with viable LBC	1.5	2.5	12	79
Fresh medium	712 $\pm$ 157.29 (S.D.)			

MC-D = Cultures derived from methylcholanthrene induced
sarcoma (line D) of Gp13
LBC-1, 2, 3 = Lymphoblast like cell cultures

caused with the supernatant of cultures not treated by freezing and thawing which contained viable LBC.

A second experiment of this nature was performed with L929 cells because they are claimed to be the most sensitive targets for lymphotoxin (504) (using again the microplate method for quantitation). The results of this experiment are given in Table 14, from which it is clear that even the undiluted supernatant did not affect the growth of L cells; hence it may be concluded that no lymphotoxins were released into supernatants of LBC cultures.

F. Characterization of lymphoblast like cells

Morphologically LBC resemble transformed lymphocytes (lymphoblasts and plasma cells) as shown in Figure 21 (E, F). The nucleus is located eccentrically in the abundant cytoplasm and a prominent nucleolus is visible. Staining these cells with methyl green-pyronine-Y, a strong pyroninophilia was revealed suggesting an intensive RNA synthesis. LBC incorporated tritiated thymidine and uridine upon stimulation by MC-D as detected by autoradiography*. All examinations for the detection of immunoglobulins in the cytoplasm or on the surface of LBC, using immunofluorescence, mixed hemadsorption, and the antiglobulin consumption test employing rabbit antiserum to guinea pig F(ab)2 and with the corresponding antibodies to rabbit globulins, gave negative results.

A certain percentage of LBC, which was higher in older cultures, always exhibited cytoplasmic vacuoles (Figure 23), some

* These experiments were done in cooperation with Dr. J. Clancy of the Department of Anatomy, Faculty of Medicine, University of Manitoba.

Table 14.

INVESTIGATION OF LBC SUPERNATANTS FOR CYTOTOXICITY ON L929 TARGET CELLS

Supernatant	No. examined	Average cell number in wells with supernatants							
		diluted to							
		1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
MC-D	3	298±79	175±25	185±56	218±5	163±54	156±31	166±55	347±114
LBC	8	257±55	260±13	237±41	265±41	242±43	251±78	226±30	208±46
Average number of L929 cells in fresh medium							326±70		

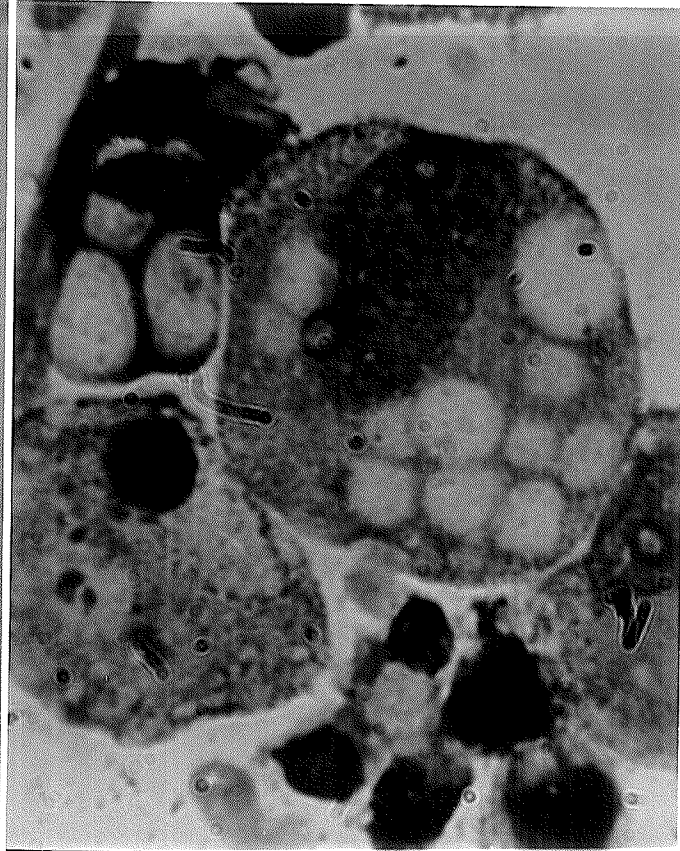
Figure 23. CYTOPLASMIC VACUOLES IN LBC.

(Wright stain, approximate magnification left: 400x
right: 1000x)

Left



Right



of which were very big, being symptomatic of cell degeneration. Immunofluorescent microscopy performed with such preparations did not reveal immunoglobulins in these vacuoles. At the time of conclusion of these experiments, no additional information was available regarding the nature and possible function of these vacuoles.

G. Investigation of LBC supernatants for immunoglobulins and mediators of cellular immunity

(i) Immunoglobulins: Search for immunoglobulins in LBC supernatants was done several times by inhibition of hemagglutination, using, as specific reagents, rabbit antisera to guinea pig F(ab')₂ and sheep anti Gp globulin sera. A total of 34 supernatants collected over a year of experimentation were examined by this method and no immunoglobulins could be detected in any one of them.

(ii) Migration inhibitory factor (MIF): A mixture was prepared from equal parts of 6 LBC supernatants and investigated in a capillary tube migratory system for its possible effect on the migration of PEC, induced by light paraffin oil in normal Gp13 and GpH. Migrations of supernatants of MC-D cells alone and in fresh CMEM were used as a basis for comparison. The results of an experiment performed with Gp13-PEC are given in Table 15, from it is evident that a strong MIF activity was exerted by LBC supernatants, which was observed on both syngeneic and allogenic PEC, and that supernatants of MC-D cell cultures had an enhancing migratory effect.

Table 15.

INHIBITION OF PERITONEAL CELL MIGRATION BY LBC-SUPERNATANTS

Supernatants	Dilution	Migration $\pm$ S.D.
MC-D	-	116 $\pm$ 10
MC-D	1 : 2	153 $\pm$ 14
MC-D	1 : 4	122 $\pm$ 9
LBC	-	57 $\pm$ 9
LBC	1 : 2	83 $\pm$ 15
LBC	1 : 4	93 $\pm$ 10
Fresh medium		100 $\pm$ 7

Peritoneal exudate cells were induced in strain 13 guinea pigs by paraffin oil as described in the text.

MC-D = Cultures derived from methylcholanthrene induced sarcoma (line D) of Gp13

LBC = Lymphoblast like cell cultures

(iii) Skin reactive factor: Supernatants of LBC and MC-D cultures were injected intradermally into normal guinea pigs (0.05 ml per site) and the skin thickness was measured at the injection sites at various times after injection. The results are presented in Table 16. Macroscopical investigation of the sites as well as the skin measurements revealed little if any reaction. A histological picture of the site which had been injected with the supernatant of LBC-3 (sample No.3) (Figure 24) revealed a mild cellular infiltration; however, it should be noted that this was the strongest reaction observed. Thus, it may be concluded that under these conditions no significant amount of skin reactive factor could be detected in supernatants of LBC.

H. Inhibition of tumor growth by LBC in vivo

In this experiment the effect of LBC, which had been induced in mixed lymphocyte-MC-D cultures, was tested in vivo on tumor cells obtained by trypsinization of solid tumors. Four animals were given 5×10^5 MC-D cells s.c. on the left flank and 5×10^5 tumor cells mixed with 10^6 LBC s.c. on the right flank, in a total of 1 ml CMEM. In addition four normal animals were injected on both flank with 5×10^5 tumor cells only. (This group served as a control for the preimmunized animals previously described, and also for the experimental group described here). The mean diameters (tumor growth $\pm$ standard error) final weights of tumors (mean $\pm$ SE) at different times after injection are shown in Figure 25, from which it is obvious that a significant

Table 16.

EXAMINATION OF LBC SUPERNATANTS FOR SKIN REACTIVE FACTOR

Experi- ment	Number of animals	Time after injection	Skin thickness at the injection site of supernatants from cultures of			
			MC-D	LBC-1	LBC-2	LBC-3
I	3	0 hr	4.00	4.00	3.50	3.76
		24 hr	4.10	4.26	3.73	4.46
II	2	0 hr	3.35	3.37		
		3 hr	3.40	4.12		
		6 hr	3.35	3.80		
		24 hr	3.40	3.75		

MC-D = Cultures derived from methylcholanthrene induced sarcoma (line D)
of Gp13

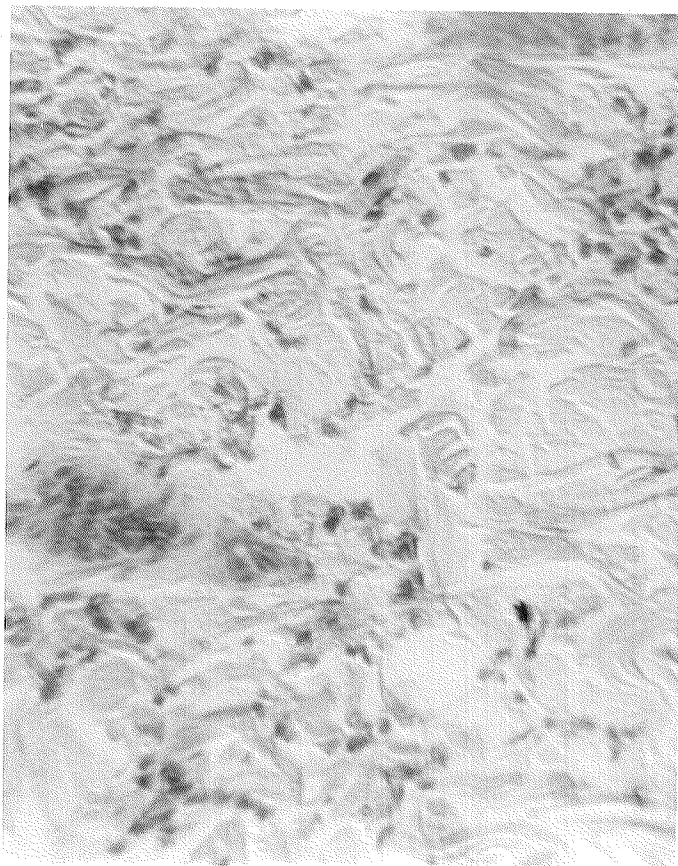
LBC-1, 2, 3 = Lymphoblast like cell cultures

The skin was lifted up at the injection sites and the thickness of the resulting wrinkle was measured by a caliper

Figure 24. HISTOLOGICAL PICTURE OF THE INTRADERMAL INJECTION SITE OF
LBC SUPERNATANT IN Gp13.

A mild cellular infiltration is visible. (Hematoxylin and
eosin, approximate magnification on left: 100x, right 400x)

Left



Right

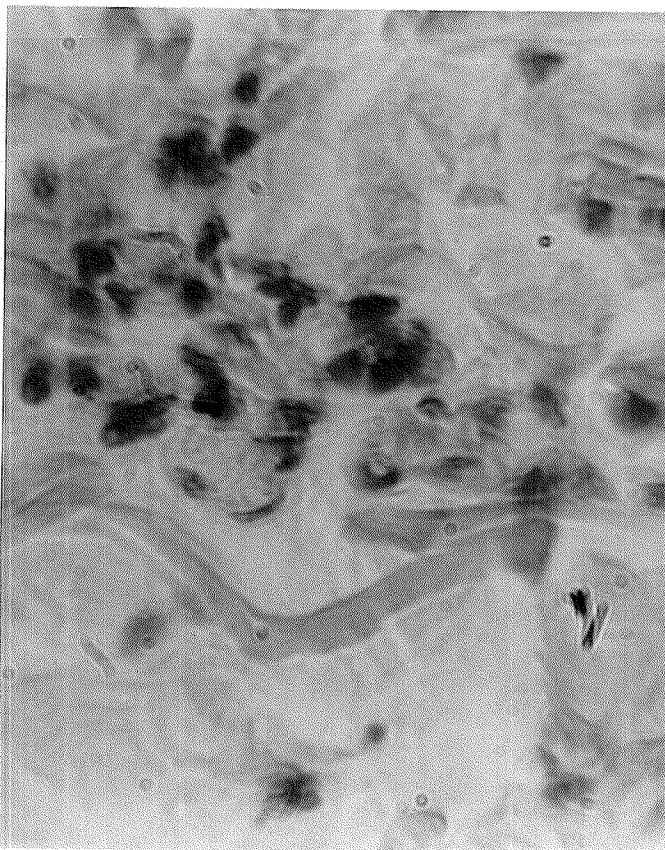
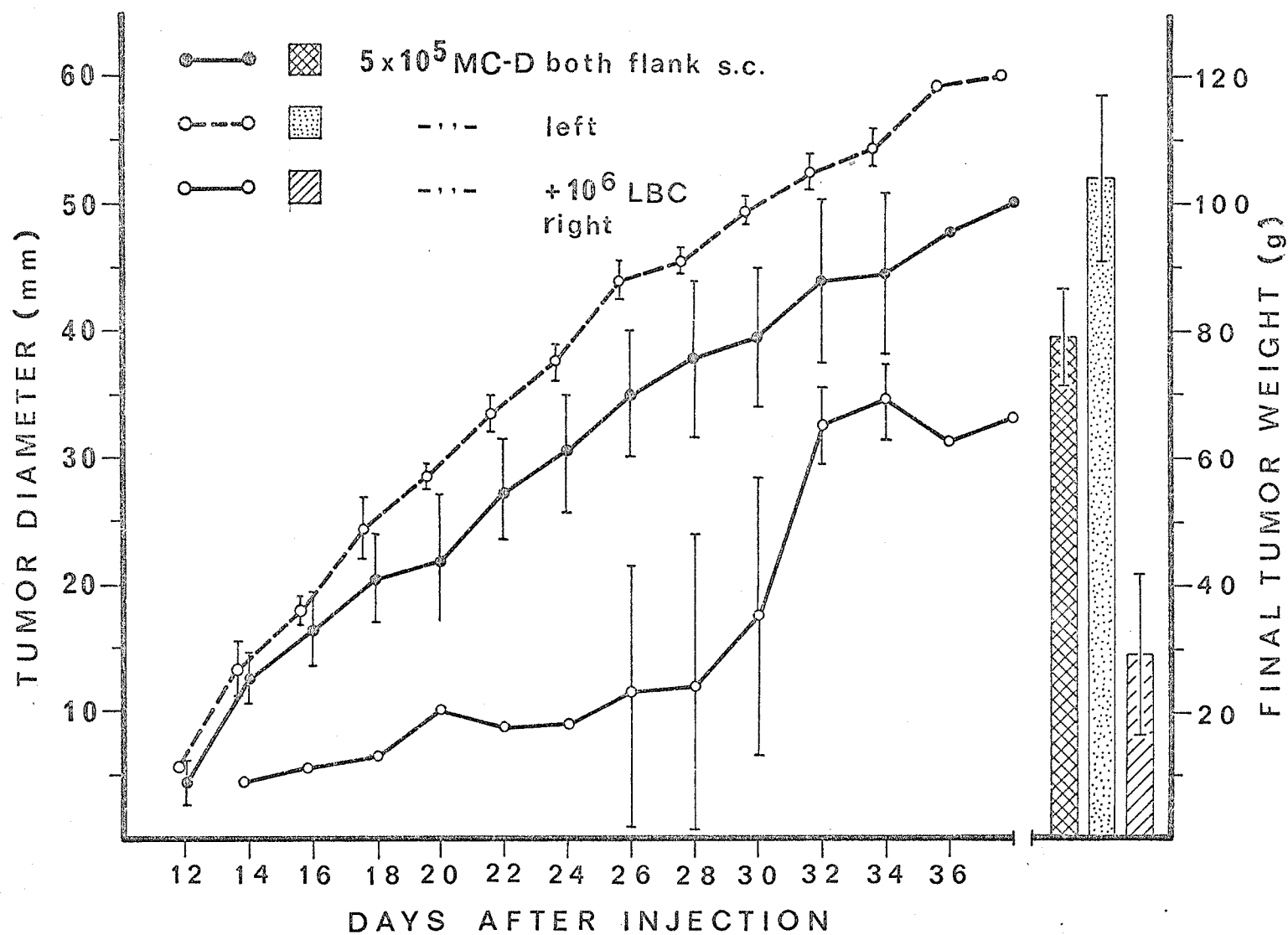


Figure 25. INHIBITION OF TUMOR GROWTH BY LBC IN VIVO



local inhibitory effect was exerted by LBC on the in vivo growth of MC-D tumor under these conditions. Tumor growth on the other flank of the animals injected with LBC, however, was not inhibited at all; in fact, if any systemic effect occurred, it seems to have led to enhancement of tumor growth.

I. Discussion and conclusions

Preliminary experiments have shown that the MC-D tumor in Gpl3 represents a real syngeneic tumor-host system, and that this methylcholanthrene-induced sarcoma is strongly antigenic, which is in agreement with the findings of Oettgen et al. (658). Therefore, it is reasonable to assume, that some lymphocytes were triggered in mixed lymphocyte-MC-D cultures by the tumor antigens to a state of continuous proliferation and cytotoxicity (probably by an immune mechanism). The LBC emerging from such cultures, as well as from primary tumor cultures, possessed some of the characteristics of immune lymphocytes (e.g. pyroninophilia, production of substance(s) with MIF activity, incorporation of radioactive nucleotides upon stimulation, and cytotoxicity). However, these cells did not exhibit absolute immunological specificity, since they were capable of destroying not only MC-D cells, but also testis and kidney cells of Gpl3 and of GpH, as well as murine cells (Table 12). To explain this relative lack of specificity one could invoke the evidence that immune lymphocytes acquire the potential to kill antigenically unrelated cells after stimulation by specific antigen (769; 808);

the main feature in favour of this mechanism is that in this system LBC were more strongly cytotoxic for MC-D target cells than for other cell types.

Alternatively, the apparently broad cytotoxic activity of LBC may be due to antigenic crossreactivity among different target cells, which may be manifested under in vitro culture conditions. Some preliminary results obtained by radioimmunoassay suggest such an antigenic relationship, as shown in Table 17. It appears that syngeneic guinea pigs immunized against MC-D and developing resistance to the tumor do have circulating antibodies (anti-MC-D serum) which react with all cell lines tested. The "positive" control serum used was produced in Heston guinea pigs with monthly trocar injections of MC-D and had a hemadsorption titer of over 1:1,000. ^{131}I -labelled rabbit IgG antibodies to guinea pig F(ab')₂ were used to detect guinea pig antibodies reacting with various cell lines. Incubation with normal guinea pig serum ("negative serum") resulted in much less binding of radioactivity to the cells, however, some radioactivity appeared to be bound nonspecifically on cells which had been exposed to this control serum in comparison with the counts detected in the tubes containing cells which had not been incubated with guinea pig serum at all. Therefore, it seems, that the idea of antigenic cross-reactivity of MC-D with tissue culture cell lines, derived from other guinea pig tissues and perhaps also with mouse cell lines is not entirely unfounded. Further experimentation would reveal whether this tentative interpretation is correct or not.

Table 17.

RADIOIMMUNOASSAY OF VARIOUS CELL LINES WITH A SYNGENEIC ANTI MC-D SERUM

Experi- ment	Cell line	Serum di- lution	Counts per 10 minutes in tubes with			
			anti MC-D serum	positive serum	negative serum	no serum
I	MC-D	1:5	3222	2505	573	ND
	"	1:15	3025	2566	413	"
	"	1:45	1854	1434	ND	"
	"	1:135	1085	753	"	"
II	MC-D	1:10	2875	3017	1441	821
	Gp13-Te	"	3453	2796	1172	461
	Gp13-Ki	"	4038	3193	878	383
III	MC-D	1:10	3966	3619	465	206
	L929	"	4015	3393	799	438
	None	"	733	450	393	234

MC-D = methylcholanthrene induced sarcoma (line D) in strain 13 guinea pigs
 Gp13-Te = tissue culture line of cells derived from the testis of normal strain 13 guinea pig
 Gp13-Ki = tissue culture line of cells derived from the kidney of normal strain 13 guinea pig
 L929 = tissue culture line of cells of C3H mouse origin
 ND = not done

Although the factors determining the development of the cytotoxic effects of LBC have not been elucidated, it is clear that these cells require target cells, which are presumably the carriers of the antigenic stimulus, for maintenance of their proliferation and cytotoxicity. It is suggested that LBC, which emerged from primary MC-D cultures without the addition of lymphoid cells to the system, may represent the progeny of lymphocytes reacting against tumor antigens in vivo. The existence of such concomitant immunity in tumor bearing animals is also indicated by the experiments on the dose-growth relationship of MC-D tumor in Gpl3 (Table 10). In support of this interpretation, one may also cite the fact that cytotoxic LBC were never observed in primary cultures of kidney or testis cells, i.e. no sensitized lymphoid cells were present in these organs, whereas the MC-D tumor appeared to be infiltrated by lymphocytes which were apparently activated to a cytotoxic state - presumably - by the tumor antigens. Clearly, further experimentation is required to unravel the detailed mechanism underlying the emergence of cytotoxic lymphocyte-like cells in the presence of tumor cells and the precise nature of these cytotoxic cells.

Chapter V

General discussion *

Basic immunology and related fields have been developing in recent years at an accelerating rate, but the vast amount of accumulated information cannot as yet be integrated into a clearly defined pattern, because of the complexity of the biological mechanism underlying the immune response. By way of generalization, one may state with a fair degree of confidence, on the basis of sound experimental evidence, that for the induction of an immune response, apart from the presence of an immunogen, the cooperation of various cell types is necessary. So far 3 interacting cell types have been distinguished, namely A (accessory) cells, B (bone marrow derived or, more precisely, Bursa equivalent) cells and T (thymus derived) cells (668).

A cells are radioresistant, do not have specificity for the immunizing antigen, adhere to solid surfaces in culture, and their function is considered to be the handling, processing and probably the presenting of antigen to T and B cells, which are the specific responder immunocytes. Two subpopulations are suggested for A cells, one of them consisting of phagocytic cells which recirculate (macrophages), and the other of sessile cells which may not be phagocytic.

The major function of B cells is antibody production;

* It is not possible to cite all the relevant references here; for a detailed discussion of various statements, please refer to the corresponding chapters of this Thesis.

these cells exhibit specificity for the immunizing antigen (i.e. have receptors which are most likely surface bound immunoglobulins), are radiosensitive and nonadherent. In addition, B cells recently have been claimed to have receptor sites for the Fc fragment of immunoglobulin(s) and also for complement, and were found to participate in antibody dependent and complement dependent cell-mediated target cell lysis (84; 106; 686). It is not clear whether or not cytotoxic and antibody forming cells belong to the same population, or represent different subpopulations of B cells. For antibody forming cells, however, the existence of two subpopulations has been suggested, one of these requiring the cooperation of T cells for the initiation of the humoral response (thymus dependent) the other being independent of T cells (thymus independent) (702).

The major characteristics of T cells are more or less similar to those of B cells, e.g., they do bear specific receptors for the recognition of antigen (IgX, IgT, IgM?), are radio-sensitive, and nonadherent. However, they do not produce antibodies and are sensitive to cortisone (B cells are not). Triggered T cells are strongly pyroninophilic (356; 601). No immunoglobulin or complement receptor sites have been detected so far on T cells. T cells seem to have a very important function in the immune response. They are capable of cytotoxicity and of producing a number of biologically active substances (mediators of cellular immunity), which are able to amplify greatly the cellular immune response mediated by T cells, and they seem to control the humoral immune response to a vast majority of antigens as well (thymus

dependent antigens). Evidence points to the direction that immunological tolerance and memory to the thymus dependent antigens are also the functions of T cells (701). Recently, as for B cells, the existence of two subpopulations has been suggested for T cells, one of which is cortisone sensitive, the other cortisone resistant (178).

Besides A-, B- and T-cells, other cell types may also participate in the induction phase of the immune response. For instance it has been noted that eosinophil granulocytes proliferate in mice upon antigenic stimulus (590). Mast cells were also shown to proliferate in rats after antigenic stimulation (604).

Cell interaction during the effector phase of the immune response seems to be even more complex and may involve multiple and alternative pathways. Antibodies may serve as specific receptors for most if not all cell types of the leukocyte series. It is well established that human basophils and mast cells have specific receptor sites for the Fc portion of IgE (homocytotropic antibody) (450), monocytes and macrophages for IgG₁ and IgG₃ (cytophilic antibody, opsonizing antibody) (432; 433) and neutrophils also for IgG₁ and IgG₃ (583). In all these cases the Fc portion of the immunoglobulin molecule was shown to combine with the binding site on the cell surface. Furthermore, human monocytes were shown to have receptors for C'3 which may function either independently or cooperatively with IgG binding sites in the induction of phagocytosis (opsonization) (432).

Lymphocytes of the B population were also claimed to exert immunologically specific cytotoxicity, triggered by immuno-

globulins combined with cell surface antigens, or by fixed complement (C'7) (84; 106; 384; 686; 687).

During the complement reaction a biologically active material, capable of antigen-independent histamine release from mast cells (anaphylatoxin), is produced, as well as other factors which are chemotactic for polymorphonuclear leukocytes (729).

Finally the majority of the mediators produced during cellular immune reactions by T cells may be classified as amplifying factors, which act on other leukocytes, inducing thus cooperation which greatly increases the (defensive) power of the reaction. Chemotactic factors for macrophages (216; 924) neutrophils (923) and eosinophils (183), mitogenic factors acting on other lymphocytes (470; 471; 410; 708), the transfer factor (525) and the skin reactive (inflammatory) factor (629) may be listed in this category. Until recently the basophil was considered to be the only type of leukocyte not affected by a specific factor; however, it has been suggested that T cells may produce such a material in relation to cutaneous basophil hypersensitivity (246).

The mechanism of regulation of this labyrinth of immunological pathways remains essentially undefined. Immunological tolerance in terms of the clonal selection theory of Burnet (125) served as an explanation for many phenomena including self tolerance. In a recent development of this hypothesis, it has been proposed that all lymphocytes which express "self" reactivity during differentiation in the thymus are destroyed in situ and that, whenever the thymic function is disturbed, "forbidden clones"

capable of auto-reactivity may arise and cause autoimmune disease (126). Although one may cite supporting evidence for this hypothesis, it is to be noted that autoantibodies to various tissues are frequent in man and that autoimmunity is readily inducible in experimental animals. Furthermore, many of the induced autoimmune conditions are self-limiting and subside spontaneously if the provocative treatment is ceased (21). Thus self reactivity and some kind of a compensating mechanism seems to exist. It may be envisaged that such a mechanism is vital for survival, since otherwise immune reactions analogous to the devastating effects of autoimmunity would be ubiquitous in the body. All the intracellular parasites (viruses, bacteria etc.) and a great variety of tissue injuries may be considered, and many of them were in fact shown to induce new antigens on the cell surface. Such antigens are able to trigger immune reactions (see under virus induced tumor antigens) in the host. It was shown that cells which are antigenically converted in this manner can easily be killed in vitro by immune reactions directed to the newly induced antigens. A variety of hypersensitivities and allergic tissue injuries manifest themselves in an analogous manner in vivo. In the majority of cases, however, autoimmunity is not triggered after a virus infection or tissue damage, and full recovery follows, pointing again to the existence of a regulating mechanism.

The weight of accumulated evidence indicates that the course of many autoimmune conditions does not appear to be correlated with the presence of humoral antibodies and it is worth noting that

normal individuals may also have autoantibodies (21, 700, 785). This lack of correlation between the presence of autoantibodies and pathological changes strongly suggests that antibodies are not always responsible for pathogenesis. If one assumes that some autoantibodies are able to protect against autoimmune damage in a manner similar to that of graft or tumor enhancement, the lack of correlation with pathological changes is understandable. For example, it is well documented that experimental allergic encephalomyelitis is transferable by sensitized lymphoid cells and that humoral antibodies are able to interfere with the manifestation of the disease in the presence of autoaggressive lymphocytes (21). A similar serum-mediated protection of antigenic kidney grafts has been achieved in experimental animals (305; 423; 851) and also in human (65).

It was recently suggested that a number of immunological phenomena which were formerly believed to represent true examples of immunological tolerance were in fact governed by analogous serum mediated immunological mechanism, i.e. a cell-mediated immune response is present and tends to cause tissue damage, but humoral factors (antibody) protect the tissues from injury. This was described for allophenic (tetraparental) (691; 930) and F_1 hybrid mice (694), and for mice made chimeric at birth with the neonatal injection of F_1 hybrid spleen cells (400).

Such antibody mediated tissue protection is known as immunological enhancement, which may be regarded as the counterpart

of rejection, whereby an antigenic tissue is not destroyed and may remain intact structurally as well as functionally for long periods of time, despite the presence of immune reactions against it. Both cell-mediated and humoral immunity are detectable in these situations.

Until a few years ago it was commonly believed that most "autochthonous" human tumors were not antigenic and could, therefore, progress readily in the host. However, recently, a convincing body of evidence has been adduced to undermine this belief and the present consensus is that most tumors, whether spontaneous or experimentally induced, are in fact antigenic. In spite of the mounting of both humoral and cell-mediated immune responses by the host, which are readily detected, the tumor continues to grow causing eventually the death of the host. Therefore, it seems reasonable to assume that there must be an escape mechanism which permits tumor growth. Of the various mechanisms discussed in Chapter II, immunological enhancement appears to be the most frequent mechanism for the escape of antigenic tumors. Therefore, one is led to conclude that the same mechanism, which is presumably vital for protection against autoimmune tissue damage, causes fatal disaster by protecting the neoplastic tissue from immune destruction.

It was proposed on theoretical grounds that antibodies may interfere with tissue damage centrally by acting on the reacting lymphocytes, which are located in the lymphoid organs, or peripherally by covering up the (foreign) antigenic determinants on the tissue, thus preventing recognition by sensitive lymphocytes

(86). For the peripheral mechanism, an ideal candidate capable of protecting the tissue would be an antibody which does not agglutinate or precipitate with the antigen and which is unable to fix complement and hence to cause damage on combining with tissue antigens. Such antibodies designated as "incomplete" antibodies, have long been known to exist in various autoimmune conditions to erythrocytes (700). Passive enhancement, however, can be induced with microliters, or even smaller quantities of antisera, and hyperimmune sera are usually more effective (464), which suggests a central, rather than a peripheral mechanism. For the numerous experimental data available it is clear that there is no general rule for enhancement. Factors capable of shifting the immunological balance from cell-mediated to humoral response and of changing the molecular composition of the humoral antibodies, which in turn affect the relative vulnerability of the tumor to complement mediated cytotoxicity, are all important regarding the end result (464).

Experiments on the minimal dose required for tumor induction revealed a complex relationship, i.e., in certain cases tumor take is higher at a low dose of cells than at a medium dose, and the incidence increases again with increasing the cell dose (439; 661). This phenomenon resembles low and high zone tolerance, as observed in studies of basic immunology. However, it is not possible to conclude on the basis of this similarity if indeed similar immune mechanisms are responsible for these two phenomena. Theoretically, one would expect that growing tumors would reach at a certain stage

an antigen level which would cause "high zone tolerance" or "immunological paralysis", and in actual fact "unresponsiveness" to progressing tumors in both animals and human have been described. Circulating soluble antigens have been also found in hosts bearing various viral leukemias and other tumors, which may be considered as deaggregated antigens (not sedimented by 100,000 G for 1-2 hours), which are known to induce immunological tolerance (931). Finally, the condition designated as immunological inertia of cancer patients, which manifests itself in general immunological unresponsiveness might be mediated by mechanisms involving antigenic competition.

It is well known that tumors are very diverse, both biologically and antigenically, and hence the immunological factors governing tumor-host relationships are probably unique to each type of tumor. In addition, they represent a dynamically changing antigenic stimulus, which is seldom investigated in basic immunology. Episodes of regression followed by progression, appearance and disappearance of delayed skin reactivity and of cytotoxic antibodies and enhancing serum factors may be observed. Obviously, the best strategy for the immunotherapy of cancer would be to eliminate the undesirable (e.g. enhancing) immune reactions and reinforce those which tend to protect the host, i.e. cell-mediated immunity in the case of solid tumors. Presently there is no safe method to achieve this and immunological enhancement remains the greatest hazard in the development of an immunotherapeutic approach to the destruction of tumors.

In this study attempts were made to develop immunological reactions against cancer, which can be controlled. One approach consisted in attaching new antigens to the cells of a solid tumor and directing cellular immunity against these antigens, in the hope that tumor destruction would result from the interaction of sensitized lymphocytes with this artificial neo-antigens located on the surface of tumor cells. The second approach involved the development of an in vitro system of cytotoxic reactions for the destruction of tumors, which would permit the study of various immunological parameters of tumor-host relationships and which may have the potentiality of an effective therapeutic method.

For the study of tumor destruction by the proposed indirect, cell-mediated immune mechanism, an antigenic polyoma virus-induced tumor of C57Bl/6J mice was used as a model system. Tumor specific antibodies were prepared in rabbits and syngeneic mice were sensitized to rabbit immunoglobulins (RIg) in the form SRBC-RIg complexes in complete Freund's adjuvant (CFA). The growth of Po cells coated with tumor specific rabbit antibodies was inhibited in vivo, when the Po cells were mixed with sensitized lymphoid cells and injected s.c. to syngeneic animals. The inhibition was immunologically specific.

Regression of primary or secondary cutaneous tumors in patients has been successfully achieved by a similar indirect immunological mechanism, namely by inducing a delayed hypersensitivity reaction locally in the tumor foci with sensitizing agents, such

as trenimon (395; 486; 941), dinitrochlorobenzene (486, 844, 941), BCG (624), vaccinia virus (441), or nontoxic extracts of hemolytic streptococci (638). Immunotherapy of basal cell carcinomas with trenimon or dinitrochlorobenzene was claimed to be as effective as with the best chemotherapeutic agent (941).

More recently, similar results were obtained in experimental animals. Thus, guinea pigs injected with a lethal dose of tumor cells were protected when a delayed hypersensitivity reaction was elicited locally in the site of injection with BCG, tuberculo-proteins or with antigenically unrelated tumor cells. However, it is to be noted that close contact between sensitized cells, sensitizing agents and antigenically unrelated tumor cells was required to suppress the growth of these tumor cells. Tumor graft rejection without delayed hypersensitivity skin reactions did not cause suppression of antigenically unrelated tumor (963; 966).

None of these agents, however, is able to reach tumor nodules in deep tissues and, therefore, they are not effective for systemic treatment. In addition in the case of local treatment the antigen used to elicit delayed hypersensitivity reaction may be applied in optimal doses, while in the model system developed in this study, the density of artificial neo-antigens on the surface of Po cells was limited by the number of antigenic determinants accessible to tumor specific antibodies. Yet, despite this limitation, tumor specific antibodies were shown to be capable of functioning as neo-antigens, and were able to activate sensitized

lymphoid cells so as to induce tumor inhibition or even the complete rejection of a lethal inoculum.

The cardinal problem in this model system is the production of tumor specific antibodies which are also the carriers of new antigenic determinants. As has been shown production of tumor specific, heterologous antibodies by absorption is very difficult, because of the inherent danger that the tissues used for absorption may possess low concentrations of the tumor-associated antigens and may thus remove all the required antibodies. Moreover, there is the possibility that the proteins used for coating the tumor cells may be strong antigens and might not favour the type of sensitization which would lead predominantly to the production of killer cells required for tumor rejection. In this connection it is worth mentioning that studies in mice, in which chemically modified tumor cells were used for immunization against a subsequent inoculum of intact tumor cells, revealed that tumor cells coupled to "strong" antigens provided no protection, or actually induced enhancement, while those coupled to "weak" antigens elicited protective immunity (35; 709). Hence, it is obvious that the proper choice of neo-antigen for this indirect model system is one of the important factors which will determine the final outcome of the reaction. Antibodies coupled to haptens, or to compounds which could serve as haptens and also for a variation on this theme, one may suggest the coupling of cytotoxic agents (e.g. trenimon) to antitumor antibodies, which could act as haptens for the induction of delayed hypersensitivity.

With such improvements of the model, it is anticipated that neo-antigens may be used as 'homing' devices for localizing in tumor foci sensitized lymphoid cells capable of unleashing a local inflammatory reaction leading to tumor destruction. In principle this procedure should prove applicable on a systemic basis for the treatment of tumor foci located in deep tissues which could be thus reached ~~and~~ by antitumor antibodies and the appropriate sensitized cells.

In the second phase of this study the possibility of mounting an in vitro immune reaction against tumor cells was studied. A methylcholanthrene induced sarcoma (MC-D) in inbred strain 13 guinea pigs was used as a model system. Lymphoblast-like cytotoxic cells (LBC) were initiated in mixed lymphocyte tumor cell cultures. Interestingly, LBC also emerged from some of the primary tumor cultures without the addition of lymphoid cells. From the overall features of this in vitro system, one may conclude that it possesses some of the characteristics of other cell-mediated immune reactions, induced in vitro against allo- or xenoantigens, i.e.

(i) LBC incorporated tritiated thymidine and uridine upon stimulation by MC-D as detected by autoradiography (551).

(ii) LBC were strongly pyroninophilic as revealed by staining with methyl green pyronil-Y (899).

(iii) No immunoglobulin was detected either on the surface of LBC or in the medium of MC-D cultures lysed by LBC (166; 899).

(iv) Cytotoxicity was detected after 6-7 days of incubation (38; 177; 338; 391; 421; 500; 823). Most of the investigators working with other systems claimed specific cytotoxicity; however,

in two studies the cytotoxic effector cells produced in mixed lymphocyte cultures with mouse (421) and human (823) cells exerted nonspecific cytotoxicity.

(v) Production of substance(s) with MIF activity was detected in LBC supernatants.

On the basis of the above results the thymus derived lymphocyte appears to be a likely precursor for the LBC detected in this study (601). Although LBC possessed some of the characteristics of immune lymphocytes, they did not exhibit absolute immunological specificity since they were capable of destroying not only MC-D cells, but also testis and kidney cells of syngeneic or of outbred Heston guinea pigs, as well as murine cells (Table 12). In this context it should be noted, however, that recent studies revealed similar cytotoxic behaviour of "effector" cells produced in a mixed lymphocyte culture (421; 823), which is a system considered to represent an in vitro correlate of cell-mediated immune reactions involving an immunologically specific proliferation of lymphocytes (551). In this respect one may quote the finding that lymph node cells from inbred rats with cell-mediated immunity to tuberculo-protein, bovine serum albumin, or egg albumin produced progressive destruction of monolayers of rat embryo fibroblasts in tissue culture, which was apparent at 48 hours and maximal at 72 hours. This effect was specific for the sensitizing antigen and did not depend on the genetic difference between lymph node cells and target cells (769). Damage of rat embryo fibroblasts in the experiments was shown to be closely correlated with delayed skin reactions in

the animals from which the lymph node cells were taken, but not with Arthus reactivity. In animals sensitized with picryl conjugates of ovalbumin or of human serum albumin, skin reactivity and the in vitro cytotoxic effect could be elicited only with the homologous conjugate or the hapten carrier alone, but not with picryl conjugates of heterologous proteins (770). Essentially, similar results were obtained by the candidate's colleagues in a contemporary study with lymph node cells from guinea pigs sensitized to a state of delayed hypersensitivity to mouse or rabbit gamma globulins. Such sensitized lymphoid cells induced lysis of mouse sarcoma I cells, when the target cells were coated with the corresponding globulins in the form of anti-sarcoma I antibodies. Similar lysis of target cells was induced by immune lymphoid cells pretreated with the sensitizing antigen, or in the presence of free antigen in the medium containing immune and target cells. The cytotoxic effect was immunologically specific with respect to the antigens used for induction of delayed hypersensitivity, but was nonspecific with respect to the target cells, and no soluble cytotoxic mediators were detected (808).

Although it is noteworthy that the mechanism of in vivo reactions is very complex and almost never clear cut, there is some indication for the occurrence of nonspecific cytotoxicity in vivo. Thus, (Lewis x BN) F_1 hybrid rats normally do not reject Lewis kidney, but they do so if Lewis rat spleen cells are inoculated under the capsule of a freshly grafted Lewis kidney. It would, therefore, seem that the donor lymphoid cells can be immunologically stimulated by circulating host leukocytes and the interaction of

these two cell populations results in nonspecific damage to kidney parenchyma (263). Skin grafts from rats were transplanted to isogeneic, allogeneic, xenogeneic or specifically sensitized allogeneic recipients, and after 24-28 hours were retransplanted to the original donor. Whereas 92% of iso-autografts were accepted, allo-autografts and xeno-autografts were accepted in only 45% and 36% respectively (109). These results taken in conjunction with the findings of the previous experiment suggest that rejection of allo-autografts or xeno-autografts could be due to a local reaction between lymphoid cells of the graft donor and allogeneic or xenogeneic lymphocytes, which had infiltrated the graft ("passenger leukocytes").

In summary it may be concluded that there is evidence indicating that cell-mediated immune reactions triggered by the specific antigen exert nonspecific cytotoxicity. This supports further the general conclusion made by Perlmann and Holm (686), on reviewing the mechanisms of in vitro target cells lysis, that two steps may be involved in cytotoxic reactions mediated by lymphoid cells. The immunological specificity resides in the first step, in which contact between effector cells and target cells is established, presumably by the specific antibody-like receptors. The second step involves the lytic reaction, which is exhibited by viable and metabolically active effector cells only.

Alternatively, the apparently broad cytotoxic activity of LBC observed in this study may be due to antigenic cross-reactivity among different target cells in tissue culture. Some preliminary results obtained by radioimmunoassay using a syngeneic anti MC-D serum, as a specific reagent indicate that such an antigenic relationship may actually exist. Although more experiments are needed for the elucidation of the factors governing this system, it is tempting to speculate that MC-D antigen(s), which are recognized *in vivo*, may be expressed *in vitro* on cell lines derived from normal syngeneic guinea pig tissues and also on xenogeneic cell lines. Although the experimental evidence does not support the view that the observed crossreactions may have been due to common virus induced antigens, this possibility cannot be categorically ruled out at this time.

So far in one study by Sinkovics et al. (813), a similar phenomenon to that described in this thesis has been reported. Large number of lymphocytes appeared in primary cultures of neoplastic tissue from cancer patients, which eventually led to the destruction of tumor cells. These findings are considered to be analogous to some of the results of this investigation, since in both cases lymphocytes emerged from cultures of neoplastic tissue and were cytotoxic to the tumor cells as well as to established cell lines. These results suggest that both transplantable tumors in animals and autochthonous human tumors may be infiltrated by sensitized lymphocytes and that, although these lymphocytes do not seem to be capable of suppressing tumor growth *in vivo*, they are capable of proliferation and complete tumor destruction in vitro. This

striking difference indicates the possible importance of tumor enhancing mechanism(s) operating in vivo which may involve humoral antibodies. Moreover, it is worth noting that immunoglobulin production could not be detected in culture; hence it would appear that similar interference by humoral antibodies was not manifested in vitro. Clearly, it would be useful to study the effect of serum of guinea pigs carrying the tumor on the activity of LBC in vitro. Finally the observation that LBC were able to partially inhibit the growth of tumor cells in vivo when administered locally indicates the potential usefulness of such cells, for the development of immunotherapy of tumors. However, further studies are needed for the realization of this possibility.

Chapter VI

Conclusions

The following conclusions can be drawn from the experimental findings of this investigation:

(i) Tumor destruction by a cell-mediated immune reaction directed against artificially induced neo-antigens on the tumor cell surface is feasible. Tumor specific antibodies foreign to the tumor bearing host may be used as neo-antigens. The humoral response by the host to these neo-antigens may interfere with cell-mediated tumor destruction.

(ii) The conjoint application of polyoma cells and normal syngeneic spleen cells by the intraperitoneal route results in tumor enhancement, but not when given subcutaneously.

(iii) In mixed lymphocyte-tumor cell cultures from syngeneic guinea pigs, cytotoxic cells with the main characteristics of transformed (thymus derived) lymphocytes can be produced. Also from primary tumor cell cultures similar cytotoxic cells emerge in about half of the cases. These cytotoxic lymphoid cells are capable of inhibiting tumor growth in vivo. It was shown that this tumor was infiltrated by sensitized host lymphocytes which although incapable of rejecting the tumor in vivo, were capable of proliferating and destroying the tumor in vitro.

Claims to Originality

- (i) The basic idea of this study of using tumor specific antibodies for the destruction of cancer by an indirect cell-mediated immune reaction had not been proposed before. Clearly such a system allows the localization of a tumor destroying cell-mediated immune reaction in (metastatic) tumor foci practically anywhere in the body.
- (ii) No extensive study of the possibility of mounting a tumor destroying cell-mediated immune reaction in vitro has been reported until now. The development of such system in this study opens up the possibility of establishing the immunological parameters involved in tumor-host relationships and offers a new approach to the immunotherapy of cancer by immune lymphocytes produced in vitro.

APPENDIX ITHE IMMUNE SYSTEM AND ITS FUNCTIONIntroduction

Ever since Charles Darwin realized the obligatory need for every living organism to be able to comply with its environment, this fundamental biological law has been considered to be the most powerful factor of selection during evolution. In other words, in a given environment only those biological systems will perpetuate, which can fully meet the complex requirements of survival. One aspect of successful accommodation is the possession of an effective defence mechanism against harmful organisms and compounds. Even the most primitive living creatures, such as bacteria, have to face many of these noxious environmental factors including destructive viruses, which represent also some of the pathogenic agents in higher organisms. The discovery of antibiotics served as the most fascinating example of the operation of such defence mechanism(s). It is also remarkable that many of these protective compounds, produced by fungi or other primitive organisms, are able to function equally well in highly evolved biological systems including Homo Sapiens. The enormous advance in chemotherapy, as achieved with antibiotics illustrating that alternative solutions for the same problem are possible and they have developed during evolution. Production of antibiotics illustrates an extracellular defence mechanism, while resistance to them reflects an intracellular reaction.

Intracellular recognition of foreignness (incompatibility) was clearly observed in Protozoa, which does not exist in Metazoa

(872; 350). On the other hand, species specific aggregation of sponges which is mediated by cell surface glycoproteins represents an example of primitive extracellular recognition (439). Transplant failures in Coelenterates were attributed to the existence of a defence mechanism similar to that of graft rejection in higher animals (135). Similarly, earthworms possess the ability of destroying both allografts and xenografts, and this immune reaction is characterized by specificity and anamnesis. Adoptive transfer of xenograft immunity has been also achieved with immune cells called coelomocytes in these animals (155; 194; 195). Insects can develop immunity rapidly with the appearance of non-protein antibacterial factors produced in response to antigenic stimulation, while in general arthropodes fail to recognize allografts and some xenografts as foreign (419). Although our knowledge of the immune responses of invertebrates is rudimentary, it appears that they possess recognition "factors" for antigens which, however, may be produced at a very low rate. The rapid adaptive change to antigens, which involves the synthesis of specific proteins (antibodies), has been encountered only in vertebrates (819); this immune response is of vast importance for their survival constituting their principal means of defence against pathogenic microorganisms and possibly also against neoplastic cells (127).

At this juncture it seems appropriate to define some of the terms used in immunology. An antigen is a substance which, under appropriate conditions, is capable of inducing the production

of effector lymphocytes (cellular immunity) and/or the formation of antibodies (humoral immunity), and of reacting specifically with these cells or humoral factors. A complete antigen possesses two properties: (i) immunogenecity, i.e., the capacity to stimulate the immune system, and (ii) the ability to react specifically with antibodies or immune lymphoid cells. Substances, which are not immunogenic by themselves, but do react with appropriate antibodies in a highly selective fashion, (i.e. specifically) are designated as haptens.

The term antibody refers to a globular serum protein, formed in response to an antigen and reacting specifically with it and, to variable extent, with substances of similar structure, i.e. possessing "crossreacting" molecular features. In response to an antigen are formed different classes of antibodies, designated as immunoglobulins (Ig). Antibodies specific to numerous antigens, are present in the serum (and in other body fluids) even without any known exposure to the corresponding antigens. These immunoglobulins are referred to as natural antibodies; it is not clear if these antibodies are formed without an antigenic stimulus, or if they represent a response to crossreacting environmental antigens.

The thymus and the bursa of Fabricius (the latter organ is present only in birds) constitute the primary lymphoid organs, in which lymphopoiesis is dependent on the availability of stem cells and is not antigen dependent. These organs are regarded as "maturation" sites of lymphocytes from stem cells supplied by the bone marrow.

Some of the lymphocytes generated in the thymus migrate out (T cells) to become part of a pool of long-lived small lymphocytes, which populate the paracortical area of lymph nodes and periarterial lymphocyte sheaths of the spleen (thymus dependent areas). Bursa (or bursa equivalent) derived lymphocytes (B cells) are released similarly into the circulation and home to germinal centers in the various lymphoid organs. Lymphopoiesis in the spleen, lymph nodes (lymph nodes are absent in birds), tonsils and other lymphoid follicles involves immunocompetent lymphocytes and is antigen dependent. These organs are defined as secondary lymphoid organs (601). A model of lymphocyte dynamics designed for the mouse (223), is schematically illustrated in Figure 26. Evidence is rapidly accumulating to suggest that the situation is similar in other species, including man (944). The bone marrow, primary and secondary lymphoid organs and the recirculating pool of lymphocytes constitute the immune system. It has been established that mature lymphocytes are equipped with antigen receptors on the cell surface, which are extremely diverse, and therefore it has been postulated (125; 167; 558; 782; 970; 971) that there exist numerous subpopulations of cells referred to as clones, each of which being sensitive to a distinct antigen.

All the changes elicited in the immune system by antigenic stimulation may be defined as the immune response. Two major events are are characteristics of this reaction:

(1) Rapid proliferation of lymphoid cells and (2) production of effector cells and synthesis of humoral antibodies.

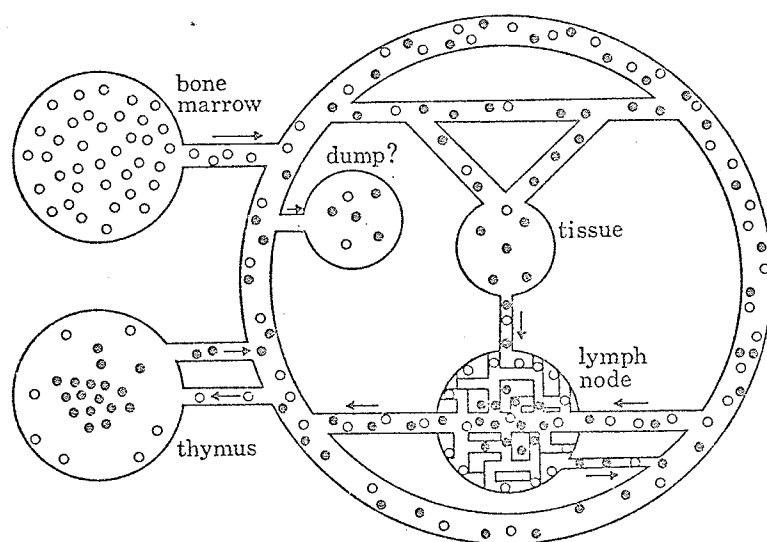


Fig. 26. An idealization of the situations in mice in which lymphocytes are found and the primary links between them. Cells are to be thought of as thymus-derived (•), or bone-marrow-derived (o). The arrows indicate the usual direction of flow of cells.

Davies, A. J. S., E. Lenachars, V. Wallis, M. J. Doenhoff:
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Animals responsive to a given antigen become immune, i.e. develop an elevated number of specifically altered, recirculating lymphocytes, which are sensitive to the antigen, as well as high level of antibodies in their blood and/or other tissue fluids, including mucosal secretions. This status results in a heightened resistance to infectious agents or toxins, if the immunity is directed to them, or in general in a more efficient clearance and destruction of the antigens which may be regarded as foreign and potentially harmful.

It is clear that only antigens which come into contact with immunocompetent lymphocytes, i.e. which may penetrate into body tissues deeply enough, will elicit an immune response. If this process is achieved by injection of the antigen or by entry of the antigen into the host's system, as is the case with bacteria and viruses, it is referred to as active immunization. Vaccination is a special term reserved for immunization against infectious agents. The first well documented vaccination was achieved by Jenner (1798) against small-pox with cow-pox (*variola vacca*) virus. The term vaccination used by Jenner in his pioneering work on immunity was coined to describe the procedure and the term vaccine for the corresponding antigen. Certain materials, which are used mainly as vehicles for the antigen and do not contribute to the specific antigenic stimulus, but have the potential to amplify the immune response, are called immunological adjuvants.

From this short survey of the immune response, it is obvious there are two basic manifestations of immunity: i.e. humoral and cellular immunity. Humoral immunity is transferable with antibodies or serum (passive immunization) from immune to normal animals, while cellular immunity can be transferred to histocompatible (syngeneic = genetically identical) hosts only with immune lymphocytes (adoptive immunization). Both forms of immunity are elicited by most antigen, but the absolute and relative intensity of each of these manifestations varies greatly according to the type of antigen involved.

If an animal is exposed repeatedly to the same antigen, usually an accelerated and stronger immune response occurs, this is referred to as secondary response and is due to the proliferative stimulation of memory cells (i.e. immunological memory or anamnestic reaction) formed in and preserved from the first encounter with the antigen, which is termed primary response.

Under normal (physiological) conditions no immune reaction occurs to self- constituents of the body; this status of unresponsiveness is termed tolerance (self- tolerance). Immunological unresponsiveness may be also induced to various antigens under certain circumstances to be discussed later. The concept of immunological enhancement or facilitation is derived from tumor immunology and refers to a situation when an antigenic tissue (recognized as not -self) induces an immune reaction which does not result in the destruction but in the protection of the tissue or

cells in question. Although it is clear that specific antibodies are involved in immunological enhancement, the exact mechanism underlying this reaction is still obscure (464). Immunological enhancement is regarded by some as a physiological mechanism contributing to the astonishing success of Nature's graft, the conceptus.

Antigens

Antigens have two essential characteristics: immunogenicity, i.e., the capacity to stimulate proliferation of appropriate lymphocytes, and the ability to react specifically, i.e. in a highly selective fashion, with the resulting immune or sensitive cells or with the corresponding antibodies, which could be free or cell-bound. In contrast to the restricted classes of proteins that possess antibody activity, an enormous variety of macromolecules can behave as antigens (e.g. virtually all proteins, polysaccharides, nucleoproteins, lipoproteins). Under appropriate conditions, even small molecules (haptens) which cannot act by themselves as antigens may acquire immunogenicity if they are suitably linked to macromolecular "carriers" such as proteins or synthetic polypeptides. Thus, it appears that a necessary requirement for most antigens is that they be presented to the immune system in the form of macromolecular compounds. However, immunogenicity is

not an inherent property of a macromolecule, since "self" constituents are not immunogenic. One cardinal condition is that the putative immunogen i.e. the molecular form or metabolized product of the antigen which is responsible for the stimulation of the immune response, be recognized as alien (i.e., not self) by the responding organism. The restricted portions of the macromolecular antigen that determine the specificity of antibody-antigen interactions are the antigenic determinants. A considerable body of experimental data indicates that these determinants are limited in size, occupying a volume equivalent to 6-8 amino acids or sugars (224; 340; 461).

Haptens are not immunogenic but they react selectively with antibodies of the appropriate specificity. In general they are small molecules and have to be covalently linked to proteins or polypeptides (i.e. to macromolecular carriers) in order to become immunogenic (conjugated proteins). A number of well-defined chemicals, like pyrcil chloride, dinitrochlorobenzene (DNCB), dinitrofluorobenzene (DNFB), 2-ethoxymethylene-5-oxazolone, etc. (897) and merthyolate (740) can elicit immune reactions in contact with skin or other tissues (contact sensitizing agents). Although these compounds are not immunogenic, they have the capacity to react with self proteins in the skin which serve as carriers, and the resulting hapten-protein conjugates involving an alteration of the body constituents are recognized by the immune system as foreign.

It was stated already that antigens are normally macromolecules, however, in recent years a few small molecules with immunogenic properties have been discovered. Thus, the nonapeptide of L-lysine conjugated with dinitrophenol (DNP) as a hapten was shown to be immunogenic (793). Similarly arsanilic acid coupled by diazotization to L-thyrosine was capable of inducing hapten specific delayed hypersensitivity (534). L-thyrosine-azobenzene-p-arsenate (ABA-Tyr) was shown recently in guinea pigs to function as a carrier for a macromolecular "hapten" (16) as well as for DNP if sufficiently spaced from each other through one or more 6-amino-caproyl spacers ($8 \text{ \AA}$), but one ABA-Tyr group did not function as carrier for another ABA-Tyr when linked to each other through 6-amino-caproyl groups. Modifications of ABA-Tyr either at the arsonate or thyrosine positions showed that other charged groups (sulfonate and trimethyl-ammonium) could substitute for arsonate without loss of immunogenicity. Removal of either the amino or carboxyl group from the side chain of thyrosine did not abolish immunogenicity, but immunogenicity was lost upon removal of both (16). These data indicate that ABA-Tyr is able to stimulate cell-mediated immunity exclusively and unable to induce antibody formation. Contact sensitivity to DNCB was induced in guinea pigs by immunization with large amounts (0.5-1.0 mg) of dinitrophenyl and dinitrophenyl sulphonyl(DNP-S) groups linked to single amino acids, i.e. ϵ -DNP-L-lysine, DNPS-glycine, DNP-S-glutathione and DNP-S-cysteine (929).

It is generally believed that protein antigens should consist of L-amino acids, and that polymers of D-amino acids are not immunogenic. However, it was shown in the mouse that D- and L- synthetic polypeptides have immunogenic properties, and that only an extremely small dose of a poly-D-peptide was capable of eliciting an immune reaction, and that higher doses induced immunological "paralysis" (454). This is reminiscent of the immunogenicity of the polysaccharide of pneumococci, i.e. administration of less than 10 μ g of the polysaccharide was immunogenic, but doses larger than 100 μ g proved paralytic for the immune system of mice (286). The postulated reason for this dose effect is that these antigens are not susceptible to enzymatic breakdown and are, therefore, able to persist for very long periods in high (paralytic) concentrations in the body if administered in large doses.

Tissue antigens: So far antigens have been considered in molecular terms only. However, much bigger particles like viruses and bacterial, plant or animal cells behave also as antigens. Antigens of highly differentiated animal tissues are termed as tissue antigens. Many of these antigens are used for genetic purposes as markers, since tissues with identical antigens are immunologically compatible (i.e. syngeneic or isogeneic tissues) while tissues from genetically non-identical hosts may have, and usually do have different antigens, are therefore rapidly rejected if transplanted to different members within the species (allogeneic tissues) or to members of a different species (xenogeneic tissues).

Tissue antigens which segregate within the species are termed as alloantigens or isoantigens. Other tissue antigens which are present in all members of the species (species specific) may be recognized as foreign by xenogeneic animals only and are coined as xeno-antigens.

The antigenicity of various tissues within the same species was originally detected by transplantation tests and, therefore, the corresponding antigens were termed as transplantation or histocompatibility antigens. A system of strong antigens has been shown to be present in each species examined so far, and to be chiefly responsible for graft rejection; the respective systems have been designated as HL-A for human, H-2 for mouse, DL-A for dog and Ag-B for rat. All these histocompatibility systems consist of numerous antigens with different specificities, which are organized into diverse patterns. Consequently, the probability that two individuals will have identical antigens is very low (1 in 30,000 for man). Identical twins and highly inbred animals possess identical histocompatibility antigens and accept each other's grafts permanently. Transplantation antigens are present on the surface of most, if not on all the cells of the body, but they vary in quantity and in some cases even in quality according to the type of tissue (215; 805; 855; 856).

Blood group antigens present on red cells, on many epithelial cells and virtually on all endothelial cells, but absent from connective tissue cells, constitute another well known system of alloantigens (224).

Several alloantigens have been described for mouse lymphoid cells. Thus, Reif and Allen (734) discovered a thymic antigen of AKR mice which was also present on the brain tissue. Later on it turned out that this thymic antigen, termed as Θ , is determined by a single genetic locus with two alleles: one is Θ -AKR found also in RF mice, the other is Θ -C3H which is present in most other inbred strains of mouse. Thymocytes and thymus-derived lymphocytes may be distinguished from other lymphoid cells with the aid of Θ -specific antiserum (718). A similar alloantigen present on thymus cells of certain mouse strains and also on some leukemias of thymic origin is named as thymus-leukemia (TL) antigen (105). Mouse strains may be classified as TL-positive or TL-negative. TL-negative mice can form TL antibody. Leukemias may also be classified as TL-positive and TL-negative. A unique feature of the TL antigen is their appearance in leukemias of strains that do not have TL in their normal tissues. Two other TL antigens (TL^2 and TL^3) belonging to the same system have been described but these have not been found in leukemias of TL^2 and TL^3 negative strains. Three phenotypes are distinguishable in normal mice: TL-negative, TL^2 -positive and those possessing all three TL antigens. The genetic determinant for TL (Tla locus) is in the linkage group IX near the D end of H-2 (cross over frequency 1-2%). There is a close relationship between TL and histocompatibility antigens on the cell membrane, since TL was not separated from H-2 through a series of purification steps (105; 221). The Ly series represent another

group of alloantigens of thymocytes and T-cells (865). Yet another alloantigen present on plasma cells has been designated as PC. The gene Pca (plasma cell antigen) which determines PC.1 alloantigen segregates as a mendelian dominant gene, not closely linked with H-2. PC antigens are present on the cells of liver, kidney, brain, lymph nodes, antibody forming cells and myelomas. The strain distribution of PC.1 does not conform to that of any immunoglobulin allotype or cell surface alloantigen previously described (864). The allotypes of immunoglobulins are further examples of antigenic differences caused by genetic variation within the species (559).

Xeno-antigens of the lymphoid system have also been described: A mouse bone marrow derived lymphocyte antigen (MBLA) was detected with rabbit antisera produced against B-cells and subsequently absorbed with mouse liver, red blood cells and thymocytes (720). Specific antisera to chicken thymus and bursa cells were also produced in rabbits by similar procedures and the corresponding antigens were designated as chicken T-lymphocyte antigen (CTLA) and chicken bursa-derived lymphocyte antigen (CBULA) (586).

A specific antiserum produced against stimulated macrophages of the rat reacted with free (peritoneal, bone marrow, blood etc.) macrophages but did not combine with fixed phagocytic cells located in the liver, lung, lymph nodes and thymus. A similar difference in the antigenic makeup has been shown to exist between the free and sessile macrophages of guinea pigs,

mice and rabbits (621).

Recent investigations revealed that thymus derived cells were necessary for a successful immunization with a number of antigens like red blood cells, most proteins and hapten-protein conjugates etc., while other antigens like flagellin of *S. adelaide*, pneumococcal polysaccharide or *Brucella* were able to induce a normal immune response in the absence of the thymus (222; 601; 723; 859; 902). Therefore, the former antigens may be classified as thymus dependent antigens and the latter as thymus independent antigens. Resistance to certain infectious agents shows a somewhat similar tendency, namely that thymic function is essential for withstanding most viruses, fungi, protozoa, mycobacteria and some other bacteria, but it does not seem to play an important role in pyogenic infections caused by staphylococci, pneumococci, streptococci or *Haemophilus influenzae* (253).

If two or more antigens are injected simultaneously or within a short interval into the same animal, a diminished immune response is observed to each antigen compared to that elicited by the same dose of each of the antigens when administered to different groups of animals separately. This phenomenon is known as antigenic competition (873). However, it should be noted that certain antigens like mycobacterial antigens, *Bordetella pertussis* vaccine, endotoxins etc. greatly potentiate the immune response to unrelated antigens upon conjoint administration and are for this reason frequently used as immunological adjuvants.

Humoral immunity

A. The bursal lymphoid system

Glick et al. (343) discovered that chickens which had been bursectomized * at hatching were unable to form humoral antibodies. This observation was confirmed by others (632) and it became clear soon that in the chicken the bursa was responsible for the generation of antibody forming precursor cells (B-cells). On the other hand neonatal thymectomy combined with whole-body x-irradiation (600 r) was associated with failure of antibody production in about 40% of chickens only (197). Bursa cells from chickens, inbred for the major histocompatibility loci, were labelled with H^3 -thymidine and transferred to x-irradiated recipients. Six to eighteen hours later bursa cells tended to localize within the germinal centers of the spleen. Bursa cells not localized within germinal centers homed preferentially to the red pulp. On the other hand, less than 0.1% of killed bursa cells were found localized in the spleen, and most of these cells accumulated in the liver and to a lesser extent in the lung. Although thymus cells homed to the spleen they were never found in the germinal centers, but localized along the arterioles (242). While bursectomy eliminates completely the capacity of specific antibody formation, a new immunoglobulin-like protein was found in the sera of such chickens. The molecular weight of this protein was 300,000, it

* The bursa of Fabricius is a primary lymphoid organ present only in birds. It develops embriologically as an invagination of the cloacal entoderm.

contained only heavy chains of IgM, but no light chains could be detected. The origin of this primordial immunoglobulin-like serum protein is uncertain (159). It was also reported that bursal lymphocytes produce hemolytic antibodies to sheep erythrocytes inoculated into the cloaca and that these cells reacted with *Escherichia coli* antigens but not with gamma streptococci (907).

On the basis of the absolute necessity of bursal function for a normal immunological reactivity in birds, it is reasonable to assume that a bursa-equivalent organ must exist in mammals. However, no conclusive evidence is available so far as to what organ would have the attributes of the bursa. The two likely candidates are the gut-associated lymphoid tissue and the bone marrow. Peyer's patches and other gut-associated lymphoid tissue were considered to have bursa equivalent function in rabbits (196; 685). Based on the fact that human bone marrow cells responded to pokeweed mitogen and anti-immunoglobulin sera (B-cell stimulants), but were unresponsive to phytohemagglutinin (T-cell stimulants), the bone marrow was proposed as bursa equivalent in man (2).

B. Immunoglobulins (Ig)

Analysis of serum proteins by electrophoresis revealed that antibodies are members of different classes of globulins. Of the three principal electrophoretically separable globulins of vertebrate sera (α -, β -, and γ -globulins) the γ -globulins were observed by Tiselius and Kabat (889) to be elevated in the sera of

intensively immunized animals. Moreover, the purified antibodies to various antigens, isolated from specific precipitates, were found to be indistinguishable from γ -globulins, not only with respect to chemical properties (electrophoretic mobility, solubility, amino acid composition), but also with respect to antigenicity. Hence following Hereman's suggestion, all proteins that have antigenic determinants in common with antibody molecules, are now called immunoglobulins (224).

The subject of immunoglobulins has been extensively treated from many aspects in several reviews (182; 185; 251; 531; 572; 654; 855). Serum immunoglobulins comprise a vastly heterogeneous population of proteins composed of antibodies of various molecular sizes and widely different specificities. The term immunoglobulin, as distinct from the gamma-globulin, is commonly used in a somewhat broader extent to describe serum proteins with characteristics which include γ -globulins, but extend into the β and α_2 range of electrophoretic mobility. In humans, there are at least five major classes of immunoglobulins known as IgA, IgD, IgE, IgG and IgM, each class being characterized in terms of its specific antigenic determinants as revealed with antisera produced in different species. All five classes of immunoglobulins coexist in human sera, but occur in different concentrations, differ in antibody content, rates of synthesis and catabolism, placental passage and in physico-chemical and immunochemical properties as shown in Table 18 (251; 450; 572; 891). The three major classes

IgG, IgA and IgM comprise more than 95% of the serum immunoglobulins, while IgD and IgE represent two minor classes (251).

Antibody heterogeneity manifests within a fundamental structural framework common to all immunoglobulins. This basic structure consists of two identical light (L) and two identical heavy (H) chains linked by disulphide bonds and noncovalent interactions in a Y-shaped molecule as shown in Figure 27. This tetrachain structure is designated as the monomer unit. The L-chains of all immunoglobulins fall into two chemically and antigenically different groups, known as kappa (κ) and lambda (λ). Conversely, the H-chains of each immunoglobulin molecule belong to a specific antigenic class and designated by the corresponding Greek letters as α , δ , ϵ , γ and μ chains. Each class (isotype) is common to all members of the species. Furthermore these H-chain members may be divided into subclasses according to specific determinants located in the carboxyl-terminal portions of the chains.

The first indication of the multichain structure of immunoglobulins was provided by an analysis of the products obtained following reduction of disulfide bonds of IgG with mercaptoethanol and subsequent alkylation. Starch gel electrophoresis revealed the presence of two type of bands (251); the faster moving bands were the L-chains and the slower were the H-chains. The basic four chain structure of immunoglobulins was confirmed in latter studies that were concerned with the cleavage of rabbit IgG by various proteolytic enzymes. This structure is schematically represented in Figure 27.

The region in the vicinity of the inter-heavy chain disulphide bond is susceptible to enzymatic attack and is referred to as the hinge region. The digestion of rabbit IgG with papain (activated by cysteine) produces three fragments (molecular weights 40-50,000) which may be separated by chromatography on carboxymethylcellulose (706). One of these fragments the Fc fragment consists of the carboxyl halves of the H-chains still linked by a disulphide bond. The Fc fragment is crystallizable, and although it possesses no antibody activity, it does have important biological functions, e.g. complement fixation, cytophilic and cytotropic activity, passage through membranes and excretion are all mediated by this portion of the molecule, as it will be discussed later. Most of the carbohydrate moiety of IgG is attached to the H-chain via β -carboxyl group of an aspartic acid residue in the Fc portion of the molecule. However, the other two fragments designated as Fab, which are similar in size to Fc but differ in charge, each possess one antibody combining site and therefore are univalent. They each consist of an L-chain linked to the amino terminal segment of an H-chain.

The action of pepsin on IgG resembles that of papain in that it cleaves IgG in a similar region of the molecule, the hinge region, but results in a divalent 5S F(ab')₂ fragment (molecular weight 100,000) and a series of smaller peptides (904). These small peptides arise from the digestion of Fc region by pepsin. The divalent F(ab')₂ fragment can be further cleaved

by sulfhydryl agents to yield monovalent $F(ab')$ fragments resembling the Fab fragments obtained with papain (653). Similarly the $F(ab')_2$ is sensitive to attack by papain and may be split into two univalent Fab fragments plus a disulphide linked peptide (143). However, the univalent $F(ab')$ fragments differ slightly from the Fab in amino acid composition and molecular weight. The peptic fragment has a molecular weight of about 46,000, whereas the molecular weight of the papain fragment is approximately 41,000. Treatment of IgG with trypsin or cyanogen bromide (926) also results in $F(ab')_2$ fragments. A unique feature of the immunoglobulins is their linear arrangement and periodic distribution of inter- and intra-chain disulphide bonds. Amino acid sequence analysis has shown that the H-chain may be joined by one to five disulphide bonds depending on the partial subclass of IgG (295; 299). Approximately 60% of rabbit IgG molecules can be dissociated into half molecules after reduction of one disulphide bond per molecule. The other 40% are more resistant to reduction probably because more than one inter-heavy chain disulphide bond is present. H-chains are also linked via disulphide bridges to L-chains either through the carboxyl-terminal cysteine residue in the case of kappa chains or the penultimate carboxyl-terminal cysteine residue of lambda chains. The disulphide bonds linking the H-chains are more labile to reduction with mercaptoethylamine than those joining L-chains to H-chains. In addition to H-H and H-L disulphide bonds, L and H chains possess respectively a total of 2 and 4 intra-chain disulphide bonds each enclosing loops containing about 60 amino

acids (Figure 27). The basic pattern of repeating intra-chain disulphide loops is apparently fundamental to the function and rigidity of the molecule and has been conserved during evolution. This pattern could result by gene duplication from a smaller precursor gene (251).

Although less information is available about the immunoglobulins in species other than man, evidence is increasing that birds and mammals have the major classes of immunoglobulins present in primates (313; 448; 891). An evolutionary scheme of immunoglobulin genes is present in Figure 28 (559). Serum levels of immunoglobulins in germ free animals are much lower than in conventional animals, i.e. germ free mice were reported to have only 2.8% of the normal IgG level, and to produce antibody at a slower rate than conventional animals (444). Similarly, antibody producing cells of germ free mice were about 8% of those found in normal mice and increased to 33% on immunization with human IgG and *Salmonella typhimurium* (666). Similarly, the γ -globulin level of germ free rats was 10-15% of the level present in normal rats and it approached the normal level within 4 weeks after exposure to normal intestinal flora (371). These and many similar observations indicate that most, if not all, of the immunoglobulins present in the serum at the "normal" level are produced in response to stimuli by various environmental antigens, which could be exogenous (food constituents, bacteria, viruses etc.) or endogenous (neoplastic cells, for instance). Therefore natural

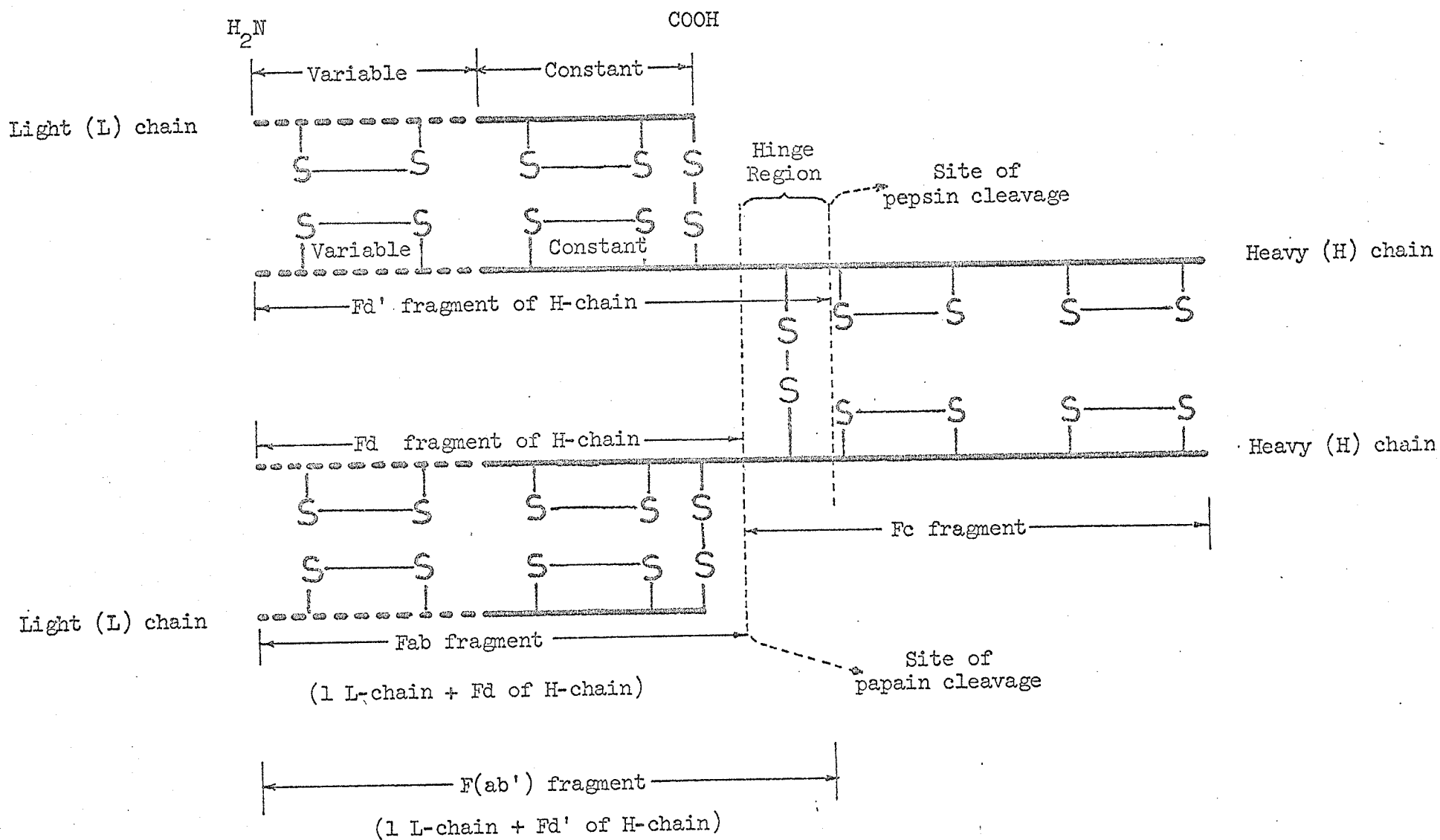


Figure 27. The scheme of IgG structure.

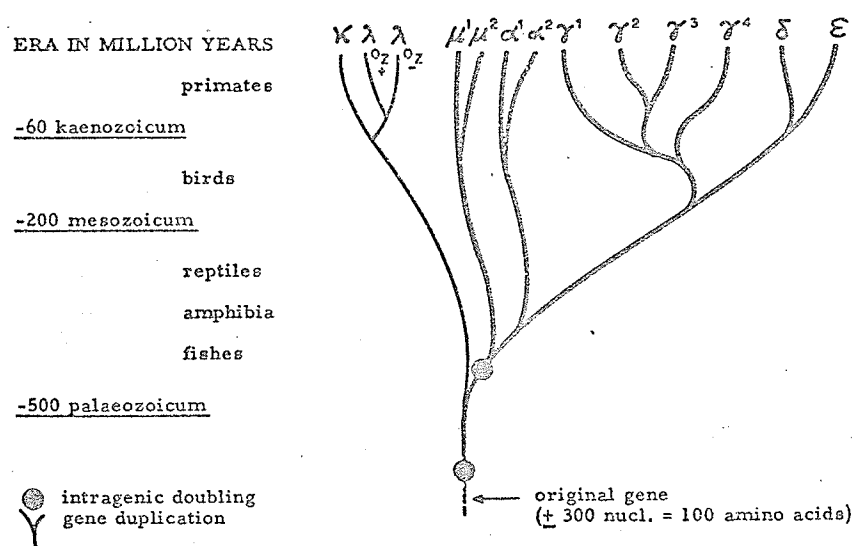


FIGURE 28. Evolution of immunoglobulin genes.
 (Van Loghem, Ann. N. Y. Acad. Sci. 190:136, 1971)

antibodies, which are detectable in serum to a great variety of antigens, may be regarded as being the result of the immune response to environmental antigens rather than due to a spontaneous or inherited capacity to produce immunoglobulins. However this latter possibility cannot be completely excluded. The important role of immunoglobulins in the defence mechanism of the body is emphasized by the fact that children born with congenital agammaglobulinemia are not able to resist pyogenic infections and Haemophilus influenzae, although they may survive till middle childhood (253). Furthermore individuals with IgA deficiency, which occurs in 0.2% of the population, have an increased sensitivity to mucosal, especially to respiratory infections (187).

During the past few years it became clear that the various immunoglobulin classes were different not only in terms of their physicochemical characteristics but also as regards their different biological functions. As already stated, the variable (amino terminal) regions of H and L chains constitute the antigenic combining site in all classes of antibodies. On the other hand, the Fc portion, which is unique to each class, is responsible for the biological behaviour of these different immunoglobulins, e.g.: complement fixation, cytotropic or cytophlyic activity, passage through membranes, excretion, etc. The major characteristics of human immunoglobulins are summarized in Table 18 (251; 448; 572).

IgA is present practically in all mucous membrane secretions (bronchial, small intestine, vaginal), in parotid saliva, amniotic and lachrymal fluid, and in the colostrum. It is not derived from

Table 18.

Major characteristics of human immunoglobulins.

	IgA	IgD	IgE	IgG	IgM
Synonyms	$\gamma A, \beta_2 A, \gamma_1 A$	γD	$\gamma E, IgND$	$\gamma G, 7S\gamma, \gamma_2, \gamma_{SS}$	$\gamma M, 19S\gamma, \beta_2 M, \gamma_1 M$
Heavy chain class	α	δ	ϵ	γ	μ
Light chain class	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ
Sedimentation constant (S _{20,w})	6.3-6.9	6.2-6.8	8	6.5-7	18-20
Molecular weight	180,000	183,000	196,000	150,000	900,000
Total carbohydrate(%)	5-7.5		10-11	2.9	11.8
Subclasses known	2			4	2
Serum concentration (mg%)	390 $\pm$ 90	0.3-30	0.0017-0.006	1240 $\pm$ 220	120 $\pm$ 35
Approximate total globulin (g/kg/70 kiloman)	0.1-0.25	0.01-0.05		0.75-1.9	0.05-0.19
Synthesis rate (mg/kg/day)	20.9-55	0.03-1.49		20-40	3.2-16.9
Catabolic rate (% i.v. pool/day)	13-34	18-60	50	4-7	14-25
Distribution (% i.v. pool)	40	63-86		48-62	65-100
Antibody activity	+	+	+	+	+
Skin sensitization in					
-heterologous spec.	-		-	+	-
-homologous species	?	-	+	-	-
Complement fixation	-		+	+	+
Placental passage	+	-	-	+	-
Presence in cerebro-spinal fluid	+			+	-

* through alternate pathway (449)

serum by simple transudation, but excreted against a concentration gradient. In contrast to serum IgA, which is a monomer with a sedimentation coefficient of 7S, the secretory IgA has a dimeric form (as illustrated in Figure 29) (281), the Fc portions of two IgA molecules being linked to a secretory "piece" (transfer-, T component); for this reason the sedimentation coefficient (S^{20}_w) of secretory IgA is 11, and its molecular weight 385,000. The secretory piece is a glycoprotein with a sedimentation coefficient of 4,2 (Molecular Weight 58,000) and a 9.5% hexose content, and is linked to α chains by both covalent (disulfide) and noncovalent forces. The secretory piece may be present by itself in saliva. The primary site of synthesis of the IgA portion of the secretory molecule is in plasma cells lying in intimate anatomical relationship to mucous membranes or glandular tissue. It is not known whether dimerization is achieved inside or outside the plasma cell. Roughly five times more IgA is produced in the secretory system than in the secondary lymphoid tissues such as spleen and lymph nodes. There is very little correlation between the IgA level in serum and saliva. Myeloma patients with extremely high serum levels have normal level in saliva. In infants salivary IgA is produced earlier than serum IgA, and it also approaches adult values faster. Although the biological significance of IgA is as yet not completely established, the available data strongly suggest that it is the host's first line of defence against infections and other harmful agents which come into contact with mucous membranes. Secretory IgA contains natural antibodies to numerous infectious agents and reinfections with

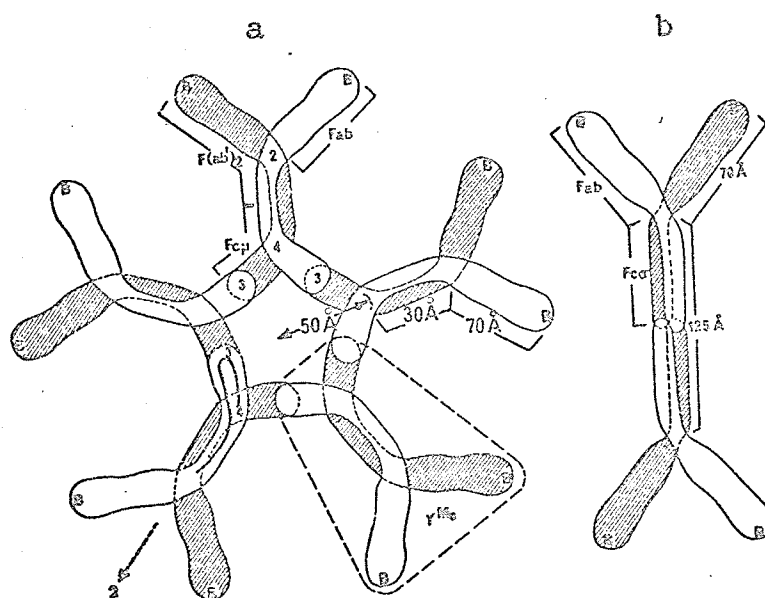


FIGURE 29. Diagrams of (a) a γM pentamer and (b) a γA dimer. The drawings, which are approximately to scale, are based on electron micrographs and incorporate data derived from chemical and physical analyses. The antigen-binding sites are represented by B. The positions of the inter- and intrasubunit cystine bridges in γM are shown by the numbers 2, 3 and 4. The numbering, which runs from the N terminus, is that used previously. A portion of the folded polypeptide chain is shown in one protomer at the bottom left. One of the five polar twofold symmetry axes of the γM molecule is shown.

respiratory viruses, rhinoviruses and type 1 parainfluenza are better correlated with a lower level of mucosal antibodies than of antibodies present in sera (187;365; 891). It was also proposed that IgA protects other immune systems from excessive stimulation via the gastrointestinal tract.

IgD is a minor component of serum immunoglobulins and no unique function has been revealed so far for this immunoglobulin. A significant elevation of IgD was found in sera of pregnant women collected at delivery. Sera collected at other stages of gestation and from women using oral contraceptives showed considerable variation in IgD levels but with less significant elevation when contrasted with nonpregnant controls. It remains to be elucidated, whether IgD has a physiological role during gestation(484).

IgE is present in the serum in even lower amounts than IgD (Table 18), but it plays a central role in allergic diseases as "reaginic" or "skin sensitizing" antibody. It was only recently realized that human reaginic antibodies belong to distinct immunoglobulin class (447). Tonsils and adenoids possess the greatest number of IgE plasma cells, which form even germinal centers. Bronchial and peritoneal lymph nodes as well as the respiratory and gastrointestinal mucosa contain IgE plasma cells, but very few can be found in the spleen and subcutaneous lymph nodes, none in peripheral blood and bone marrow. Such distribution of plasma cells suggests that IgE may be formed locally in response to antigen.

Tissue mast cells and basophil leucocytes, but not other granulocytes or lymphocytes have receptors for the Fc portion of IgE. The fixation of IgE on these cells is readily impaired by

maintaining a reaginic serum at 56°C for $\frac{1}{2}$ - 4 hours and by treatment with mercaptans at low concentrations. Basophils sensitized with IgE release histamine upon exposure to either allergen * or anti-IgE antibody. The initial step for triggering a reaginic hypersensitivity reaction appears to be the combination of the antigen with cell-bound IgE molecules, which is considered to result in structural changes in the Fc portion of the antibody molecules leading to activation of membrane fixed enzymes and to the release of histamine and/or slow reactive substance of anaphylaxis (SRS-A) as illustrated in Figure 30 (184; 447; 450). Following challenge with the appropriate allergen of human lung tissue, which had been passively sensitized with serum from a ragweed-sensitive donor, the anaphylactic diffusate has been shown to contain, in addition to histamine and SRS-A, a factor selectively chemotactic for human eosinophils. This factor was distinguished from histamine, bradykinin, serotonin, the prostaglandins PGE_1 , PGE_2 and $\text{PGF}_{2\alpha}$ and SRS-A (475).

Serum concentration of IgE was significantly raised in some patients with allergic asthma and hay fever, but was normal in others. Children and Africans suffering from parasitic infestation had significantly raised levels of IgE (447). The strong IgE response to parasites suggests that this immunoglobulin may have a special function in reactions providing protection against parasitic diseases. Besides the pathological aspects

* The term allergen is used to denote an antigen responsible for induction of an allergic state, i.e. of immediate hypersensitivity characterized by appearance of reaginic antibodies.

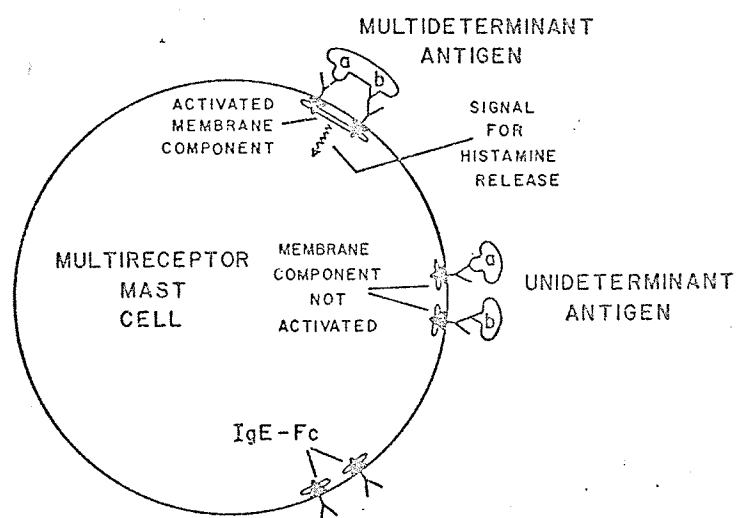


FIG. 30. A model for activation of histamine release.

Cohn, M., Ann. N.Y. Acad. Sci. 190:529, 1971.

little is known about the role of IgE in the immunological defence mechanism. It is believed that the main function of IgE is to equip basophils and tissue mast cells with antigen specific receptors (homocytotropic antibody), and that these cells are then able to participate actively in immune reactions leading to an inflammatory reaction. The recently discovered "cutaneous basophil hypersensitivity", to be discussed later, may represent such an inflammatory process.

IgG is known as the major class of immunoglobulins comprising about 85% of the total immunoglobulins in man. About 50% of IgG is located extravascularly; therefore, it is also the major component of humoral immunity in the tissues. Studies on G myeloma proteins revealed that at least four subclasses of IgG can be distinguished. These four subclasses share common antigenic determinants, specifying the IgG class of immunoglobulins and each subclass possesses unique antigenic determinants not shared by any of the other subclasses. The subclasses were designated IgG₁, IgG₂, IgG₃ and IgG₄ which were denoted also as γ 2b, γ 2a, γ 2c and γ 2d by Terry and Fahey, and as We, Nc, Vi and Ge by Grey and Kunkel (447). The number of disulfide bonds between the H chains varies among these four subclasses, i.e. 2 for IgG₁, 4 for IgG₂, 5 for IgG₃ and 2 for IgG₄. The L chain is linked at different positions to γ 2, γ 3 and γ 4 chains. There is increasing evidence that these four subclasses of IgG have different biological activities as indicated in Table 19 (447; 605); however, the relation

Table 19.

BIOLOGICAL ACTIVITIES OF HUMAN IgG SUBCLASSES

	IgG ₁	IgG ₂	IgG ₃	IgG ₄
1. Complement fixation	+	+	+	-
2. Cytophilic antibody	+	-	+	-
3. Fixation to guinea pig macrophages	+	-	-	-
4. Skin sensitization in heterologous species	+	-	±	+
5. Presence in cord blood	+	+	+	+
6. Reaction with rheumatoid factor	+	+	+	+
7. Antibody activity to				
- thyroglobulin	+	+	+	?
- Rh factor	+++	-	+	-
- factor VIII (clotting factor)	-	-	-	+++
- diphtheria toxoid	+	+	+	?
- dextran	+	+++	+	-
- teichoic acid	+	+++	+	-

between their function and structure is not settled yet. IgG is less efficient in complement fixation and agglutination than IgM, thus, the neutralization of soluble antigens both in the extravascular and intravascular space may be considered as its main function.

IgM is a pentamer of five basic units as was mentioned earlier having a sedimentation coefficient of 19S and a molecular weight of 900,000. A recent molecular model is presented in Figure 29, which has been derived on the basis of electronmicroscopic and physical properties of this immunoglobulin (281). Because of the large molecular size (M stands for macroglobulin), IgM is localized almost entirely in the vascular space and the titer in the tissue fluid is only 1/16th of that in the blood (376). IgM is 100 times more efficient in lysing red cells than IgG, and also a very efficient agglutinator suggesting that it is specially well adapted for handling bacteria and other particulate antigens (572).

Three groups of alloantigens (allotypic markers) referred to as Gm, Am, Inv are known for human immunoglobulins and have been shown to be coded by one particular gene locus, respectively, as illustrated in Table 20. It appears that there is linkage of genes for the heavy polypeptide chains, but this gene cluster is not linked to the loci of genes for light chains. In summary, there are three main principles responsible for the great variation of antibodies found: (i) antigen binding specificity; (ii) class and type variety; (iii) allotype variety (559).

TABLE 20.
GENETIC MARKERS OF HUMAN IMMUNOGLOBULINS

Light Chains			Heavy Chains		
Inv	Gm			Am	
κ	γ_1	γ_2	γ_3	α_2	
	Fd	Fc	Fc	Fc	
l	f	a	n	b ⁰ g	
a	z	x		b ¹ s t	
b				b ³ c ³	
				b ⁴ b ⁵ c ⁵	

Van Loghem, E., Ann. N.Y. Acad. Sci. 190:136, 1971.

Despite the advanced stage of the immunochemistry of antibodies, operational terms such as cytophilic antibody, cytotoxic antibody or enhancing antibody are still used frequently in the immunological literature, without any reference as to the class and subclass of the antibodies involved.

Cytophilic antibodies are immunoglobulins which are capable of combining with macrophages prior to combining with the antigen and may be eluted from macrophages at 37°C. Cytophilic antibodies belong in rabbit to the IgG class, in guinea pig to IgG₂, in mouse to IgM, IgG₁, IgG₂ (but not IgG₃) and to IgG₁ in primates. In the mouse alveolar, splenic and peritoneal macrophages, Kupffer cells, microglia, and blood monocytes take up cytophilic antibodies to an apparently identical extent. Guinea pig, and rabbit alveolar macrophages adsorbed more cytophilic antibodies than their peritoneal cells. Oil stimulated mouse peritoneal macrophages take up considerably more cytophilic antibodies than unstimulated macrophages. Nonspecific cytophilic antibodies are able to compete against the specific cytophilic antibodies for binding sites. The biological significance of these antibodies is not entirely clear. Opsonization or localization of antigen on dendritic macrophages may enhance the primary immune response. The bulk of available evidence indicates no relationship to delayed hypersensitivity skin reactions. Activated macrophages for cytotoxicity could be "disarmed" by trypsin treatment and "rearmed" by exposure of these treated cells to either immune serum or eluates from activated macrophages. Cytophilic antibody

may play a role in resistance to *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Salmonella typhimurium* (649; 890).

Another frequent operational term is enhancing antibody.

It was realised long time ago that the enhancement of growth of allogeneic tumors is mediated by antibodies produced in response to tumor antigens (463). Based on the fact that enhancement was obtained with allogeneic tumor cells coated with antibodies in vitro in the absence of circulating antibodies in the host (617), it was logical to assume that enhancing antibodies act by masking (covering up) tissue antigens and that they would not be cytotoxic, i.e. would not fix complement (64). Further experiments revealed, however, that immunological enhancement is a very complex phenomenon and that it might be governed by multiple mechanisms, the effect of humoral antibodies being dependent on the system in question. Although most frequently IgG has been shown to be responsible for enhancement. IgM despite it's complement fixing properties may also enhance tumor growth in certain cases and inhibit it in other cases (464). Another system affected by enhancing or blocking antibodies appears to be autoimmunity. Thus, the course of many autoimmune conditions does not correlate with the presence of humoral antibodies, and it is worth noting that normal individuals may also have autoantibodies. In addition a number of experimental autoimmune conditions are self-limiting and they subside spontaneously if treatment is ceased (21; 700; 785). This lack of correlation

between autoantibodies and pathological changes strongly indicates that antibodies are not always responsible for the pathogenesis. However, if one assumed that at least some autoantibodies are able to protect against autoimmune damage in a manner similarly to that of graft or tumor enhancement the lack of correlation with pathological changes is easily explainable. For example, it is well documented that experimental allergic encephalomyelitis is transferable by sensitized lymphoid cells and that humoral antibodies are able to interfere with the manifestation of the disease in the presence of autoaggressive lymphocytes (21).

It is obvious that red blood cells are potentially one of the most vulnerable targets for autoantibodies, since hemolysis or even agglutination by antibodies would result in failure of the circulatory system. Nevertheless, erythrocytes have been shown to carry autoantibodies on their surface, which do not cause complement-mediated lysis and which are unable to agglutinate the cells, hence, these immunoglobulins were designated as "incomplete" antibodies. Previous exposure of erythrocytes to these antibodies results in inhibition of clumping of the cells by agglutinating antibodies and therefore, the antibodies in question have been also referred to as blocking antibodies. Coombs and associates (193) demonstrated that an antiserum against human globulin would induce agglutination of erythrocytes coated with the incomplete antibody (Coombs test). Later on incomplete antibodies were shown to acquire agglutinating capacity after exposure to 2-mercaptoethanol (a reducing agent which disrupts disulfide links), which indicates

that incomplete antibodies are (at least) bivalent and that one of the combining sites of these immunoglobulins is not functional because of unique steric constraints of the molecule (700). "Warm" acting erythrocyte antibodies are generally IgG immunoglobulins, some of which are nonagglutinating and presumably inefficient in complement fixation (527). Warm acting erythrocyte autoantibodies can be produced in normal healthy individuals without premature erythrocyte destruction (139; 527; 700). Since the autoimmune response to erythrocytes represented by hemolytic or agglutinating antibodies, constitutes a circulatory disaster, which can be prevented by blocking antibodies, it is not surprising on teleological grounds that the latter antibodies are produced by healthy individuals. Indeed, the characteristics of incomplete antibodies are those of a ideal blocking antibodies which would be expected not to agglutinate erythrocytes or fix complement, but to cover up the antigenic determinants on the red cells on a competitive basis. The fact that the presence of these antibodies can be detected only by an antiglobulin test or by inhibition (blocking) of the effects of other antibodies, would support the view that similar antibodies with a blocking (or enhancing) function might be present in other systems and more frequently than suspected.

The presence of an immunoglobulin, designated as γ 1a, was regularly demonstrated in dog sera hyperimmunized with bovine serum albumin (BSA). This hyperimmune serum containing mainly γ 1a did not show distinct precipitation reaction and passive hemagglutination,

but had the capacity to inhibit the precipitin and passive hemagglutination reactions of a rabbit antiserum to BSA (313). Nonprecipitating antibodies of the IgG class were also isolated from rabbit antisera to ovalbumin. The F(ab')₂ fragments of these antibodies did also not precipitate with antigen, and it was suggested that their non-precipitating property was due to a particular configuration of the molecule that made it impossible for one antibody to combine simultaneously with two different molecules of antigen. These non-precipitating antibodies did not fix complement, could not produce a reverse Arthus reaction but were active in passive cutaneous anaphylaxis (57). Non-precipitating (blocking or incomplete) antibodies may also be found in other sera. They are bivalent, and some of them will precipitate if complement is added. Some of them have unusually low affinities, but in other cases purified nonprecipitating antibodies of the γ G class have been shown by equilibrium dialysis to be bivalent and of high affinity. Antisera vary a good deal with respect to the fraction of their total antibody that is nonprecipitable (224).

Pathological immunoglobulins are produced by spontaneously proliferating antibody forming cells (benign, or malignant myelomas) without apparent stimulation by antigen. They resemble the known immunoglobulin classes, i.e. there are A, D, E, G and M (could be monomer) myeloma proteins, and even separate chains may be produced ("heavy chain disease"). Some myeloma patients excrete the light immunoglobulin chains in the urine in high concentration. This protein has unusual thermostability properties, i.e. it precipitates on heating to 45 to 60°, redissolves on boiling, and precipitates

again in cooling; it is called Bence Jones protein after the physician who first studied it in the mid nineteenth century. The myeloma protein produced by a particular patient is relatively homogeneous and the view is held that they are produced by a clone representing the progeny of one antibody forming cell. Therefore, these proteins are termed monoclonal, while others which are more heterogeneous may be termed as polyclonal (224; 669). Certain pathological immunoglobulins have antibody activity and may have also abnormal physicochemical characteristics (e.g. cold agglutinins). A condition called idiopathic monoclonal proliferation may be present for years, sometimes as long as 13-17 years, before malignant myeloma develops (572; 669).

No attempt has been made to present a detailed review of the physical chemistry of antigen-antibody reactions, since this topic has been covered systematically in excellent textbooks (224; 460; 461; 942). Suffice it to say, as already mentioned that antibodies are bivalent or polyvalent and that noncovalent forces are involved in antigen-antibody interactions and overall magnitude of the cooperating combining forces being referred to as affinity. Antibodies are normally heterogeneous with respect to their affinity for the homologous antigen and this is increasing during the course of immunization.

C. Complement (C')

The complement system is a group of 9 serum proteins (C'1-C'9) which can be activated by the Fc portion of complement fixing antibodies after reaction with antigen (729). The properties

of some purified components isolated from human serum are summarized in Table 21 and the sequence of reactions in immune hemolysis is illustrated in Figure 31 (224). The overall reaction between C' and red cells requires Ca^{2+} and Mg^{2+} , and the rate of the reaction is much greater at 37° than at 0° . These observations suggest that at least some of the C' components are enzymes. Activation of the various components involves a chain reaction, the activated components in the reaction being denoted by the letter a (Figure 31). Many of the activated components (C'4a, C'2a, C'3a) become rapidly inactive ("i") in the serum, if not bound to the antigen-antibody-complex. Following the reaction with C'8 and C'9 the red cell (referred to at this stage as E *), develops small holes, each about $100 \text{ \AA}$ in diameter, and is finally lysed (224). Some biological reactions, other than hemolysis are also mediated by various C' complexes as follows: immune adherence; agglutination and opsonization by M-A-C' 1a, 4, 2a, 3 (M = cell membrane, A = antibody); anaphylatoxin and C' dependent histamine release by M-A-C' 1a, 4, 2a, 3, 5; leucocyte chemotaxis by M-A-C' 1a, 4, 2a, 3, 5, 6, 7. It has been established that C'3 and C'5 fragments are generated during the reaction and function as chemotactic factors for polymorphs and anaphylatoxins. The active substance of anaphylatoxin appears to be a polypeptide (224; 461).

In the absence of antigen, both IgM and IgG antibody combine reversibly (weak interaction?) with the C'1q component of complement, but the chain of events leading to the lytic action

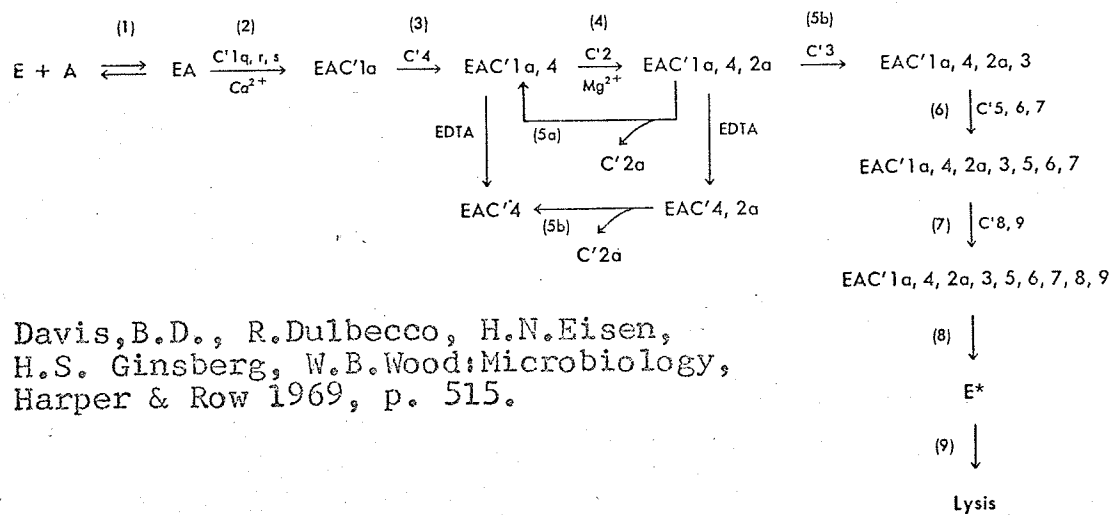
TABLE 21. PROPERTIES OF SOME PURIFIED COMPLEMENT COMPONENTS
ISOLATED FROM HUMAN SERUM

Component	C'1q	C'1s	C'4	C'2	C'3	C'5	C'6
Synonyms	11S component	C'1 esterase	β_{1E} -globulin	—	β_{1O} -globulin	β_{1F} -globulin	—
Sedimentation constant	11S	4S	10S	6S	9.5S	8.7S	5S
Concentration in serum ($\mu\text{g/ml}$)	20-30		30-50	<10	300-400	30-50	<10
Thermolability (56° , 30 min)	+	+	0	+	0	+	0
Inactivated by		DFP	N_2H_2 , NEM	p-CMB	N_2H_2		

DFP = diisopropylphosphorfluoride; N_2H_2 = hydrazine; NEM = N-ethylmaleimide; p-CMB = p-chloro-mercuribenzoate.

From M. J. Polley and H. J. Müller-Eberhard. *Chemistry and Mechanism of Action of Complement*. In *Progress in Hematology*, vol. 5. (E. Brown and C. V. Moore, eds.) Grune & Stratton, New York, 1966.

Fig. 31. Sequence of reactions in immune hemolysis. E=sheep red cells. A=rabbit antibodies to sheep red cells. EDTA=ethylenediaminetetraacetate (Versene), a chelating agent used to remove Ca^{2+} . Both $\text{EAC}'1\text{a}, 4, 2\text{a}$ and $\text{EAC}'4, 2\text{a}$ are unstable and tend to lose $\text{C}'2\text{a}$ (reactions 5a and 5b).



Davis, B.D., R. Dulbecco, H.N. Eisen, H.S. Ginsberg, W.B. Wood: *Microbiology*, Harper & Row 1969, p. 515.

of C' are not activated (635). When immunoglobulins in the absence of antigen are simply aggregated by heating, or by other denaturing conditions, they fix C' in the same way as antibodies that are aggregated by specific combination with multivalent antigen. IgM antibodies tend to be more efficient in C' mediated lysis than IgG. The reason for this is that the binding of a single IgM molecule is able to activate the reaction while the interaction of IgG with one determinant will not activate lysis. The activation requires the linking together of two, and in some cases four moles of IgG antibody so spaced ($\leq 200 \text{ \AA}$) that C'1q, by interacting with two singly held Fc domains, undergoes a conformational change which activates the chain of events leading to lysis (443).

A disease called hereditary angioneurotic edema is due to the congenital lack of C'1 esterase inhibitor in the serum. Therefore, this enzyme, generated through C' fixation, remains active and causes edematous reactions (attacks) in various regions of the body, which occasionally could be fatal. C'2 deficiency, when only present to a low level (i.e. a few per cent of the normal level), has no pathological consequences. Autoimmune glomerulonephritis, serum sickness and perhaps rheumatoid fever are also mediated by complement. A hemolytic disorder - paroxysmal nocturnal hemoglobinuria - is probably due to an abnormality of the red cell surface which is able to activate complement (receptor) without the participation of antibodies (300).

D. Surface immunoglobulins of lymphocytes

For a long time the view has been propounded that lymphocyte receptors for antigens would be similar, if not identical to antibodies. Indeed, it was observed that rosette-formation by lymphoid cells (binding of erythrocytes on the surface of lymphocytes) of mice immunized with sheep red blood cells could be inhibited by treatment of lymph node cells with antisera to murine μ and γ chains (587). Recently, it was shown that rabbit anti-mouse kappa serum had selective cytotoxicity for B lymphocytes but not for T cells (more than 95% of mouse immunoglobulins is kappa-type) implying that there was at least a quantitative difference in the surface immunoglobulins of the two cell types (603). Hemolytic plaque-forming cells (PFC) of IgG type from mice had no demonstrable surface immunoglobulin (plasma cell), but a proportion of IgM PFC were $\kappa^+ \mu^+$. Virtually all rosette-forming cells (RFC) had surface immunoglobulin, more than 90% of them being inhibited by anti- κ , 50% by anti- μ and 10-30% by antisera to other heavy chains. No immunoglobulin was demonstrable on thymocytes. Cells that had the potential to initiate graft-versus-host reaction and were θ -positive and Ig-negative as revealed by C'-mediated cytotoxicity, thus, it was concluded that T-cells may not carry immunoglobulin (865).

However, surface immunoglobulin light chains could be demonstrated on thymocytes (14.4%) and thymus-derived thoracic duct lymphocytes (37%) with radioiodinated antisera in mouse (54). Approximately 20% of mouse spleen lymphocytes were found to carry two major γ -globulin classes γ G (7S γ 2a) and γ F (7S γ 1) on their

surface and another 10% carried γF only, as detected by a modified mixed agglutination (reverse immuno cytoadherence) test (528). In cell populations of mouse lymph nodes 95% of the mature lymphocytes possessed surface κ and μ determinants, as revealed by electron microscopy. This finding was interpreted as an indication that both T and B cells express IgM. Plasma cells did not carry immunoglobulin on their surface. In the thymus surface-associated immunoglobulin could not be detected in the vast majority (98%) of the cells, but 2% positive cells were found. T-lymphocytes may thus acquire immunoglobulin as a surface constituent shortly before or after the cells leave the thymus (379). Finally the ability of mouse spleen cells to induce graft-versus-host (GVHR) disease could be suppressed by treatment with univalent Fab and Fab' fragments of rabbit anti-mouse immunoglobulins, but not with the intact antibodies. Antibody treated spleen cells elicited antibody response to rabbit immunoglobulins in each case (754). This finding may be regarded as an indication of the functional importance of T-cell surface immunoglobulins, which are initiators of GVHR.

Surface immunoglobulins have been also detected on lymphocytes of other species (192; 689). Thus, purified suspensions of human peripheral lymphocytes underwent transformation into blast cells when incubated with anti-light chain serum (892). Human bone marrow cells were also found to respond to anti-immunoglobulin serum by blast formation (2). Surface immunoglobulins on peripheral lymphocytes from normal individuals were easily detectable by

immunofluorescence but were absent from lymphocytes of patients with Burton type agammaglobulinemia. Lymphocytes from these patients responded to phytohemagglutinin, pokeweed mitogen, Concanavalin-A, antilymphocyte serum PPD (purified protein derivative of *Mycobacteria*) and allogeneic lymphocytes, showing a normal transformation. Therefore, it was concluded that these patients had a normal T lymphocyte function. It was suggested that lymphocytes with membrane-bound immunoglobulin belong to the plasma cell series, and are B lymphocytes (311).

Using immunofluorescence as a method for detection, 15% of peripheral lymphocytes were found immunoglobulin positive in normal adults, but 0-0.5% in cases of Burton-type agammaglobulinemia. In chronic lymphoid leukemia the percentage of immunoglobulin positive lymphocytes may vary between 2 and 100. Cells of the plasma cell series may or may not have membrane bound immunoglobulins. Studies performed in rabbit have shown that all IgM-containing plasma cells have also IgM bound on their membrane, whereas some of the IgG-containing plasma cells, up to a maximum of 15%, have membrane bound immunoglobulins. In the latter case, it could be shown that the membrane-bound immunoglobulins are not IgG but IgM, an observation which suggests the possibility that the same cells shift from synthesis of IgM to synthesis of IgG (689).

Using radioactive iodine for labelling surface immunoglobulins, an immunoglobulin with a molecular weight of 200,000 could be isolated from human and murine thymocytes. The heavy chains in both cases were of the μ -type. On the assumption that all thymus lymphocytes

were equivalent and that one atom of ^{125}I was incorporated per IgM molecule detected, it was estimated that a minimum of 400 molecules were fixed per cell (567).

In summary, it may be concluded that both T and B lymphocytes have immunoglobulins on their surface and that they are present on T cells at best at a very low density being detectable only by sensitive methods. IgM monomer (?) seems to be present on T cells, which probably serve as receptor sites. Results regarding B cells are controversial and it remains to be seen what is the significance of immunoglobulins easily detectable on their surface.

Cell-mediated immunity

A. The thymic lymphoid system and its function

As already mentioned, bursectomized chickens are not able to form humoral antibodies. However, their ability to reject homografts is virtually unimpaired (34; 632). Miller demonstrated the essential role of the thymus in the development of various parameters of the immune response in mice (600). Neonatal thymectomy in birds and mammals results in lymphopenia, general suppression of cell-mediated immune responses (e.g. skin graft rejection, resistance to mycobacteria, fungi and viral infections) and also in impaired antibody formation (197; 220; 601).

Similarly children with congenital thymic aplasia (DiGeorge's syndrome) fail to develop contact sensitivity to dinitrofluorobenzene, their allograft rejection capacity is greatly impaired, and they do not have resistance to measles and other viral, fungal, and bacterial infections including mycobacteria. However, they can

produce humoral antibodies. A moderate deficiency of lymphocytes is detected histologically in the deep cortical regions of their lymph nodes and periarterial sheaths of spleen, but their cortical germinal center activity and plasma cells are normal. Such babies survive for one year at the most (253; 314; 503; 639).

Thymectomized animals are also the victim of diverse infections under conventional conditions ("runt disease"), but survive well in a germ-free environment (593; 945). The congenitally athymic nude mice have a retarded growth pattern, defects in salivary glands and a tendency to die at an early age. They do not reject grafts, their antibody response to sheep erythrocytes is depressed, but normal to *Brucella*. Autoradiography, using ^{125}I -labelled flagellin, hemocyanin, a co-polymer of Tyr. Glu. Ala. Lys and DNP-BSA (dinitrophenyl-bovine serum albumin), revealed that these mice were completely deficient of T cells, are not deficient of antigen binding cells. It was suggested that the antigen binding cell technique as routinely performed with non-primed populations of cells detects primarily, if not exclusively, B cells (248). Spontaneous (background) rosettes in non-immunized animals, formed by T cells can be inhibited in vitro by low and non cytotoxic concentrations of antilymphocyte serum (ALS) in the presence of complement, and by azathioprine. The inhibition of background rosettes from bone marrow requires much higher concentrations of either ALS or azathioprine. A deficiency of ALS- and azathioprine-sensitive spontaneous rosettes occurred in adult thymectomized mice with or without x-irradiation; this effect of thymectomy takes 5 days to develop (43).

The thymus emits a population of T cells (Θ positive in the mouse) which are mitotically relatively inactive and become part of a pool of long lived lymphocytes localizing in the paracortical area of the lymph nodes, periarterial lymphocyte sheaths of the spleen (both birds and mammals) and interfollicular tissue of the Peyer's patches (thymus dependent areas) (220; 242; 601). A large number of specific antigen binding cells ($27-103/10^6$ thymocytes) were found in the thymus (614). However, in TL-positive mouse strains it could be shown that only TL-negative thymocytes were immunocompetent and that they represented a minor (13%) population (526). Hence, it was concluded that immunocompetence was probably acquired in the thymus before the release of the T-cells. As detected by cytotoxicity test with anti- Θ serum, the mouse lymph node contains 70-80% and the spleen 30-50% T-cells. The thymus contains 97-100% Θ -positive cells (718). Thymus derived cells constitute the majority of the recirculating lymphocyte pool (60-90%) detectable in the peripheral blood (220).

T-cells have not been shown to secrete antibody (601) but they are able to mediate specific cytotoxic reactions (146; 147; 556) and produce soluble mediators of cellular immunity (217). It was shown that phytohemagglutinin (PHA) stimulates only T-cells (359). On the other hand, cortisone which acts also on T-cells does not abolish the ability of lymphocytes to induce GVHR (178; 179). It is interesting to note that when stimulated with PHA, lymphocytes become cytotoxic (425).

B. Manifestations of cell-mediated immunity

Robert Koch was the first to realize in 1890 that healthy individuals reacted scarcely or not at all to the s.c. injection of 0.1 ml of the boiled culture fluid of *Mycobacterium tuberculosis* (old tuberculin), but 0.01 ml injected s.c. to a tuberculous patient caused a severe general reaction associated with a rise in temperature of 2° to 4°C, starting 4-5 hours after injection and lasting for 12-15 hours. The injection site also became red and painful. However, the "normal" human gave a similar reaction on injection with a higher dose (0.25 ml) of the material and no reaction could be detected in the normal guinea pig even on s.c. injection with as much 2 ml of the culture fluid (499). This reaction was coined as the "Koch-phenomenon" and was often confused with other forms of "allergy", until 1942 when Landsteiner and Chase pointed out that the passive transfer of contact sensitivity can be achieved successfully with lymphoid cells, but not with serum in the guinea pig (513). In 1945, Chase demonstrated that tuberculin sensitivity fell into the same category of immune reactions. Thus, viable spleen or lymph node cells, or peritoneal exudate cells harvested 48 hours after i.p. injection of paraffin oil into sensitized donors were able to transfer immunity (153). On the basis of these fundamental observations, all immune conditions transferable by viable lymphoid cells, but not with serum, to syngeneic recipients are considered to be due to cell-mediated immunity, in contrast with humoral immunity which is transferable with serum or with purified antibodies. A great variety of thymus dependent antigens,

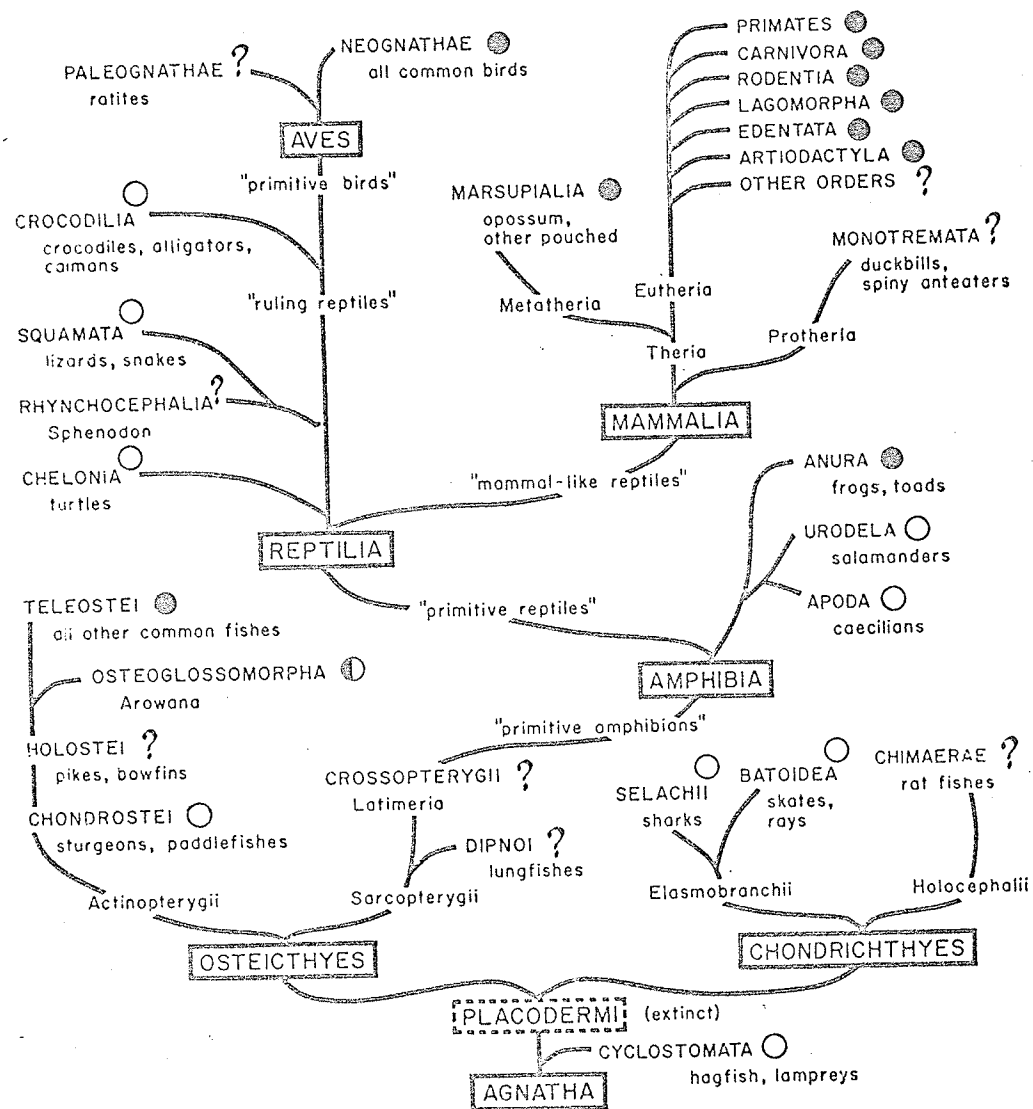
including viral, bacterial, fungal, protozoal and tissue antigens, as well as simple chemicals (contact sensitivity) are able to elicit cell-mediated immunity (897). As an example of the phylogeny of cell-mediated immunity evolutionary trends in the rapidity of allograft rejection in vertebrates are illustrated in Figure 32 (181).

In relation to cell-mediated immunity induced by painting of the ears of guinea pigs with ^{14}C -DNFB (dinitrofluorobenzene), it was shown that 72 hours after, 25% of the total radioactivity resided in lymph nodes and was bound to lipid and protein components (677). This finding supports the view that small lymphocytes become stimulated in the periphery by passing through the site of antigen application or deposition, where they pick up the antigen, and migrate from there to the lymph nodes, where they find the correct milieu to transform into immunoblasts (580).

The characteristic cytological changes induced by antigens which elicit cellular immunity are transformation of small lymphocytes to large pyroninophilic cells in thymus dependent areas and mytosis for the production of further small lymphocytes (356;601). The cells localized in the draining lymph node of mice four days after the application of a skin homograft were studied by electron microscopy. Unlike plasma cells, these cells contained no rough endoplasmic reticulum, although the cytoplasm was very rich in ribosomes and the Golgi apparatus was well developed (87).

The behaviour of lymph nodes during a primary response to various types of antigenic stimulation is shown schematically in Figure 33 (897). The small lymphocytes generated in the thymus

Figure 32. EVOLUTIONARY TRENDS IN THE RAPIDITY OF ALLOGRAFT REACTION



KEY: RAPIDITY OF REJECTION MEDIAN OR MEAN SURVIVAL TIMES (MST)

● - ACUTE	< 14 DAYS
① - SUBACUTE	~ 20 DAYS
○ - CHRONIC	> 30 DAYS
? - NOT STUDIED	

Temperature at which allograft rejection in ectothermic species was studied was ~ 20-25°C in most cases.

Cohen, N., M. Borysenko-Transplant. Proc. 2:333, 1970.

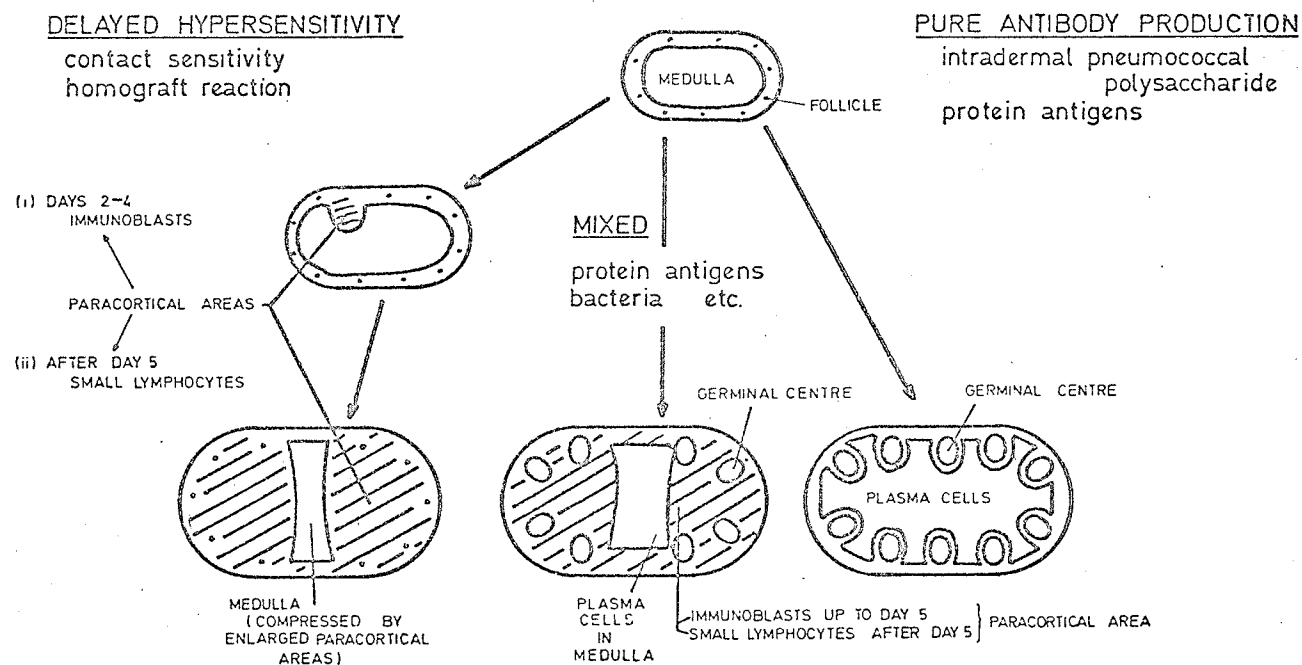


Fig. 33. Diagram of the behaviour of lymph nodes during various types of antigenic stimulation; primary response.
 Turk, J.L.: Delayed Hypersensitivity. 1967. p.152.

dependent areas of the primary lymphoid organs recirculate. Upon deposition of the antigen in the skin, or in other tissue, a local inflammatory reaction occurs in "sensitized" animals, which usually starts about 6 hours after the injection of the antigen and reaches a maximum between 24-48 hours. Because of the latency of the onset of this manifestation this type of immune response is known as delayed hypersensitivity, by contrast with the rapid occurrence (within 30 minutes) of serum reagin mediated inflammatory response, which is referred to as immediate hypersensitivity (200; 897; 901). Delayed hypersensitivity is frequently used as an alternative term for cell-mediated immunity. However, throughout this thesis, the term delayed hypersensitivity will be used to denote a sense of delayed inflammatory reaction to antigenic challenge in vivo. Histologically, delayed hypersensitivity skin reactions are characterized by a cellular infiltration which is predominantly, but never exclusively composed of mononuclear cells (lymphocytes plus macrophages); polymorphonuclear leukocytes participate also in this reaction especially in the early stage (8 hours). The inflammatory reaction is qualitatively similar to nonspecific inflammation and the participating mononuclear cells are blood borne as revealed by autoradiography (828). If the antigen is injected into the cornea, vascularization occurs first and positive corneal reactions occur afterwards, indicating the important role of blood vessels in the reaction (794; 901). Autoradiographic studies on rats exhibiting cell-mediated immunity to *Listeria monocytogenes* revealed that the protective small lymphocytes in the lymph have a rapid turnover and a short lifespan in circulation.

It was also shown that the committed cells were generated at the site of bacterial implantation in the regional lymph nodes and that they entered the blood by way of efferent lymphatics and the main lymphatic ducts, and participated in the inflammatory process (591; 592).

Uhr and coworkers (900) described the induction of delayed hypersensitivity in guinea pigs by means of antigen-antibody complexes, precipitated in antibody excess (4:1), injected in incomplete Freund's adjuvant. However, this type of delayed reaction did not fit into the category of classical delayed hypersensitivity reactions because of its early appearance and transient nature (721) and Nelson and Boyden proposed for it the term, Jones-Mote reaction, after the two physicians who had described similar reactions in patients injected intradermally with heterologous sera. After a great deal of debate it was shown that there was histologically qualitative difference between this reaction and the classical type of delayed reaction, inasmuch as the Jones-Mote reaction was due predominantly to basophilic leukocytes, the lymphocyte was also an important contributor to the reaction, but not the macrophage. (There was no evidence for histamine release). Consequently, recently the term "cutaneous basophil hypersensitivity" (CBH) was proposed to distinguish this reaction from delayed hypersensitivity (244; 751; 752).

Delayed reactions elicited in animals sensitized with protein antigens in complete Freund's adjuvant included also the participation of basophils when tested early after sensitization;

however, at later intervals this component became less prominent. Delayed lesions of contact sensitivity to DNFB and DNCB similarly contained a prominent basophil component that was not diminished when testing was delayed for several weeks following sensitization. CBH was passively transferred with lymph node cells but not with serum (247). Inhibition of macrophage migration is not present in CBH (63). Further studies revealed that basophils participate in skin allograft rejection in guinea pigs (243) and in delayed reaction to vaccinia virus (245). Intravenously administered ovalbumin in doses which induce tolerance for classical delayed hypersensitivity and antibody formation actually stimulated CBH and PCA antibody production. Similar immunogenic-tolerogenic requirements and the prime role played by the basophil suggested a developmental or functional relationship between CBH and PCA antibody response (750).

In patients with allergic contact dermatitis to a variety of allergens, the microscopic findings in 3-day biopsies differed significantly from the classical descriptions of tuberculin hypersensitivity and showed, in addition to mononuclear cells and the characteristic epidermal changes of contact dermatitis, a substantial infiltration of basophilic leukocytes and evidence of vascular permeability with vascular compaction, dermal edema, and fibrin deposition. Serial biopsies from one individual showed that the initial lesion consisted of perivascular accumulation of lymphocytes, this was followed by an influx of basophils and, subsequently, of eosinophils (246).

From all these investigations it is clear that basophilic polymorphonuclear cells are regular participants in practically all types of cell-mediated immune reactions. It remains to be seen what is their function, and whether this participation is mediated by homocytotropic antibody and/or some mediators of cellular immunity or some other, so far unknown mechanism is involved. In this context it is of interest, that mast cells, which are very similar, if not identical, to that of basophils were shown to proliferate after antigenic stimulation in adult rats (604).

When a sufficient amount of antigen enters the circulation of a delayed hypersensitive individual, fever and lymphopenia occur. In the guinea pig and rabbit, fever begins at 1-2 hr after administration of antigen, reaches a peak by 6 hr, and ends by 6-10 hr. In man, maximum fever may not occur until 8 hr, with slow subsidence over the following 24 hr. Antibody does not appear to play any role in this systemic delayed hypersensitivity reaction. The fever was shown to be associated with a circulating endogenous pyrogen in rabbits. If the dose of systemically administered tuberculin is increased beyond that required to obtain maximal fever, then a syndrome referred to as tuberculin shock may occur. In this case, after a minimal rise in temperature, there is a progressive hypothermia beginning at 2-3 hr. Loss of appetite, muscular weakness, hyperventillation, decreased blood pressure, intermittent convulsions, loss of consciousness and death may follow 4-30 hr after the tuberculin injection. Pathological changes

are: swelling, edema, and occasionally hemorrhages of lymph nodes and thymus; congestion and minute punctuate hemorrhages of the viscera; and hemorrhage at the sites of previous skin tests or recent trauma. Histologically, the viscera show infiltration with mononuclear cells, the lung contains an extensive cellular infiltrate of polymorphonuclear and mononuclear cells, and the liver has large areas of necrosis. There is a striking diminution in the number and volume of splenic follicles. The tuberculin-sensitive human shows a similar type of systemic reaction. The relative systemic reactivity to tuberculin of mouse, guinea pig and man is 1:10:100, respectively, on the basis of the dose requirement per unit body weight. There is considerable evidence that susceptibility to shock is not well correlated with skin sensitivity (200; 901).

In general cell-mediated immune reactions occur earlier at a weaker antigenic stimulus, and are then followed by a humoral response in most cases. Injection of small antigen doses intradermally or s.c. in complete Freund's adjuvant favours the induction of cell-mediated immunity (74; 901). If guinea pigs are sensitized with a sufficiently small amount of protein antigen to stimulate delayed response but without antibody formation, the animals are "primed" to develop a secondary antibody response when next exposed to the antigen despite the fact that they had never a manifested primary immune response (784). Because requirements are somewhat different for the induction of humoral and cellular immunity, the system can be manipulated. Large dose of protein antigen (10 mg bovine gamma globulin) given i.v. or s.c. to guinea

pigs resulted in the loss of delayed hypersensitivity and in the suppression of IgG₂ antibody formation, without any effect on IgG₁ formation. This phenomenon was termed immune deviation (32).

Man's reaction to mosquito bites (*Aedes aegypti*, *Anopheles maculipennis atroparvus*) may be regarded as a common example of the dose and time relationship of immunological reactivity. The usual pattern of sensitivity on continued exposure was established to consist of about 5 phases: no reaction - delayed reactivity - immediate and delayed reactivity - immediate reactivity alone - no reactivity. It was suggested that, as circulating antibodies appear, delayed reactivity gradually disappears and finally the subject may become completely desensitized. The sensitization was immunologically specific depending on the strain of mosquito used (582).

Most germ-free guinea pigs could not be sensitized with picryl chloride in contrast to conventional animals. When injected with a fully antigenic substance, such as bovine gamma globulin (BGG), none of the germ-free animals acquired BGG-specific delayed hypersensitivity. Complete Freund's adjuvant did not develop the characteristic induration or erythema normally seen at injection sites. Germ-free guinea pigs given competent lymphoid cells from highly sensitized conventional animals were unable to translate adoptive cell-mediated immunity into dermal reactions (536). These data emphasize again the role of environmental antigens in the development of normal immunological reactivity.

As discussed in the section dealing with antigens, the actual immunogenic capacity of antigens is necessary for the induction of the immune response, which involves the stimulation of immunocytes (T and B lymphocytes), while haptens are not able to do so by themselves. It follows that immunogens but not haptens will be able to elicit cell-mediated immune reactions as well (the "carrier specificity" of delayed hypersensitivity), since this reaction involves again the stimulation of sensitive T-cells. A few exceptional examples of antigens have already been discussed.

C. Cytotoxic effect of lymphoid cells

The local lesions discussed above represent an effector mechanism which appears to have a counterpart in some of the experimental and human autoimmunities, in various allograft reactions, in some form of tumor rejection and in immunity to a variety of microbes. During recent years, a number of in vitro models have been established in order to investigate the mechanism of tissue damaging reactions due to cell-mediated immunity. It has been shown that lymphoid cells from sensitized donors will destroy tissue culture cells carrying the antigen to which the cell donor is sensitized. The cells which are destroyed upon addition of lymphoid cells are called target cells or targets, the cells which achieve this effect are called effector cells. The term sensitized cell or sensitized lymphocyte designate the antigen reactive lymphocytes with antibody-like receptors believed to be produced by the committed cell which carry them.

A variety of methods have been used for the investigation of cytotoxic cells. A common feature of these methods is that target cell damage is evaluated after a certain period of exposure to lymphoid cells either qualitatively, e.g. plaque formation on a monolayer or quantitatively by cell counting, inhibition of colony formation, isotope release from labelled target cells (^{51}Cr is used most frequently) or by inhibition of isotope incorporation. A critical evaluation of these methods is given in a recent review by Perlmann and Holm (686).

Two steps may be recognized in cytotoxic reactions mediated by lymphoid cells. The immunological specificity resides in the first step in which contact between effector cells and target cells is established presumably by the specific antibody-like receptors. The second step involves the lytic reaction, which is exhibited by viable and metabolically active effector cells only. Although cytotoxic factors may be released in this cell to cell interaction, most of the evidence suggest that cell to cell contact is a principal requirement for the lytic step. Phagocytosis does not seem to play a major role. When macrophages, polymorphonuclear leukocytes, and antibody-secreting cells are present, target cell destruction may occur along a number of different pathways (686).

At least one form of cytotoxicity, which is regarded as one of the most important factors of cell-mediated immunity is exhibited by T-lymphocytes. When irradiated (800r) DBA/2 mice were reconstituted with 50×10^6 thymus or bone marrow cells i.v. from

A, C3H or C57Bl/6 donors, and the cytotoxicity of pooled spleen cells was measured 5 days later against ^{51}Cr -labelled mastocytoma grown in DBA/2, thymus cell recipients showed significantly higher cytotoxic activity (146). Furthermore, anti- θ serum eliminated cytotoxic cells to sheep erythrocytes from sensitized spleen populations, but plaque forming cells were unaffected (147). Cytotoxic cells obtained from mice immunized with skin allografts were also shown to be θ bearing cells (556). Exogenous complement is not required for cytotoxic activity (682; 686). Experiments with highly purified T cell populations indicated that only thymus derived cells were involved in the killing of target cells by allogeneic immune cells in vitro, which had specific receptors for the target (351).

If allogeneic lymphocytes are mixed and cultured together for 5-7 days, a greatly increased blast formation and DNA synthesis occurs in comparison to non-mixed or syngeneic cultures. This phenomenon is known as the mixed lymphocyte reaction which is regarded as an in vitro correlate of cell-mediated (allograft) immunity (551). It was recently observed that mixed lymphocyte cultures produce effector cells capable of cytotoxicity (391; 421; 823). Effector cells generated in mixed lymphocyte cultures induced second set skin graft rejection and severe graft-versus-host reaction in rats (GVHR) (266; 354). Another way to induce cell-mediated immunity in vitro is by culturing lymphoid cells with allogeneic or xenogeneic non-lymphoid target cells (44; 338). The lytic potency of rat lymphocytes sensitized against mouse cells in vitro

was 5-10 times greater than those sensitized in vivo as measured by ^{51}Cr -release. Rat lymphoid cells sensitized in vitro to mouse cells were also able to distinguish strain-specific and species-specific target cells, whereas lymphocytes from rats sensitized in vivo to mouse were unable to distinguish murine transplantation antigens during the lysis of mouse target cells in vitro. While normal rat lymphoid cells were unable to induce a significant GVHR in newborn mice, rat lymphocytes presensitized to mouse antigens in vitro gave strong GVHR in newborn mice similar to the reaction caused by injection of rat (antimouse) cells presensitized in vivo. On the other hand, lymphocytes from normal rat or from rats sensitized in vivo to murine cells, or in vitro to alloantigens were unable to cause an accelerated rejection of mouse skin grafts on rats, whereas the same number of (antimouse) lymphoid cells sensitized in vitro significantly accelerated rejection of these grafts. It was suggested that the xenograft reaction induced in vitro is an immunological phenomenon and that it may be more intense than the heterograft reaction induced in vivo (79). "Autosensitization" of rat and mouse lymphoid cells was also achieved under identical culture conditions. Moreover, the addition of small amount of glucocorticoids (1 $\mu\text{g}/\text{ml}$ prednisolone) to the medium, increased the degree of sensitization. Both mouse and rat lymphoid cells "autosensitized" in vitro induced GVHR in syngeneic newborn animals. Furthermore "autosensitized" rat, but not mouse, lymphoid cells were able to produce immunospecific lysis of syngeneic target fibroblasts in vitro (175; 176).

As already mentioned the first step in the cell-mediated immune cytotoxic reaction involves the specific interaction of antigens by the sensitive lymphoid cells with the aid of surface receptors. As would be expected, there is strong experimental evidence, indicating that in an immunocompetent lymphocyte population, there exist numerous sub-populations (clones) sensitive to different antigens. If a particular sub-population is killed by toxic nucleotide analogs during in vitro stimulation (782; 970; 971) or adsorbed out by target cell monolayers (167; 558), the remaining cells are not able to respond when stimulated with identical antigens, but the response is virtually unaffected if unrelated antigens are used for stimulation. There is no evidence, however, that the antibody-like receptors of the recognition step also participate in the subsequent lytic reaction which probably lacks specificity in an immunological sense (686). Spleen cells or blood lymphocytes from guinea pigs immunized with Tubercle bacilli lysed PPD coated chicken erythrocytes, i.e. after 24 hr. of incubation ^{51}Cr -release from PPD coated cells was 2-4 times higher than the background release from non-coated cells. On other hand, antimycin A, an inhibitor of respiration, inhibited the cytotoxic reaction (688). Splenic lymphocytes of guinea pigs, which had been sensitized with DNP-human gamma globulin (HGG) caused (carrier) specific lysis of P815 mastocytoma cells of DBA mice when the latter were coated with DNP-HGG. Covalently coupled to erythrocytes with the aid of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (409; 410).

In the presence of specific antigen, lymph node cells from inbred rats with cell-mediated immunity to tuberculo-protein, bovine serum albumin and egg albumin produced progressive destruction of monolayers of rat embryo fibroblasts in tissue culture, which was apparent at 48 hours and maximal at 72 hours. The effect was specific for the sensitizing antigen and did not depend on the genetic difference between lymph node cells and target cells (769). Damage of rat embryo fibroblasts in these experiments was shown to be closely correlated with delayed skin reactions in the animals from which the lymph node cells were taken, but not with Arthus reactivity. In animals sensitized with picryl conjugates of ovalbumin or of human serum albumin, skin reactivity and the in vitro cytotoxic effect could be elicited only with the homologous conjugate or the protein carrier alone but not with picryl conjugates of heterologous proteins (770). Essentially similar results were obtained in the author's laboratory. Lymph node cells from guinea pigs sensitized to a state of delayed hypersensitivity to mouse or rabbit globulins induced lysis of mouse sarcoma I cells, when these cells were coated with the corresponding globulins in the form of anti-sarcoma I antibodies. Similar lysis of target cells was induced by immune lymphoid cells pretreated with the sensitizing antigen, or in the presence of free antigen in the medium containing immune and target cells. The cytotoxic effect was immunologically specific with respect to the antigens used for induction of delayed hypersensitivity but was nonspecific with respect to the target cells. No soluble cytotoxic mediators were detected (808).

Nonspecific cytotoxicity was also observed with mouse and human lymphoid cells sensitized in mixed lymphocyte culture (421; 823) as well as with rat lymphoid cells sensitized to rat or mouse fibroblasts in tissue culture (173). Although it is noteworthy that the mechanism of *in vivo* reactions is very complex and scarcely clear cut, there is some indication for the occurrence of non-specific cytotoxicity in vivo. Thus (Lewis x BN) F_1 hybrid rats normally do not reject Lewis kidney, but they do so if Lewis rat spleen cells are inoculated under the capsule of a freshly grafted Lewis kidney. It would, therefore, seem that the donor lymphoid cells can be immunologically stimulated by circulating host leukocytes and the interaction of these two cell populations results in nonspecific damage to kidney parenchyma (263). Skin grafts from rats were transplanted to isogeneic, allogeneic, xenogeneic or specifically sensitized allogeneic recipients, and after 24-48 hours were retransplanted to the original donor. Whereas 92% of iso-autografts were accepted, allo-autografts and xeno-autografts were accepted in only 45% and 36% respectively (109). These results, taken in conjunction with the findings of the previous experiment (263), suggest that rejection of the allo-autografts or xeno-autografts could be due to a local reaction between lymphoid cells of the donor of the graft and allogeneic and xenogeneic lymphocytes, which had infiltrated the graft ("passenger leukocytes"). Thus these studies provide strong evidence that target cells can be destroyed by an "indirect" cell-mediated mechanism involving effector cells activated by an immunologically

specific reaction to antigens other than those of the target cells.

Another form of target cell destruction may be designated as antibody dependent lymphoid-cell mediated cytotoxicity. Certain antibodies are capable of inducing this reaction at very high dilutions and with highly purified lymphocytes as effector cells from normal donors. The reaction resembles that of direct cytotoxicity of lymphoid cells from sensitized donors. Antibodies possessing this capacity may occur in xeno-, allo-, and autoimmune situations. Cytotoxicity does not require differences in histocompatibility between lymphoid cells and target cells. The reaction is most easily induced by antibody bound to antigen on the surface of the target cells which are destroyed. The lytic reaction requires contact between effector cells and the antibody-target cell complex, and the lytic process is energy-dependent.

Lymphocytes from Campbell ducks tolerant to Peking duck target cells became cytotoxic if heat inactivated antisera from Campbell ducks was added; a good correlation was found between the ability of these antisera to induce lymphoid cell-mediated cytotoxicity in vitro and their graft rejecting capacity in vivo (117). The capacity of lymphoid cells from non-sensitized mice to lyse antibody-coated target erythrocytes in vitro did not require the presence of thymus-derived or thymus-dependent lymphocytes. However, prior treatment of spleen cell populations with antibody to kappa chains diminished this function, suggesting a role for bone marrow-derived lymphocytes (106). It was also shown that

spleen cells from thymectomized and x-irradiated adult rats were fully competent to destroy xenogeneic target cells (Chang liver of human origin) in the presence of rat antibodies to Chang cells. Therefore cytotoxic cells in this system could function in the absence of thymic influence; however for the production of specific antibody T cells must participate (384).

Although in the systems reviewed above no direct proof for the involvement of C' components was adduced, target cell bound complement may also trigger lymphocytes for cytotoxicity. Thus, for example ^{51}Cr -labelled chicken erythrocytes treated with rabbit anti-Forssman antibody and the first seven components of human C' released the isotope upon exposure to purified human lymphocytes. Lysis of target cells was suppressed by treatment with antimycin A. Monocyte enriched leukocyte preparations lysed antibody coated target cells if the first 3 or the first 7 components of C' were added to the system (687) and it may be visualized that the lytic factor in the form of C'8 may be provided by the effector cell (686). In the mouse θ bearing lymphocytes were shown to be distinct from lymphocytes possessing receptor for complement (84).

From this survey, it follows that B cells are involved in both the antibody dependent and the target cell bound complement induced cytotoxicity. These reactions may represent important alternative pathways of cell-mediated target cell destruction, the biological significance of which is incompletely understood at the present time.

Finally, the nonspecific cytotoxicity of lymphoid cells activated by phytohemagglutinin (PHA) or other stimulants is also a well established phenomenon. Common for all models is the requirement of metabolically active effector cells, susceptible to stimulation. Cytotoxicity is an energy -requiring process. Blast transformation and DNA synthesis per se are not required for cytotoxicity. There is no relationship in immunological specificity between stimulating agents and antigens of the target cells; therefore, cytotoxicity is nonspecific. Autologous, syngeneic and allogeneic target cells may be affected. Direct contact between effector cells and target cells is not needed for activation of the lymphoid cells. However, contact seems to be necessary for target cell destruction at least during early phases of interaction. Mechanisms for target cell destruction by immune cells are also applicable to this model (425; 686).

D. Mediators of cellular immunity

In addition to their cytotoxicity, sensitized lymphocytes when stimulated by specific antigen or by appropriate mitogens produce a variety of soluble substances that have different biological activities depending on the method of assay (217; 218; 524; 760). These substances may be classified into two major groups. The first group represents substances which act on other leukocytes, amplifying thus the effect of the initial cytotoxic reaction and leading to an inflammatory reaction; this function of sensitive lymphocytes may be more important than their capacity to exert direct cytotoxicity on target cells. The second group of mediators is considered to act directly on target cells and to inhibit their proliferation or destroy them.

So far only a few mediators have been isolated in a pure form and, therefore, it is conceivable that a single substance may have more than one biological activity or that more than one kind of biologically active molecule may be involved in eliciting a particular biological response. The classification presented here is based on biological effects and should not be regarded as a classification of various mediator substances.

(i) Chemotactic and other amplifying mediators

(a) Migration inhibitory factor (MIF)

MIF is produced within the first 24 hours of culture by sensitive lymphocytes in the presence of antigen. It inhibits the migration of normal peritoneal macrophages. Its production is inhibited by inhibitors of protein synthesis. MIF is heat stable at 60°C for 30 minutes and is nondialyzable (216). Its activity is associated with an acidic α -glycoprotein with a molecular weight of 40,000 which migrates on acrylamide gels in the prealbumin region. Chymotrypsin and neuraminidase destroys its biological activity; the latter enzyme is known to degrade sialic acid. The factor(s) responsible for MIF activity could be clearly separated from the factor(s) endowed with leukotactic activity (741; 742). A MIF-like substance produced in mixed culture of guinea pig lymphocytes (Strain 2 and Strain 13) caused delayed skin reactions in guinea pigs when injected intradermally in a concentrated form (61). MIF activity can be detected in supernatants of established human lymphoid cell lines (341; 358).

The detection of MIF by macrophage migration test is regarded as one of the most reliable in vitro correlates of cell-mediated immunity (548). It has been suggested that MIF has "carrier" specificity (511).

(b) Chemotactic factor for macrophages

This is a nondialyzable, heat stable material, which has been shown by sedimentation in a sucrose density gradient, to be present in fractions with a broad spectrum of sedimentation coefficients extending from less than 4.5S to 7S. The fraction with chemotactic activity can be regularly separated from MIF by discontinuous electrophoresis on acrylamide gels. In contrast to the relative species-specificity of MIF the chemotactic factor is not species specific, and it appears to be resistant to treatment with neuraminidase (217; 741; 742; 924).

(c) Chemotactic factor for neutrophils

A guinea pig chemotactic factor for neutrophils is also present in the medium of antigen stimulated lymphocyte cultures. This factor appears to be distinct from the macrophage chemotactic factor. On centrifugation in sucrose density gradients it resides in a slightly lighter fraction than the former factor on electrophoresis in acrylamide gels at pH 9.1 it remains at the original point of application (217; 924).

(d) Chemotactic factor for eosinophils

Supernatants of cultures of lymphocytes from delayed hypersensitive guinea pigs in presence of the specific antigen

react with immune complexes in vitro to generate a factor which is chemotactic for eosinophils. This factor appears to be unique, since previously described chemotactic factors for other cell types require for their generation either immune complexes or substances released into lymphocyte cultures, but not both. In this case, the interaction between the substance elaborated by the lymphocyte and the immune complex appears to be antigen-specific, inasmuch as the immune complex must contain the same antigen as that used to activate the lymphocyte cultures. Although this factor was generated in an in vitro system, it has been shown to possess in vivo as well as in vitro activity (183).

(e) Chemotactic factor for lymphocytes

Lymph node cells of guinea pigs sensitized to O-chloro-benzyl-bovine γ -globulin conjugates were found to elaborate upon exposure to the specific antigen factor(s) chemotactic for neutrophils, and mononuclear cells including rat lymphocytes. This is the only product discovered so far to be chemotactic for lymphocytes. Lymphocytes did not respond to agents that were highly attractive for eosinophils and mononuclears, e.g. to *E. coli* culture fluid or chemoattractants of the complement system (924).

(f) Mitogenic (blastogenic) factor

A soluble mediator was discovered in human mixed lymphocyte cultures (MLC), which was involved in the transformation of lymphocytes (564). At least two types of stimulating activity could

be detected in human MLC, i.e. a specific blastogenic factor (histocompatibility antigen) and a nonspecific stimulatory factor ("potentiating factor") (471; 471). The fraction possessing this mitogenic action isolated from guinea pig lymphocytes had a sedimentation coefficient of 4S, was not precipitated by 50% $(\text{NH}_4)_2\text{SO}_4$ and its electrophoretic mobility in acrylamide gel was that of albumin (952). This factor was inactivated by trypsin, but was resistant to treatment with DNAase or RNAase. It was also released from lymphoid cells by PHA stimulation and the suggestion was made that it was a nonspecific recruiting agent (41).

(g) Autosensitizing factor

Purified human peripheral blood lymphocytes release a factor in mixed cultures, or when exposed to concanavalin-A, which induces increased DNA synthesis of fresh autologous lymphocytes or of lymphocytes from genetically similar donors (siblings). The maximum mitogenic effect occurs after 3 days exposure and it is increased by the dose of the factor. An inhibitor for this autostimulating factor appears to be present in fresh autologous serum. This factor differs from all the mitogenic factors previously described inasmuch as it acts only on lymphocytes, which are histocompatible with cells from which the factor was released. In the one-way MLC reaction only one kind, in two way MLC two kinds of autostimulating factor can be detected. Such a substance cannot be an antigen (708).

(h) Transfer factor

Extracts of leukocytes from patients with delayed hypersensitivity to microbial extracts are as effective in the adoptive

transfer of delayed sensitivity as viable cells; the factor involved is called transfer factor. This activity of leukocyte extracts is not abolished by endogenously liberated nucleases and lysosomal hydrolases, or by the addition of exogenous DNAase, RNAase, or DNAase plus trypsin. It does not function as antigen or superantigen, on repeated injections into rabbits or man. The factor is dialysable having a molecular weight of less than 10,000 and a polypeptide/polynucleotide composition. The transfer factor is inactivated by heating at 56°C for 30 minutes, but its activity is preserved on lyophilization (525).

Incubation of sensitive blood leukocytes for less than 30 minutes with specific antigen (e.g. PPD = purified protein derivative of *Mycobacterium tuberculosis*) results in desensitization of the cells and in the liberation of the preformed transfer factor into the cell-free supernatant. Although, true transfer factor does not cause any discernible effects on nonsensitive lymphocytes in culture, as measured by thymidine uptake or autoradiography, it converts these cells to an antigen-sensitive state, as revealed by the transformation of such lymphocytes to lymphoblasts and by the release of MIF after, but not before, the addition of the appropriate antigen (674). Similarly, administration of the transfer factor to negative recipients in vivo, in addition to initiating specific delayed cutaneous reactivity, confers on the recipient's lymphocytes the capacity to respond to the appropriate antigen with transformation and clonal proliferation in vitro (525).

The tuberculin transfer factor from human leucocytes was shown to convert to nonsensitive mouse spleen lymphocytes in vitro to a specific antigen-responsive state detected by transformation and thymidine uptake in the presence of tuberculin (7).

Recently a transfer factor has been isolated from lymphoid cells of guinea pigs sensitised to DNCB. The lymph node and oil induced peritoneal exudate cells were collected 14 days after sensitization to DNCB and incubated for 4 hours in Hanks solution at 37°C without addition of antigen. The transfer factor released into the incubation fluid, was dialysed and concentrated by lyophilization. On i.p. injection, this factor conferred contact sensitivity on normal guinea pigs, which was detectable 48 hours after administration; some recipients displayed contact sensitivity even 3 - 6 months after transfer. The molecular weight of this factor was estimated to be 1-2,000 and the material had a polypeptide-polynucleotide composition. It was not retained on passage through a DNP-specific immunosorbent column. On the basis of the extended duration of the adoptive sensitivity, the small molecular weight and its polypeptide-polynucleotide composition, this transfer factor was considered to function as a derepressor molecule (123).

(i) Skin reactive (inflammatory) factor

Peritoneal exudate cells sensitized to PPD release a factor on exposure to the specific antigen in vitro, which is able to induce a local reaction analogous to delayed hypersensitivity

skin reactions at the site of intradermal injection to normal guinea pigs. Erythema and induration occurs with a peak between 3 and 6 hours after injection. Histologically the lesion is characterized by infiltration of polymorphonuclear cells and a smaller number of mononuclear cells. Production of this agent in culture is depressed by actinomycin and inhibited by puromycin (692; 898). Normal guinea pig lymphocytes exposed to PHA in vitro release an inflammatory material similar to the skin reacting factor (693). Pharmacological studies disclosed that certain plasma fractions had an enhancing effect on the action of skin reacting factor, and certain inhibitors of the kinins and of the coagulation system had a depressing effect on its activity. The substance with a suggested molecular weight of 39,000 could not be identified with any of the known mediators of inflammation (563).

(j) Interferon

Interferons from various sources consist of small proteins unusually stable at low pH, and fairly resistant to heat. They are produced by cells infected by almost any virus (either DNA or RNA) or exposed to certain chemicals (interferon inducers). Interferons are not virus-specific, but species specific, e.g. chick interferon M.W. 38,000; mouse and human interferon M.W. 26,000. Interferons are able to inhibit viral multiplication in uninfected cells of the species of origin by interfering in the combination of viral messenger RNA with cellular ribosomes to form functional polysomes, on which the viral proteins are normally synthesized.

Thus, the proteins required for the replication of the viral nucleic acid are not synthesized and the viral nucleic acid is not replicated (224). As recently discovered, interferons are also produced during various in vitro cell-mediated immune reactions (92) including the mixed lymphocyte reaction (333), and also large quantities appear in supernatants of continuous lymphoid cell lines (341). Furthermore mouse interferon preparations enhanced the specific cytotoxic action of sensitized lymphocytes on allogeneic tumor cells. The factor responsible for the enhancement could not be dissociated from the antiviral activity of interferon by standard physicochemical means. Thus, in addition to its well known antiviral activity, and its effect on cell division, interferon also appears to enhance a specialized cellular function. It was suggested that a common mechanism of action underlies these seemingly different biologic phenomena, and that interferon may play a role in the regulation of fundamental cellular processes (549).

(ii) Effector mediators acting on target cells

(a) Cytotoxic factors (lymphotoxins)

Destruction of target L cells by either immune or PHA-stimulated normal mouse lymphocytes was shown not to be the direct result of the sole interaction of aggressor and target cells, but to consist of two steps one involving lymphocyte activation on contact with the targets and the second the release from these lymphocytes of a soluble nonspecific cell-toxin designated as lymphotoxin (LT). Lymphoid cells from a variety of animal species i.e., mouse, rat,

rabbit, human could be activated with PHA to release LT in vitro in the absence of target cells. Production of LT was reported also by established human lymphocytic cell lines (358). Only viable, metabolically active cells of lymphoid origin could be stimulated in vitro to release LT. Neonatal or adult murine thymic lymphocytes or small lymphocytes obtained from patients with chronic lymphocytic leukemia or Hodgkin's disease did not release LT. Interference with protein synthesis or with the general cellular metabolism blocks LT release. By contrast blocking of RNA or DNA biosynthesis reduced but did not stop its release (357; 690). Some properties of human murine (497) and avian LT (217) are summarized in Table 22. It is clear from this table that the LT of each of these three species is different, except for the fact that all three factors are proteins. Human and murine lymphotoxins were claimed to affect a great variety of cell types from various species (357; 943), but L cells seem to be most suitable for assay (504). Cell destruction by LT is nonspecific, the sensitivity of the target cells and rate of damage is concentration dependent, it does not seem to bind or enter the target cell and various polyanions can compete with it for membrane sites (943).

Highly purified lymph node and spleen lymphocytes and lung macrophages from tuberculin sensitive guinea pigs released factors cytotoxic for L cells when cultured with PPD for 24 hours. Sensitized lymphocytes released LT only upon exposure to the specific antigen. In contrast, purified macrophages from both normal and sensitive animals cultured with PPD released a factor(s) which was

Table 22.

Some characteristics of lymphotoxins.

	Human	Murine	Cavian
Molecular weight	80-85.000	85-95.000	25-50.000
Buoyant density in CsCl ϕ_{25}	1.33 (protein)	1.33 (protein)	1.34 (protein)
Heat sensitivity	85°C, 15 min.	100°C	60°C, 30 min.
Stable at pH	5 - 8	2 - 12	
Electrophoretic mobility	slow β - or fast γ -globulin	albumin	albumin
DNAase	stable	stable	
RNAase	stable	stable	
Trypsin (chymotrypsin)	stable	stable	stable
Neuraminidase			stable

cytotoxic for L cell. Macrophage and lymphocyte cytotoxins could not be distinguished by gel filtration, antibody neutralization, heat sensitivity, toxicity, or by inhibition of macrophage migration and the capacity to elicit skin reaction (394).

It ought to be pointed out that there is no general agreement on the mechanism underlying the release of cytotoxic factors from sensitized lymphocytes on interaction with the specific antigen. In most cases cell-to-cell contact seems to be essential for target cell lysis, and no free soluble toxic factors can be detected in the system (686). It is also difficult to visualize, what would be the biological significance of such toxins in vivo, which would kill indiscriminately all cells, including those of the host, which would be present in the range of effective concentrations. Nevertheless, it is not possible to dismiss entirely the view that cytotoxins may occur during in vivo immune reactions, since as discussed earlier, nonspecific cytotoxicity does exist.

(b) Proliferation inhibitory factor

Unstimulated and PHA stimulated human peripheral leukocytes, containing 30-60% polymorphonuclear cells, released a soluble factor which had an inhibitory effect on the proliferation of HeLa cells. Supernatants of stimulated cells were more potent in this and the inhibitory effect on proliferation of HeLa cells could be reversed by replacing the supernatants with fresh or conditioned medium. However, it is to be noted that although highly effective on HeLa cells, the inhibitory factor had no effect on replication of mouse L cells (45).

Cell cooperation in the immune response

A. Cell cooperation in humoral immunity

Soon after the discovery of the importance of the function of the thymus in the immune response (600) it was realized that, in addition to the complete abolition of cell-mediated immunity, thymectomy seriously impaired antibody formation in both birds and mammals (197; 601). Antibody formation in thymectomized animals is deficient only with respect to thymus dependent antigens, which represent the majority of antigens, i.e proteins, hapten-protein conjugates, tissue antigens, fungal, viral and certain bacterial antigens. Among the few exceptions one may cite polymerized flagellin of Salmonella, Brucella antigens, pneumococcal polysaccharides; these antigens which are referred to as thymus-independent antigens, since the antibody response induced by them is virtually unaffected by thymectomy. It should be noted, however, that the response to thymus dependent antigens is never completely abolished in thymectomized animals (352; 766).

It was clearly demonstrated that in a lymphoid cell population from mice primed with SRBC the number of rosette forming cells was reduced by anti- θ serum without affecting the number of antibody forming (plaque-forming) cells (791). Pretreatment with cytotoxic anti- θ serum reduced the capacity of mouse spleen cells to respond by the formation of direct and indirect plaque forming cells (PFC) to SRBC in vitro, but did not affect PFC, their precursors or hemopoietic stem cells. However, the response of

spleen cells treated with anti- θ serum could be restored with thymus cells incubated in vivo with sheep erythrocytes (149; 789). Analogous situation was found in vivo in thymectomized animals, which could be reconstituted for a normal response to SRBC by T cells (607). T cells acted synergistically with spleen cells during the response to SRBC in immunologically suppressed (x-irradiated) animals, but did not cooperate in a similar manner with bone marrow cells except when the dose of the latter was increased by a factor of 40-150 (i.e. $5-15 \times 10^6$) with respect to that of the spleen cells (1.5×10^5). On the other hand a low dose of marrow alone was able to generate significant PFC response (85 if 5×10^4 marrow cells were given). It was proposed that B cells in the spleen differ from those in the bone marrow, i.e. that bone marrow contains the majority of cells not requiring T cell cooperation (B_1 cells), while most of the B cells present in the spleen do require T cell cooperation (B_2 cells) to make PFC. Perhaps B_1 cells can make only IgM, and the T-dependent B_2 cells may be involved solely in the formation of IgG₁ antibodies. SRBC may have non-thymus dependent antigenic determinants, or alternatively similar determinants may be recognized by B_1 and B_2 . The two types of B cells may be at different stages of B cell maturation or they may represent quite different functional types of antibody forming cells (702).

When cell populations primed with 4-hydroxy-3-iodo-5-nitrophenyl acetic acid-bovine serum albumin (NIP-BSA) were transferred to x-irradiated recipients, which were injected 1 day later with NIP-BSA,

a secondary antibody response occurred. A similar effect was demonstrated if cells primed with NIP-chicken γ -globulin (NIP-CGG) and with BSA were mixed with each other prior to transfer and the animals challenged with NIP-BSA. The response to NIP was decreased when BSA specific cells were killed by Θ antibodies and unaffected when NIP specific (primed with NIP-CGG) cells were destroyed with anti- Θ (719). From these and other experiments it was concluded that although T cells do not produce antibodies, they have a helper function necessary for B cells to differentiate towards an antibody response. With hapten-carrier conjugates as antigens, T cells were shown to respond to the carrier (carrier effect) and to cooperate with B cells for the formation of hapten-specific antibodies by the latter cells (550; 723).

Two types of spleen cells may be distinguished on the basis of adherence to plastic surfaces; adherent and nonadherent. It was found that both adherent and nonadherent cells were necessary for the induction of antibody formation to SRBC in vitro. Exposure of adherent cells to antigen for brief periods of time was sufficient to initiate a maximal in vitro response (630). Thymectomized mice had a deficiency in the nonadherent cell population, which could be corrected by thymus graft. The cooperative function of adherent cells was radioresistant, while one or both of the nonadherent cell types (e.g. thymus derived and non-thymus derived) were highly sensitive to x-irradiation (631). Hence at least three types of cells were necessary for the induction of an in vitro antibody

response: T cells, B cells and a nonproliferating class of cells (348; 867). Adherent cells obtained from normal or immunized mice were x-ray resistant and equally effective in enhancing the response of nonadherent cells. Adherent cells, therefore, did not appear to increase in number or change in function following in vivo antigenic stimulation (199). The requirement for a radiation resistant cell in the immune response in vivo was also demonstrated (353). There is strong evidence that this adherent cell population is represented by macrophages (422; 701) and will be referred to as A cells (accessory cells) according to the suggestion of Osoba (668). The major characteristics of A, B and T cells are summarized in Table 23.

B. Cell cooperation in cell-mediated immunity

Cultures of mixed human peripheral blood lymphocytes differing in histocompatibility antigens do not react with increased DNA synthesis, if the glass adherent cells are removed from the population. Addition of adherent cells of one phenotype resulted in marked DNA synthesis of isogeneic (same sex) as well as allogeneic lymphocytes. Although both adherent and non-adherent cells have the capacity to provoke a reaction as antigen, it was concluded that the function of adherent cell is not simply an antigenic stimulus (539; 761).

Carrageenan (a high molecular weight sulfated polygalactose) was administered i.p. to guinea pigs at the time of sensitization

Table 23.

MAJOR CHARACTERISTICS OF CELLS COOPERATING IN THE IMMUNE RESPONSE

	A-cells	B-cells	T-cells	References
Probable synonyms	adherent cells accessory cell radioresistant cell, antigen processing cell, macrophage	marrow derived cell, plaque for- ming cell, ro- sette forming cell, antigen sensitive cell	thymus derived cell, antigen reac- tive cell	(422, 668)
Antigen receptors	? cytophilic antibody	present	present	(167, 558, 782, 970, 971)
Specificity for immu- nizing antigen	none	exhibit	exhibit	(199)
Antibody formation	none	precursors	none	(601)
Production of MIF	none	none	precursors	(601)
Requirement for pro- liferation	none	yes	yes	(631)
Radioresistance	resistant	sensitive	sensitive	(353, 631)
Adherence to plastic	adherent	nonadherent	nonadherent	(630)
Specific mitogen	none	pokeweed mitogen	PHA	(2, 359, 455, 672)

Response to cortisone	resistant	resistant	sensitive	(178, 179)
Receptor for immuno- globulin	present	present	none	(433)
Receptor for comple- ment	for C'3	for C'7	none	(433)
Antigenic markers in - mouse - chicken		PC, MBLA CBuLA	θ, Ly series CTLA	(865) (586)
Presence (% of total mononuclear) in mouse - spleen - lymph node - blood - thymus - bone marrow		55-58 27-30 33-34 0 36-43	29-32 58-63 68-75 >99 0	(720)
Density	low	medium-high	high	(668)
Peak sedimentation velocity	3.6 mm/hr	3-3.2 mm/hr	3 mm/hr in thymus 4 mm/hr in spleen	(668)
Subpopulations	2	2	2	(178, 179, 327, 621, 702)

to BSA in Freund's adjuvant. No cell-mediated immunity was developed in animals treated with carrageenan. When administered around the time of skin testing, carrageenan suppressed the expression of delayed hypersensitivity reactions in sensitized animals which was, however, recovered completely 1 week after drug administration. The suppressive activity was not correlated with anticomplementary or anticoagulant activity of carrageenan. Moreover, carrageenan in vitro did not appear to be cytotoxic for lymphocytes either structurally or functionally and did not possess mitogenic properties or interfere with PHA induced lymphocyte transformation. Carrageenan is known to be toxic for macrophages in vitro by inducing lysosomal damage and release of intracellular enzymes. It was suggested that carrageenan exerted its effect in vivo on immune phenomena as a result of its toxicity for macrophages (799).

Neither thymocytes nor bone marrow cells alone from parental donors caused graft-versus-host reaction (GVHR) in x-irradiated F_1 hybrid mice, but they produced it when administered together (57). Spleen and femoral lymph node cells also acted synergistically with thymocytes in the induction of GVHR. Thymus and spleen cells obtained from 1 week old mice were separately inactive but produced moderate GVHR when combined in equal proportion. It was demonstrated that in both cell populations a genetic disposition was required to produce synergy in GVHR (136).

Highly purified T cells obtained from x-irradiated F_1 hybrid recipients of parental thymocytes fully retained their

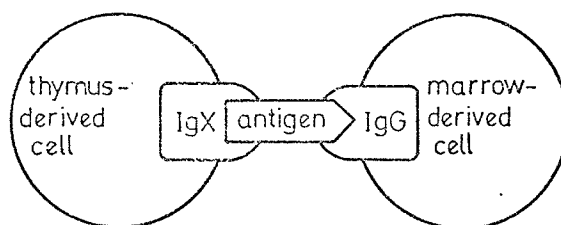
specific cytotoxic activity. This evidence strongly suggests that only T cells are involved in the killing of target cells by allogeneic immune cells in vitro (351). It was demonstrated, however, that the lytic activity of rat thymocytes sensitized in vitro to mouse embryo fibroblasts was increased when admixed with normal spleen cells. The presensitized T cell directed antigenic specificity (557).

C. The mechanism of cell cooperation

Early models of cell cooperation pictured the event as an antigen bridge between T and B cells, as illustrated by Figure 34A. Mitchison (613c) proposed that T cells combine with the carrier determinants of the antigen through their surface receptors of which he assumed were immunoglobulins (IgX) and present the antigen to a second cell which is triggered by the immunogenic determinants. A further development of the model was presented by Taylor (875) who suggested that either T cells or macrophages could have a helper function. If the macrophages were actually involved in this reaction one would have to postulate that cytophylic antibodies produced by T cells serve as antigen specific receptors on the former cells. Furthermore it was proposed that the helper cell supplies non-antigen-specific stimuli which were necessary for triggering marrow derived cells (Figure 34B). Lachmann (511) proposed that a carrier-specific MIF is released by T cells, which is cytophylic for macrophages, and the latter cells would then present the antigen to B cells (Figure 34C). Extracts containing MIF did, in fact, cause an

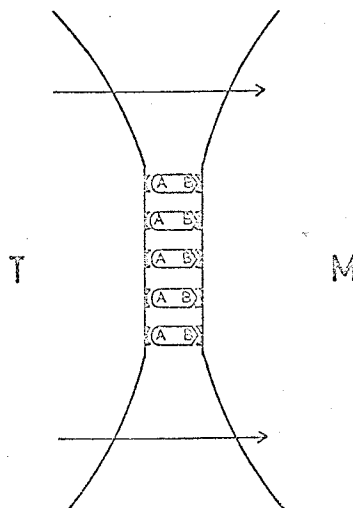
Figure 34. Theoretical models for cell cooperation.

A.



Cooperation of thymus-derived and marrow-derived cells via IgX
 Mitchison, N.A., In "Mediators of Cellular Immunity", H.S. Lawrence, M. Landy (Ed.) Academic Press, N. York & London, 1969. p.78.

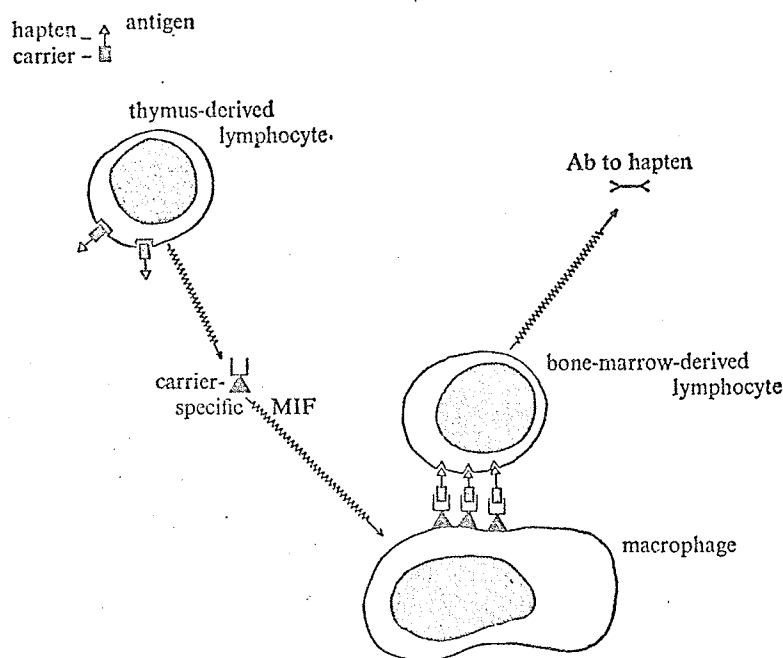
B.



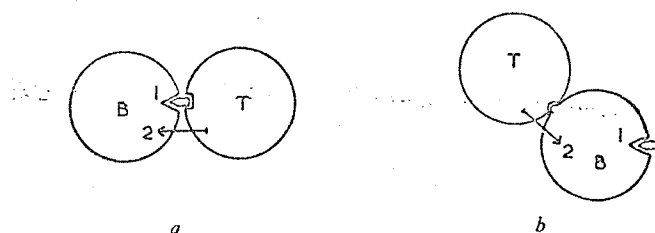
Hypothetical scheme for cell cooperation in the antibody response. Antigen AB effects cell contact between thymus-derived cell (or macrophage-bearing cytophilic antibody produced by thymus-derived cell) T specific for determinant A, and marrow-derived cell M specific for determinant B. Arrows indicate non-antigen-specific stimuli.

Taylor, R.B., Transplant. Rev. 1:114, 1969.

C.

Lachmann, P. J., Proc. Roy. Soc. London Ser. B. 176:425, 1971.

D.



Models for cell-cell interaction in the cooperation of T lymphocytes in triggering B lymphocytes to produce antibody. Cell-cell interaction effected by an antigen bridge. T and B cells have receptors specific for different determinants on the same antigen molecule. *a*, The antigen concentration-presentation hypothesis postulates multiple antigen molecules bound by T cells and presented to B cells supplying a stimulus (1) sufficient in itself for B cell proliferation and differentiation. Our cell surveillance model postulates binding of antigen by B cell receptors and subsequent recognition by T cells of exposed determinants of the bound antigen; thereupon the T cell supplies an additional stimulus (2) which is probably a soluble mitogen. *b*, Cell-cell interaction effected by histocompatibility antigens. This illustrates the system used in the present experiments to show that allogeneic T lymphocytes can replace antigen specific T cells for cooperation. The B cell requires two distinct stimuli in order to function. Antigen supplies stimulus 1 by binding to specific receptors. Stimulus 2 comes in this case from allogeneic T cells specific for B cell histocompatibility antigens with which they interact.

Kreth, H. W., A. R. Williamson, Nature 234:454, 1971.

increase in the amount of specific antigen bound to normal guinea pig macrophages. It was also demonstrated recently in a tissue culture system where carrier-primed T cells and hapten-primed B cells were separated by a membrane with 1 μ pores, that upon exposure to antigen, T cells elaborated an antigen (carrier) specific material, which diffused into the B cell compartment and cooperated in the hapten-specific antibody response. No indication was obtained for a major contribution from nonspecific pharmacological mediators similar to those released during cell-mediated immune reactions (282).

Other findings suggest, however, that the cooperative effect of T cells may be nonspecific in nature. In a culture system, where horse-RBC and SBRC were used as non-crossreacting antigens, "educated" (primed) T cells to one of the erythrocytes were able to trigger PFC against either kind of erythrocytes (388). Spleen cells from rabbits primed with either lysozyme or DNP-BSA were mixed and exposed to DNP-ovalbumin (DNP-OA) and DNP-lysozyme. No DNP-antibody was produced, with DNP-OA but the response was positive with DNP-lysozyme. Moreover, when spleen cells from rabbits primed with DNP-BSA were exposed in vitro to BSA alone, DNP-antibody was produced (853). Furthermore, the transfer of immunocompetent lymphoid cells from inbred guinea pigs to DNP-OA primed allogeneic recipients prepared these animals for a striking anti DNP-bovine γ -globulin (BGG) antibody response to an appropriately timed challenge with DNP-BGG. Parental donor lymphoid cells also elicited this phenomenon in F_1 (Strain 13 x Strain 2) guinea pigs but F_1 donor cells failed to elicit it in parental recipients.

This allogeneic effect could be elicited in recipients primed as early as 1 day or as long as 130 days prior to allogeneic cell transfer, although the effect was maximal at 21 days. Furthermore, the allogeneic effect appeared to cause expansion of antibody forming cell precursor population of the recipient as well as differentiation of such cells into antibody-secreting cells (474).

The production of a T cell replacing factor by T cells during allogeneic stimulation in vitro, which can cooperate with B cells in antibody formation to unrelated antigens was also described (790). Some batches of fetal calf sera were found to be able to replace T cells during an in vitro antibody response to SRBC, but the responding cells could not be stimulated with PHA. Thymus cells and restoring fetal calf serum did not give an additive effect but on the contrary, the response was lower. "Conditioned" media from whole thymus organ cultures had similar replacing effect. The factor is suspected to be some, as yet undetermined, thymic humoral component. It can be removed by passage of calf serum through Seitz filters but not through Millipore filters (134). In mice, like in guinea pigs, carrier specificity could be overcome by an allogeneic stimulus, suggesting that unprimed allogeneic T cells are substituting for the role played by carrier specific T cells. Allogeneic (AKR) T cells also restored antibody formation, when host T cells were killed with anti- θ serum from the population primed with DNP-OA, prior to cell transfer to syngeneic (CBA) animals. There was, however, an absolute need for specific antigenic stimulus in addition to the

allogeneic effect, which was relatively mild (CBA and AKR mice are H-2 identical). The mitogenic factor has been proposed as a plausible candidate mediating the T cell stimulus. Based on these experimental data the two models illustrated in Figure 34D have been proposed to account for cell cooperation in antibody response (505).

It is clear from the experiments discussed so far that macrophages (A cells) are essential for the induction of immune responses. Little is known, however, about the details of this important function. Antigenically, two types of macrophages could be distinguished in rats, mice, guinea pigs and rabbits, e.g. free macrophages found in the circulation, bone marrow, peritoneal cavity etc., and fixed phagocytic cells located in the liver (Kupffer cells), lung, lymph node (dendritic macrophage) and thymus (621). Whether these two types of phagocytic cells represent two stages of functional differentiation, or they are entirely different kind, is presently not clear.

The most outstanding capacity of macrophage is phagocytosis (endocytosis). In higher animals cytophilic antibody facilitates this process. It is also well documented that macrophages play an important role in the effector phase of cell-mediated immune reactions and also capable of target cell killing in vitro (647). It has been established that a portion of the injected antigen is transported to lymphoid organs and taken up by phagocytosis or pinocytosis. Intracytoplasmic localization of radioactive antigen (^3H -aniline-azo-porcine γ -globulin) was found in phagocytic

reticular cells of mesenteric lymph nodes, liver Kupffer cells, fixed macrophages of the splenic and red pulp, and "septal cells", or fixed macrophages of the lung in mice following i.v. and s.c. administration. Intranuclear localization of antigen in phagocytic (reticuloendothelial) and hepatic parenchymal cells, detectable with fluorescence microscopy was also reported (4). Uhr and Weissmann (901a) suggested that the macrophages within lymphoid organs may have evolved special mechanisms for allowing a fraction of the antigenic molecules to escape the usual fate of degradation and to make their way to the surface of the cells. Therefore, it is either antigen that has been endocytized by macrophages or antigen which escapes endocytosis that acts as immunogen.

An alternative possibility to the one described is that antigen within macrophages may stimulate the formation of an RNA which is necessary for immunization (703). Either the RNA has all the information for the amino acid sequence and, hence, confers the specificity on the emerging antibody molecule, or the RNA of an antigen-RNA complex may be the key factor in derepressing the potential antibody-forming cell and the antigen of this complex may guide the complex to the appropriate cell. A third possibility is that the macrophage RNA may act as a carrier of an immunologic fragment of antigen and thus enhance cellular uptake of that fragment by antibody forming cells.

One of the earliest studies of "immune RNA" was that of Fishman and Adler (294), who reported that a ribonucleic acid fraction obtained from rat peritoneal cells, which had been incubated

with antigen (T_2 bacteriophage) in vitro, was able to cause antibody synthesis in x-irradiated animals which had received the RNA and normal lymph node placed in diffusion chambers. When non-irradiated recipients were used, RNA alone was able to transfer the ability for specific antibody synthesis. Furthermore, the immunogenic activity was found primarily in the top layer (8-12S) of a sucrose gradient which had been divided into three fractions. Such activity could be eliminated by treating the extract with RNAase.

Transfer of cell-mediated immunity by immune RNA was also demonstrated. Accelerated rejection of skin allografts in adult rabbits and in mice, and enhanced graft-versus-host reaction in newborn F_1 hybrid mice were induced with spleen cells incubated with RNA preparations extracted from lymphoid organs of immunized animals (566; 778; 779). Moreover tumor immunity could also be transferred with immune RNA prepared from lymphoid organs of xenogeneic, allogeneic and syngeneic animals (227; 698; 699; 725).

The general consensus, based on numerous experiments of transfer of immunity with RNA is that the "processed" antigen is combined with the RNA and that this complex may be regarded as a very efficient stimulant, a "super antigen". The role of RNA in this complex is not clear (4).

More recently it was demonstrated that immune RNA to SRBC obtained from lymph nodes of rabbits homozygous for the b^4 or b^5 allele of light chain immunoglobulin allotypes could induce the formation of IgM and IgG of donor allotype when injected into rabbits homozygous for the alternate allele. It was suggested that the donor's RNA has an informational role in the synthesis of

immunoglobulins by host lymphoid cells (71).

Depression of the in vitro immune response of mouse spleen cells to SRBC by removal of macrophages could be reversed by the addition of supernatants of 24 hour macrophage cultures. The activity of the supernatant could be partially absorbed by SRBC, and the red cells treated with the supernatant were immunogenic in the absence of macrophages or supernatant. The supernatant activity was heat resistant (57°C , 30 minutes), passed through $22\text{ }\mu\text{m}$ filter, but was not stable to freezing and thawing unless serum was present in the medium. The interaction with SRBC did not appear to be antigen specific and treated red cells were not agglutinated by anti IgG and anti-kappa sera (422).

On the basis of the evidence briefly summarized in this Chapter, the author believes that the emerging conclusions as regards the immune response may be summarized as follows. At least three cell types are involved in the induction phase of humoral immunity: A cells, B cells and T cells. Furthermore, the interaction of A and T cells seems obligatory for the development of cell-mediated immunity as well (668; 701). Very likely A cells interact first with the antigen and either process it in some way and/or release factors (RNA?) which makes it possible for T cells (or B cells) to respond. Triggered T cells probably produce the soluble factors (IgX, MIF, mitogenic factor?) which induce B cells for antibody formation. It is not clear whether an antibody response to thymus-independent antigens would require the cooperation of A and B cells or not. Cooperation seems to

be a real physiological mechanism, and an antigen with two determinants one for the T cell and the other for the B cell seems to be required.

D. Cell cooperation in the effector phase of the immune response

From the survey of the literature given above, it is obvious that current knowledge of cell cooperation is incomplete. Besides A, B and T cells other cell types may also participate in the induction phase of the immune response. For instance, it has been noted that eosinophil granulocytes proliferate in mice upon antigenic stimulus (tetanus toxoid). In heavily irradiated (1100r) syngeneic recipients normal spleen cells augmented the capacity of animals to mount an eosinophil response if bone marrow was present, and a much greater augmentation occurred in mice reconstituted with splenic or thymic cells from primed animals. It was suggested that a thymus-derived or thymus-dependent mononuclear cell (T cell) population is necessary for optimal eosinophil response (590). Similarly mast cells were shown to proliferate after antigenic stimulation in rats (604).

Cell interaction during the effector phase of the immune response seems to be very complex and may involve multiple and alternative pathways. Antibodies may serve as specific receptors for most if not all the cell types of the leukocyte series. It is well established that human basophils and mast cells have specific binding sites for IgE (homocytotropic antibody) (450), monocytes (macrophages) for IgG₁ and IgG₃ (cytophilic antibody, opsonizing

antibody) (432; 433) and neutrophils also for IgG₁ and IgG₃ (583).

In all these cases the Fc portion of the immunoglobulin molecule was shown to combine with the binding site on the cell surface.

The existence of homocytotropic and cytophilic antibodies is well established in various animal species as well. Furthermore, human monocytes were shown to have receptors for C'3 which may function either independently or cooperatively with the IgG binding sites in the induction of phagocytosis ("opsonization") (433).

Lymphocytes of the B population were also claimed to have receptors for immunoglobulins as well as for fixed complement (C'7) (84; 106; 384; 686; 687). During the complement reaction a biologically active material, capable of antigen-independent histamine release from mast cells (anaphylatoxin) is also produced, as well as other factors which are chemotactic for polymorphonuclear leukocytes (729). The majority of the mediators produced during cellular immune reactions by T cells may be classified as amplifying factors, which act on other leukocytes inducing thus cooperation which greatly increases the (defensive) power of the reaction. Chemotactic factors for macrophages (216; 924), neutrophils (923) and eosinophils (183), mitogenic factors (41; 471; 708), the transfer factor (525) and the skin reactive (inflammatory) factor (692) may be listed in this category. It appears, that the only type of leukocytes which was not imputed to be affected by a specific factor is the basophil, although it was recently suggested that T cells may produce such a material in relation to cutaneous basophil hypersensitivity (246). In this context, it is interesting

to note that transfer of the same population of immune lymph node cells from rats with experimental allergic encephalomyelitis to syngeneic normal and cyclophosphamide treated rats resulted in histological lesions characterized by mononuclear infiltration in the former and polymorphonuclear infiltration in the latter group. Since mononuclear cells are more sensitive to cyclophosphamide it was suggested that polymorphonuclear cells substituted for them in treated animals as effector cells cooperating with donor lymphocytes in the pathogenesis (538).

In summary, it may be stated that the effector phase of the immune response in vivo is characterized by the participation of all types of leukocytes known to be involved in inflammatory reactions in general. The trigger mechanism for these reactions which involves antibodies, sensitized lymphocytes or both, is immunologically specific and activates a regiment of diverse cells which participate directly or by release of cytotoxic factors leading to the overall inflammatory response.

E. Cell cooperation and antigenic competition

Injection of a mixture of several antigens into an animal produces often antibodies against all the components of the mixture, and the total antibody produced may greatly exceed that which would be produced in response to the injection of one of the components alone. Nevertheless, in some situations the immunogenicity of a given antigen appears to be impaired by the previous, simultaneous, or subsequent injection of other antigen(s) (5). This interference with the immune response to a given antigen

has been termed antigenic competition (6) and may manifest itself in varying degrees ranging from complete suppression to a mere delay in the attainment of the maximum level of antibody observed in control animals. This phenomenon is affected by variables such as genetic constitution, physiological status of the animals, method of immunization, absolute amount and relative proportions of the antigens injected, past immunization history and the nature of antigen selected, and its mechanism remains obscure. Competition for a limited number of pluripotential stem cells, or the induction of some "humoral" inhibitory factors during immunization were the most frequently considered ideas.

It was found recently that the proportion of Θ positive cells in the spleen of C57Bl mice decreased to about half as a result of immunization with goose erythrocytes. Although the total number of both thymus-dependent (T) and thymus-independent (B) cells in the spleen increases as a consequence of immunization, the proliferation events are not parallel. It was suggested that immunization with the first of a pair of non-crossreacting antigens leads to a specific clonal expansion of both T and B cells, the extent of which is dependent on time. As a result of this clonal expansion, the T and B cells capable of reacting specifically to the second antigenic specificity become "diluted out", and hence their chance for encounter and interaction is decreased (477). On the other hand, Tassig and Lachmann (873) proposed, in terms of the model for cell cooperation suggested earlier by Lachmann (511, see Figure 34C), that inhibition of antibody production to the suppressed antigen is due to the competitive saturation of sites on the

macrophage membrane by antibodies to the immunogenically dominant antigen.

F. Cell cooperation and immunological memory

If an animal has never encountered an antigen, the first injection of this antigen is generally followed by the appearance of specific serum antibody. The kinetics of this "primary" response usually include a "latent period" of several days during which no antibody is detectable, followed by a moderate rise to a peak titer, with a subsequent levelling off and a decline of titer. A second injection of the same dose of this antigen produces a response which includes the following features: the latent period is shorter, the rise is steeper and the antibody titers higher than in the primary response; this second response is called an "anamnestic" or "secondary" response. This implies that the host has acquired "immunologic memory" for the antigen in question which predisposes the immune system for a faster and more powerful antibody response to the second stimulus with the same antigen. Tertiary responses usually do not differ greatly from secondary responses. Immunological memory may be retained for a very long time and it is specific for antigenic determinants; therefore, crossreacting antigens having determinants identical or similar to those of the first antigen would elicit subsequent responses resulting in the maintenance of high level of antibodies (164). It is obvious that under normal conditions the animal is being exposed constantly to a variety of environmental antigens and produces "natural antibodies" to a wide range of antigenic determinants; consequently, many responses

defined as primary should be actually considered as secondary, and it is questionable if there is a truly primary response except in germ-free animals reared on synthetic diets of nonimmunogenic materials.

All three cell types (A, B and T) were found to be necessary to initiate both IgM and IgG anamnestic responses to SRBC in vitro. Immunological memory was attributed to a significant increase in the number of both the B and T cell population (411). The in vivo indirect IgG response of primed mouse spleen cells was markedly reduced by in vitro treatment with cytotoxic anti- θ serum. However, the response of primed B cells could be fully restored to control levels by normal thymocytes, although restoration was more effective with thoracic duct cells from specifically primed mice than with cells from nonspecifically primed mice. Moreover, the activity of the reconstituting cell population from primed mice was reduced by incubation with anti- θ serum. It was concluded that memory to heterologous erythrocytes is carried by the T cell lineage, as well as by the B cell lineage, even though unprimed T cells are sufficient for expression of B cell memory (606). In a similar study, however, it was found that primed spleen cells treated with anti- θ cytotoxic antibody did not transfer response to SRBC. Normal thymocytes partially reconstituted the ability of such treated cells to transfer the primary, but not the secondary response. Incubation of the spleen cells with alloantiserum to the PC.1 antigen, which is present on antibody forming cells, did

not significantly affect the ability to transfer either the primary or the secondary response (864). The secondary response to a hapten-carrier antigen as also decreased on treatment of the carrier specific cells with an anti- θ serum, but it was not affected by this treatment of hapten specific lymphocytes (719).

In a recent review of this topic it was concluded that T cells carry specific tolerance and memory, but the cooperative effect may be nonspecific. Convincing evidence of B cell memory is nonavailable; it is absent for pneumococcal polysaccharide, which is a thymus independent antigen (701).

G. Cell cooperation and immunological adjuvants

Adjuvants include a wide variety of materials capable of enhancing nonspecifically antibody production to unrelated antigens. Some of these substances are large molecules and are antigenic, others are small organic compounds. The mechanism of action for any adjuvant has as yet not been elucidated. There is little evidence that adjuvants enhance antibody production by altering the rate of processing of antigen (482).

It was found in rats that the addition of carrier (diphtheria toxoid) primed cells, or adsorption of antigen to aluminum hydroxide, assisted hapten primed cells to initiate antibody formation. Consequently, it was inferred that antigen coated adjuvant particles may be able to substitute for T cells in cooperation with B cells (849). In mice depleted of T cells by thymectomy plus treatment with ALS or x-irradiation (850r), the

antibody response to BSA was decreased, and the disparity between normal and T cell deprived mice was greater when adjuvant had been employed (E. coli endotoxin, Bordetella pertussis vaccine, complete and incomplete Freund's adjuvant and vitamin A ('Retinal') in incomplete Freund's adjuvant) also for the primary immunization. Marked stimulation of antibody formation with adjuvant was detected in mice reconstituted with thymus graft. It was suggested that T-lymphocytes were potentiated by adjuvants for antibody formation (12). Similarly complexes of polyadenylic and polyuridylic acids (poly A:U) appeared to potentiate T cells; thus the anti-SRBC rosette forming cell response of irradiated mice was increased with thymus cells which had been exposed to poly A:U in vivo or in vitro prior to injection of bone marrow cells. This direct stimulatory action resulted in an enhancement of the rate of proliferation of antigen reactive cells in response to antigen and a shortening of the induction period for division in immunocompetent cells. However, no stimulatory effect of poly A:U on bone marrow derived antibody forming precursor cells was observed (190).

Immunological unresponsiveness

A. Immunological tolerance

An early indication of what later was to be developed into the concept of immunological tolerance was provided by one of Nature's experiments - the exchange of lympho- and hemo-poietic stem cells between twin embryos in cattle by placental anastomosis of blood vessels. A year after birth the twin cattles were found

to have admixtures of blood types, which was tolerated by the mature host (670). From the collection of data on approximately one hundred additional pairs of cattle twins, of ages up to eight years, indicated clearly that the foreign cells were not at a selective disadvantage. Placental fusions leading to similar consequences were found later in mice, rats, sheep and human. Moreover, the phenomenon was reproduced by artificial parabiosis of embryonated hens' eggs, and also by the injection of living embryonic rat cells into other rat embryos. Such animals having genetically different tissues, established either naturally or artificially, are called chimeras. Chimeric twin cattle commonly accepted skin transplants and also kidney grafts from their co-twins. An identical situation was found in artificial chimeras (671). More recently chimeras were produced by the fusion of blastomeres of genetically dissimilar mice in vitro which were replaced in the uterus of a foster mother (597; 599). In more than 50% of the cases a healthy long-lived mouse develops, carrying cells of both origins, which may differ in major histocompatibility factors; such "allophenic" mice are permanent chimeras. Appropriate skin transfers between the allophenic and its two "parent" strains demonstrated that the allophenic possessed the mosaic of histocompatibility antigens carried by each of its "parents".

Classically the essential features of immunological tolerance were defined in terms of the establishment and persistence of allogeneic tissue transplants, achieved by the introduction of

living homologous tissue cells into the embryo and neonatal individual. Unresponsiveness to self constituents of the body, which are either directly present in the body or appear as a result of the breakup of expendable cells present in the blood or lymph, was explained as a state of tolerance established before the maturation of the immune system. Antigenicity of certain body components, like thyroglobulin, lens proteins, constituents of spermatozoa etc. were attributed primarily to the fact that the components in question are normally separated in isolated organs and are inaccessible to lymphoid cells and/or they were not present as such in the body when tolerance to self components was established during embryonic life (124; 125; 671).

Another form of immunological unresponsiveness was described by Felton (286), who found that less than 10 μ g of the polysaccharide antigen of pneumococci was immunogenic in mice, while a dose higher than 100 μ g caused a long lasting unresponsiveness. This phenomenon, which was eventually elicited with high doses of a variety of soluble antigens was known later as immunological paralysis. Mitchison (613b) discovered that immunological paralysis may be induced with high and low doses of the antigen. Thus, BSA was given to CBA mice from 10 μ g to 10 mg, three injections a week for varied times. Ten days after the last injection they were bled and immediately challenged with a standard (immunogenic) dose of BSA (7 mg in complete Freund's adjuvant). From the response to this challenge, two zones of "partial paralysis" could be identified. Mice preimmunized with 10 μ g doses of BSA gave only a low-titre

delayed response, yet from an early stage in the process, these mice did not respond or responded poorly, to a standard challenge of antigen and adjuvant. Normal (secondary) immune response was observed in mice preimmunized with intermediate doses. At the other end of the scale, mice preimmunized with a large dose of antigen showed a quick rise and a quick fall in titer after challenge with the standard dose. These phenomena are known presently as low and high zone tolerance.

More recently mortality attributable to GVHR induced by grafting parental lymphoid cells into irradiated F_1 mice has been delayed and reduced by treating the donors with high and low doses of soluble recipient H-2 antigens. The phenomenon was suggested to be identical with "high and low zone tolerance" for soluble antigens (377).

Furthermore, it was shown in guinea pigs that only those compounds having immunogenic properties could induce tolerance (188). Adult mice could be rendered tolerant to bovine gamma-globulin if it was given in unaggregated form, but the same antigen in aggregated form or either with Freund's adjuvant or with bacterial endotoxin (non-crossreacting) caused the production of antibody and not the induction of tolerance (165; 241).

The cytotoxic action of mouse lymphocytes sensitized against alloantigens was abrogated in vitro by sera from the sensitizing strain, and from F_1 hybrids of the sensitized and the sensitizing strain, while sera from the sensitized strain did not. It was suggested that soluble histocompatibility antigens present in the

inhibitory sera may be responsible for this effect (112).

The induction of tolerance in adult mice was greatly facilitated by simultaneous immunosuppression (776).

When young adult mice aged 13 days received i.v. $3-6 \times 10^8$ allogeneic spleen cells in 7-12 divided doses at intervals of 3-4 days, a significant degree of tolerance was produced in a number of animals. Unresponsiveness began to develop after the 4th injection. It was estimated that the recipient spleens contained small numbers of donor cells (less than 5%) when tolerance was complete. It is of interest that tolerance in 13 day old mice could not be produced by injecting cells intraperitoneally (115; 116). Rats made chimeric by neonatal injection of F_1 or parental bone marrow cells i.v. tolerated parental skin grafts. When 6 ml of isoantiserum directed against the alien histocompatibility antigens of "incompatible" grafts was injected over a 7 day period, all incompatible grafts became inflamed and 10 of 22 (45%) was totally rejected within 49 days. Rats whose grafts survived still 50 days after administration of the antiserum were treated again with specific antibody. This increased the number of rejected grafts to 17 of 22 (77%). Although the antibody probably exerted some direct effect on the graft, the abrogation of tolerance was produced by abolition of the chimeric state of the host (561). From these and other observations it follows that the duration of the tolerant state depends upon the persistence of the antigen (164).

Under certain conditions tolerant animals may not be able to develop one type of response (delayed hypersensitivity)

but could show full competence with regard to another (humoral response) (66; 201; 438). This status is designated as split tolerance.

It was established in an in vitro system using mouse lymphocytes, that polyvalent rabbit antisera to mouse immunoglobulin could interfere with the induction of immunity and of tolerance. It was concluded that immune tolerance depends on the interaction of antigen directly with the surface of immunocompetent cells, presumably with the immunoglobulin receptors (283). The number of antigen binding (rosette forming) cells was slightly higher in tolerant animals than in normal animals and significantly lower than in immune animals (814). This observation pointed out that lymphocytes having receptors for the tolerated antigen are present at a normal level in unresponsive animals.

When an allogeneic thymus is used to reconstitute neonatally thymectomized mice it is capable of establishing tolerance if grafted early enough. Such mice will accept skin grafts from their own strain and from the strain supplying the thymus but reject any "third party" graft (600).

In mice made tolerant to BGG, tolerance persists longer in thymectomized mice than in sham operated controls. Whole body irradiation shortens the duration of tolerance, but only if the thymus is present. These experiments indicate that lymphoid cell turnover is associated with the waning of tolerance and that procedures which retard this (thymectomy) will delay the disappearance of tolerance, and procedures which accelerate this

(whole body irradiation) will accelerate the reappearance of antibody production (164).

During a series of experiments on high zone tolerance in mice it was shown that both T and B cells can be made tolerant. Induction of tolerance as well as induction of immunity in the thymus dependent B cell population seems to require the cooperation of T cells (328).

The antigen-induced DNA synthetic response of a number of different thymocyte populations was studied in the spleens of lethally irradiated recipients by incorporation of ^{125}I -5-iodo-2-deoxyuridine. The response of certain cell combinations was not only less than the sum of the responses of individual cell populations but also less than that of any one of the individual cell populations in the combination. This conclusion was based on the responses of the following combinations: i) the response to SRBC of "educated" (primed) plus normal thymocytes was less than that of educated cells alone; ii) the response of parental plus F_1 thymocytes was less than that of parental cells alone in F_1 hosts; iii) the response to SRBC of a cortisone resistant (in a low dose) plus normal thymocytes was less than that normal cells alone. The existence of a suppressor T cell population was proposed, which is capable of inhibiting the antigen-induced response of other thymocytes without the mediation of B cells or their products (i.e. antibody) (327). A strong suppressive effect on the formation of agglutinating antibody to SRBC and *Brucella abortus* was observed when 5×10^7 thymus cells from 6 week old normal chickens were given i.v. to 6 week old recipients of the same inbred line; only 1/16

of the total titer was mercaptoethanol resistant. The suppressive effect was still observed 3 weeks after the original cell transfer and after repeated immunizations. On the other hand, thymus cells transferred from bursectomized animals were more effective in their cooperation for antibody formation and a better immunological memory resulted. Thus thymus cells appear to be capable of both amplifying and suppressing the production of agglutinating antibody in the chicken. The former effect, however, was not affected by neonatal bursectomy of the thymus cell donor, whereas the latter was "bursa dependent" (240).

Tolerance to pneumococcal polysaccharide type III (SIII) in mice was not influenced by thymectomy or the transfer of thymocytes to such recipients, neither did pretreatment of the marrow with anti- θ serum abolish efficacy in transferring reactivity to SIII. Moreover, irradiated mice repopulated with marrow from SIII-tolerant donors were unresponsive and prior thymectomy did not retard the spontaneous loss of tolerance induced by 50 μ g SIII, and the ensuing PFC response was not impaired (429). These results indicate that T cells do not play a role in tolerance to thymus independent antigens.

In guinea pigs lymphatic connection between skin and regional lymph nodes was essential for the development of immunologic tolerance to dansyl chloride, which is a skin sensitizer (309).

When picryl chloride or DNFB in oil was fed to guinea pigs repeatedly over three weeks, the animals could not be sensitized by skin contact and had no antibody (154). Unresponsiveness could be obtained by an injection of as little as 50 μ g picryl chloride in 2 ml of 1% ethanol in 0.85% NaCl given directly into the mesenteric vein of adult guinea pigs on two occasions, one week apart. Orally a dose of 50 mg fed for 5 days, and a total of 15-20 feedings were necessary for inducing the same status of tolerance. Moreover, two injections of 5 μ g BGG, separated by seven days, were sufficient to induce a state of tolerance, such that the animals were unable to develop cell-mediated immunity or antibody formation, when sensitized with four intradermal injections of 2.5 μ g BGG into each footpad, 26 days after the first injection (67). These latter findings strongly indicate that the liver has a tolerogenic role, which could be of vital importance in the prevention of sensitivity to food constituents.

A tolerance inducing dose consisting of 1 mg of deaggregated HGG given i.p. to mice on the first day failed to develop tolerance in animals depleted of complement by vitamin A treatment; this finding suggests the possible participation of C' in tolerance production (40).

When tolerance is produced by the injection of nonliving antigens, it may be abrogated by the injection of normal lymphoid cells from compatible animals. In the case of thymus dependent antigens non-tolerant T-cells plus antigen will abolish the tolerant state (723; 330). Tolerance to an antigen such as bovine serum

albumin was abolished by injection of a crossreacting antigen such as human serum albumin. However, recent evidence suggested that this may not be a true "termination of tolerance", since all the antibodies produced could be absorbed out with the immunizing crossreactive antigen (931).

Immunological tolerance may be induced in adults most easily with deaggregated antigen. The phenomenon is immunologically specific; therefore, antigen-specific receptors must be involved. T-cells become unresponsive faster (at 4 days) at a lower antigen dose, and remain so longer (at least for 100 days). B cells are toleragenized much slower (15-21 days), requiring high antigen dose and recover faster (49 days). Sometimes antibody response is present during induction, which probably does not play a role. B cells become unresponsive to thymus independent antigens (E. coli lipopolysaccharide) much more rapidly in vitro. There is no antibody response to thymus dependent antigens, if either T or B, or both cell types are tolerant. Tolerance induction with higher doses of deaggregated antigen (tolerogen), is much more effective than lower doses, because to such antigen, there is no "low and high zone". Under ordinary conditions a competition of antigen and tolerogen may be the case (931).

B. Immunological enhancement

The phenomenon of enhancement was first observed by Flexner and Jobling (296) in studies of a transplantable sarcoma in rats. The growth of the tumor was favoured if the hosts were given a prior injection of an emulsion of tumor cells heated at 56°C for 30 minutes. The effect was most pronounced when injection of the

emulsion preceded tumor inoculation by ten days, and seemed to be further increased by multiple injections at ten day intervals, prior to implantation of the viable tumor. Casey (140) added the important observation that when different mouse strains were used, the effect showed substantial specificity. Kaliss and his coworkers next established the phenomenon in terms of which immunological enhancement is now defined (462; 465; 466; 467), i.e. it was shown that the potential for enhanced tumor growth could be transferred to secondary hosts by the injection of serum from mice of the same inbred strain in which immunologic enhancement had been induced. The serum was usually given at the same time as the tumor allograft and was effective in causing enhancement if given even one week prior to or after implantation of the tumor. Enhancing serum could be produced by the injection of not only tumor cells but also of normal tissue from the strain to which the tumor was syngeneic. Therefore, in the light of modern concepts, it may be inferred that involved in the enhancing phenomenon were primarily alloantigens and not tumor antigens. The demonstration that transplantation immunity could be adoptively transferred with lymph node cells (613a) revealed that graft and allogeneic tumor destruction is mediated by cellular immunity. If one postulates that humoral antibodies directed against the alloantigens of a graft are non-destructive, but can in some way block the formation or functioning of the destructive cellular response against the same alloantigens, then the enhancing phenomenon would follow as a natural consequence (821). An overwhelming amount of experimental

evidence supports that indeed this is the case (64; 463; 464; 616; 617; 618; 822).

On the basis of these experimental findings immunologic enhancement may be defined as the counterpart of rejection, whereby an antigenic tissue is not destroyed and may remain intact structurally as well as functionally for long periods of time, despite the presence of immune reactions to it in the host. Both cell-mediated and humoral immunity are detectable in animals or humans bearing antigenic grafts or tumors, and antibodies are involved in protection against tissue damage by immune lymphocytes. Enhancement and rejection are both dynamic and active phenomena with numerous intermediate situations, and occasionally rejection may turn into enhancement and vice versa. Hence transplantation and tumor immunity are governed by a delicate balance between two opposing manifestations of the immune response, one extreme leading to rejection and the other to enhancement.

Clearly, the advantage of immunologic enhancement is being exploited in organ transplantation. Thus, kidney grafts from F_1 donors survived and functioned well indefinitely in the parental strain of rats if the recipients were preimmunized against donor histocompatibility antigens (Ag-B) (305; 851). Canine renal allografts could also be prolonged by preimmunization of a subcellular soluble antigen derived from donor lymphocytes combined with postoperative treatment of azathioprine and methylprednisolone in small doses (423). Furthermore the maternal kidney graft of an 8 year old boy was also protected by Fab and $F(ab')_2$ fragments of

paternal antibodies produced against maternal leukocytes; low doses of immunosuppressive drugs were also given. When graft function was established postoperatively, all immunosuppressive drugs were withdrawn and the kidney functioned satisfactorily for three months without further immunosuppression being required (65).

An infant with severe combined immunodeficiency (the lack of both humoral and cell-mediated immune reactivity) was given maternal bone marrow and maternal plasma which contained lymphocytotoxic antibodies to paternal and patient lymphocytes and blocked mixed lymphocyte responsiveness of maternal cells against mitomycin-C treated paternal or patient cells. The infant gained weight, acquired cell-mediated immunity and produced serum IgG, IgA and IgM in higher concentrations, and survived for 14 months after transplantation. This survival time is the longest known among patients with severe combined immunodeficiency given incompatible bone marrow (120). Finally, it may be mentioned that at least in certain experimental and human autoimmune reactions antibody mediated protection may be of great importance for the preservation of homeostasis (21; 213; 700).

In contrast to the above data, it was demonstrated that lymph node and spleen cells from rats bearing enhanced kidneys were able to mount GVHR, which was quantitatively similar to that caused by cells from normal rats. Immunization of rats bearing enhanced kidneys with donor type spleen cells caused a rise in cytotoxic antibody titer but had no effect on blood urea levels. Lymphoid

cells taken from these immunized rats also produced GVHR comparable with that of controls. It was concluded that tolerance was not involved in the prolonged survival of these grafts (306).

In another study both cell-mediated immunity and the presence of serum blocking factors could be detected from such rats against donor type fibroblasts in vitro 87-127 days after kidney transplantation. Cell-mediated immunity was present in 3 of 4 enhanced recipients. Although one lacked demonstrable cell-mediated immunity, it was possible to detect serum blocking factors. Blocking factors could also be eluted from a kidney more than 4 months after transplantation. A second kidney of the same genotype was readily taken by the enhanced animal but skin graft was rejected. It was proposed that the serum blocking factors and the eluted blocking factors were actively produced by the recipient and were not a residual of the initial short course of passive immunization (850).

Three children born with severe combined immunodeficiency disease who had been fully reconstituted immunologically by bone marrow transplantation from HL-A-matched sibling donors, were examined by an in vitro assay of cellular immunity. Peripheral blood lymphocytes from these children, originated from the bone marrow graft, were cytotoxic for their respective host fibroblasts in vitro. This cellular cytotoxicity was inhibited by an autologous serum factor which contained immunoglobulin determinants. The data illustrate that human lymphoid chimeras depend upon active antagonism between humoral and cellular manifestations of the immune response (459).

An analogous situation was found in long surviving chimeric dogs

established by the transplantation of allogeneic bone marrow after lethal x-irradiation (407).

It was recently suggested that a number of immunological phenomena, which were formerly believed to represent true examples of tolerance were in fact governed by immunological enhancement. Mice made chimeric by the injection of 5×10^7 spleen cells from F_1 hybrid donors within 24 hours after birth. Tolerance was defined as the ability of approximately one month old mice to accept permanently skin grafts from the other parental strain. CBA mice tolerant to A grafts had cytotoxic lymphocytes against A fibroblasts, and the serum from the tolerant animals could nullify this cytotoxic effect. Reciprocal findings were obtained with tolerant A mice. The cytotoxic effect and blocking of sera from the tolerant mice was specific (400). Tetraparental (allophenic) mice, rendered chimeric at the eight-cell stage by joining two embryos from histoincompatible parental strains exhibited a similar mechanism, e.g. concomitant cellular immunity and a serum blocking effect, rather than tolerance through a central failure of the immune response. Preliminary studies suggested that the blocking factor may be an immunoglobulin (691; 930). Similarly, lymph node cells from F_1 hybrid mice destroyed parental fibroblasts in vitro and the serum from F_1 , but not from parental strain mice, blocked this (694).

A state of "pseudoparalysis" was achieved with type III pneumococcal polysaccharide (SIII) given in high concentration (428). Thus, no serum antibody to SIII was detected, but the number of PFC

determined with SRBC heavily coated with SIII was comparable to that of immune animals. Moreover, with the plaque assay, it was shown that under these conditions IgM antibody was synthesized as would be the case in mice in response to an immunizing antigen dose. Consequently, the lack of detectable serum antibody in "tolerant" animals was attributed to its continuous peripheral neutralization by recirculating, nondegradable antigen.

C. Mechanisms of immunological unresponsiveness

(i) Thymic censorship

Burnet proposed recently (126) that all lymphocytes which express "self" reactivity during differentiation in the thymus are destroyed in situ. Whenever the thymic function is disturbed, "forbidden clones" capable of auto-reactivity may arise and cause autoimmune disease. In support of this view, quote histological evidence that there was significant cell destruction in the form of pyknotic nuclear fragments in the thymus and calculations based on the intensity of mitosis suggested that 90-99% of the cells produced were destroyed. Furthermore, Burnet suggested that in certain autoimmune diseases, like systemic lupus erythematosus, where the autoimmune process is massive and not monoclonal in nature, a general breakdown occurs in the control function of the thymus, which may be the result of the thymus becoming a target organ for the autoimmune process.

Thymic abnormalities are indeed commonly present in association with certain autoimmune diseases e.g. germinal centers appear in myasthenia gravis and less frequently in thyrotoxicosis Hashimoto's disease, systemic lupus erythematosus, rheumatoid

arthritis. Various autoimmune conditions have also been associated with thymic tumors. It was postulated that the thymus might be a target organ in myasthenia gravis; thymectomy leads to variable results (21). Similarly, thymectomy had beneficial effects, in a 12 month old infant with autoimmune hemolytic anemia, who had been refractory to conventional treatment (prednisolone, corticotrophin, splenectomy) (469).

It was also proposed that the relative absence of epithelial cells in the thymus of NZB mice may in some way be related to the poor control of this mouse strain against autoimmune process (250). Neonatally thymectomized rats failed to develop the characteristic lymphoid cell infiltration of experimental autoimmune thyroiditis seen in sham-operated animals. Animals found to have thymic tissue remnants at autopsy showed thyroiditis equivalent to that of sham operated animals. The titers of precipitating antibody to bovine thyroglobulin were the same in both sham operated and neonatally thymectomized rats (121).

Recognition of self antigens by cells of neonatal thymus was recently described in the mouse. Neonatal thymocytes incorporated C^{14} -thymidine when cultured with adult isogeneic spleen cells. This interaction increased sharply after birth, began to decline within the first week of life and was lost in adulthood. Other isogeneic lymphoid cells could not replace adult spleen cells (430). This finding was confirmed for neonates, but thymocytes of adult mice were also found to react against adult spleen cells, even when taken from germ-free CBA mice. It was proposed

that these "self reacting cells" do not leave the thymus (in accordance with Burnet), and if they do, they are not necessarily cytotoxic (96).

Heavily labelled flagellin was found to be distributed extremely diffusely through all lymphoid tissues of newborn rats, including the thymus (609). In a similar experiment rats were sublethally irradiated with their thymuses shielded in order to destroy immunocompetent cells throughout the body and leaving the thymus intact. Immediately thereafter, groups of these rats received 20-40 mg of BGG either directly into the thymus or i.p. The first group showed neither skin sensitivity, nor antibody production, while those injected i.p. showed both manifestations of the normal response (832). In addition to these supporting data, it is to be stressed that the weight of evidence supports the view that immunological memory and tolerance are primarily governed by T cells.

According to a related hypothesis on autoimmunity, T cells are considered to play a key role in preventing autoimmunity which is postulated to involve two steps. First, T-lymphocytes are normally unresponsive to self-antigens and, therefore, would not cooperate with B cells to mount an autoimmune response. However, B-lymphocytes could produce autoantibodies in several situations. Thus, virus infection could lead to T cell sensitization and, through a carrier effect this could stimulate B cells to produce antibody; a similar result could be achieved by attachment of a

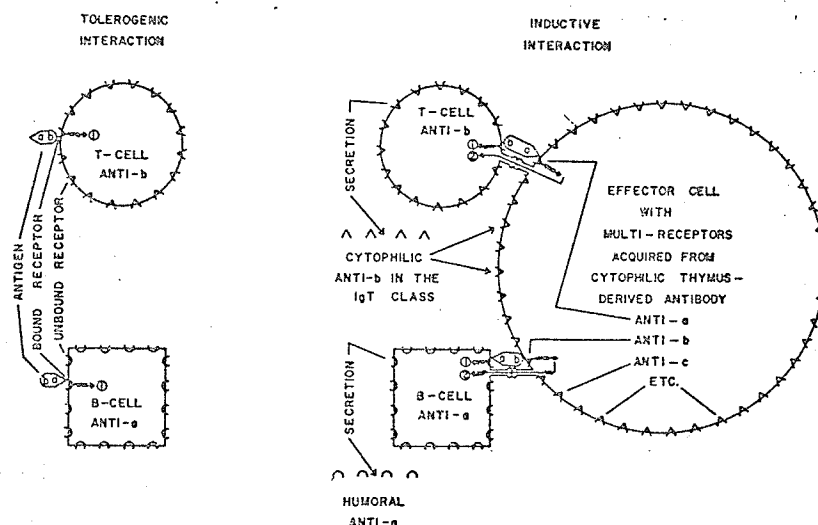
happen to a self-constituent. Nonspecific stimulation of T cells by adjuvant or by injection of allogeneic cells may eliminate the need for antigen-specific stimulation of T cells and thus cooperation with B cells may result in the production of auto-antibodies. T cells can also exert specific feedback control of autoantibody formation (14). This latter proposal as a T cell function coordinates with the suggestion for the existence of suppressor T cells discussed earlier.

Medawar (581) proposed that antigen could produce tolerance by its effect on immature cells. Immature cells form a higher proportion of lymphocytes in the fetus or neonatal animal than in the adult. It is, therefore, not surprising that it is easier to induce tolerance in the foetus or neonatal animal than in the adult. In the adult there are proportionally more mature cells than immature cells. In adult animals some cells develop tolerance whereas other cells develop sensitivity. As treatment continues the sensitive mature cells die off, leaving only tolerant mature cells derived from immature cells which have become tolerant at an earlier stage.

(ii) The signal theory of tolerance and induction

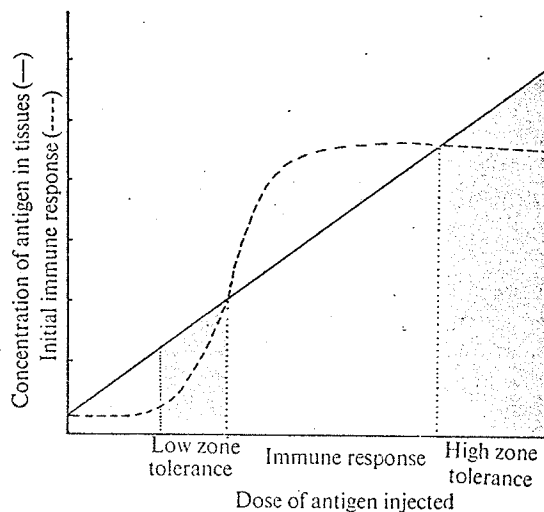
Cohn (184) proposed that the induction of immunity requires the recognition of 2 determinants on an antigen, one by an immunoglobulin produced by T cells (IgT) and another by B cells. If only one determinant is recognized by the antigen sensitive cell it results in a conformation change of the receptor leading

A.



A precise (not minimal) model of tolerance and induction.
Cohn, M., Ann. N. Y. Acad. Sci. 190:529, 1971.

B.



Relationship between dose of antigen and either concentration of antigen in tissues (—) or level of initial antibody response (---). Zones where antibody is present but not in excess of antigen in tissues represent tolerizing conditions ("low and high zone tolerance"), whereas the zone where antibody is present and in excess of antigen in tissues allows a continuance of the immunologically responsive state ("immune response"). A dose of antigen which leads to the presence of tissue antigen but no induction of the immune response does not alter the immune capacity.

Sinclair, N. R. StC., P. L. Chan, Nature, 234:104, 1971.

to the death of that cell (signal 1).

A third party effector cell (macrophage, reticulum cell, dendritic cell, thymus cell, etc.), which derives its receptors passively as cytophilic antibody (IgT) secreted by T cells is necessary for induction. This effector cell carrying receptors of many different specificities can now interact with a unipotential antigen sensitive cell of either T or B origin. The inductive interaction is made up of two events: signal 1 identical to that of tolerogenic interaction, and signal 2 involving a communication between the effector cell and the antigen sensitive T or B cell (Figure 35A). Any cell which is inducible can be tolerogenized. It is proposed that paralysis and cellular immunity are mediated by conformational signals.

The suggestion by Weigle (931) that antigens in a deaggregated form (e.g. monomeric human IgG) are tolerogenic is supported by experimental evidence and seems to justify the idea that the immunogenic and tolerogenic form of an antigen may represent different signals for T and/or B cells.

(iii) Antibody mediated unresponsiveness

Voisin (917) claims that immunity and tolerance are predominant manifestations of an active, two-way immunological reactivity, i.e. rejection and enhancement ("facilitation"). According to Voisin, the rejection reaction involves cell-mediated immunity by T cells or by complement fixing antibodies, whereas enhancement is mediated by non-complement fixing ("synergistic hemagglutinating")

antibodies produced by thymus independent cells. The factors governing both rejection and enhancement are considered to be present during an immune response, the predominant reaction type dictating the final manifestation.

The previously discussed evidence that the tolerance of transplant recipients as well as F_1 and chimeric animals is mediated by serum factors supports this hypothesis.

Another unified theory for immunity and tolerance was proposed by Sinclair and Chan (809). They state that there is a threshold dose of antigen below which no initial immune response is induced. This is followed by a zone where the production of antibody is minimal and insufficient to react with all the antigen present in the tissues. As the dose of antigen is increased further, there is a rapid increase in the amount of antibody formed initially so that antibody excess is attained. With even greater doses of antigen, the amount of antigen in tissues will be in excess even if the initial antibody response remains maximal. Thus, there would be low and high zone of antigen dose where antigen in tissues would be in excess of the antibody produced (Figure 35B). It was also proposed that tolerance could develop when an immune response is regulated by the presence of antigen-antibody complexes.

The antigen-focusing hypothesis proposed by Feldmann and Diener (284) is essentially the same, but explains further the mechanism of action of antigen or antigen-antibody complexes. According to this theory antigen and preformed antigen-antibody complexes may interlock cell-bound receptor sites into a lattice,

which depending on the degree of crosslinking may render the cell nonreactive to further antigenic stimulus.

The antigen focusing hypothesis is based on experimental evidence obtained with in vitro antibody forming cultures of mouse cells. Thus, immunological tolerance was induced by the exposure of spleen cells to mixtures of polymerized flagellin and antibody for 6 hours at 37°C; the cells were thoroughly washed and exposed subsequently to optimally immunogenic doses of polymerized flagellin. Induction of antibody-mediated tolerance was critically dependent on the ratio of antigen and antibody to which the cells had been exposed. Low zone tolerance could be induced with subimmunogenic concentrations of monomeric flagellin in amounts as low as 2 µg/ml (about 10^{-14} molar) and the appropriate concentration of antibody (a titer of 10^{-3}). In contrast tolerance could not be induced with subimmunogenic concentrations of polymeric flagellin in the presence of a wide range of antibody doses.

There was a remarkable similarity between the tolerant states obtained in vitro with tolerogenic doses of polymeric flagellin, and with immunogenic and subimmunogenic concentrations of flagellin in the presence of antibody, suggesting the same crosslinking mechanism for the cellular basis of the tolerant state (284). Further support for this hypothesis was adduced in a more recent study in which it was demonstrated that divalent $F(ab')_2$, but not univalent Fab' , fragments were capable of inducing tolerance in vitro (285).

By contrast, it was shown that for optimal in vivo immunosuppression by administration of antibody plus SRBC, it was

essential to maintain the intactness of the IgG molecule (150). Therefore, the in vitro and in vivo situation may not necessarily be identical; however, it must be noted that two entirely different antigens were used in these systems, i.e. flagellin which is thymus independent and SRBC which is thymus dependent.

Exposure of lymphocytes of mice sensitized to alloantigens to antigen-antibody complexes rendered these cells incapable of cell-mediated immunity in vitro to target cells (not treated with antibody) (495). On the other hand, stimulation of DNA synthesis in normal human lymphocytes by antigen-antibody complexes, which was followed by cytotoxicity, was reported recently (619).

Billingham et al. (86) proposed that failure to induce a response to a homograft might be accounted for by one of three possible mechanisms, i.e. it could be due to afferent, efferent or central inhibition of the reaction. Thus, the antigenic determinants of the graft or tumor may be "masked" by antibody either before (afferent) or after (efferent) the induction of cell-mediated immunity; the strongest evidence for the efferent mechanism was presented by Möller (617) who used allogeneic tumor cells and achieved enhanced growth with antibody-coated cells in the absence of circulating antibodies in recipient mice. The direct action of antigen-antibody complexes on lymphocytes and their power to induce tolerance strongly indicates the possibility of a central mechanism (284). Low and high dose inhibition of GVHR with soluble H-2 antigens has already been demonstrated, as discussed earlier (377).

It should be emphasized that the peripheral (afferent and efferent) and central mechanisms are not mutually exclusive, and in all probability each of these mechanisms operates to a different extent under different condition and their effects may be synergistic.

D. Nonspecific serum inhibitors of the immune response

(i) α -globulin

An alpha globulin is present in human serum which has the ability to suppress the immune response of mice to SRBC and also to inhibit the in vitro transformation of human lymphocytes by PHA (342; 957). A protein fraction with similar effects was isolated from bovine serum and also from bovine thymus (137).

(ii) Maternal immunosuppressive factor

A factor was detected in maternal plasma during pregnancy that suppressed the reactivity of mixed leukocyte cultures (MLC) from both maternal-newborn pairs and unrelated adult pairs. Pregnancy plasmas of longer gestation times were more inhibitory. The inhibitory activity reached a maximum at the time of delivery and disappeared a few days later. Fetal plasma also suppressed the reactivity of MLC from mother-newborn pairs. Unlike maternal plasma, fetal plasma did not significantly affect the reactivity of MLC from unrelated adult pairs (472).

A heterogeneous glycoprotein with a wide range of electrophoretic mobilities (both greater and lower than that of serum α_2 glycoprotein) was extracted from human placenta. This material inhibited the stimulation of human peripheral lymphocytes with PHA,

pokeweed mitogen or goat anti-lymphocyte globulin, but did not affect lymphocyte viability (756).

(iii) Nonspecific immunoregulatory serum factor

A factor capable of inducing almost complete inhibition of the in vitro primary response of mouse lymphocytes to SRBC (thymus dependent antigen), but not to E. coli lypopolysaccharide (thymus independent antigen) was found in mouse serum. Sera obtained 4 days after immunization contained a higher concentration of this inhibitory factor. The immunosuppressive activity could not be absorbed out with antigen, and its activity was found to be the same when spleen cells were incubated with it either in the presence or absence of specific antigen. It was nondialysable stable at 56°C for 30 minutes, but labile at 65°C for the same duration. It was postulated that this factor, present in the serum at a threshold concentration, serves as a continuously acting control agent regulating the immune response (915).

In recent years increasing evidence has been accumulating that genetic, hormonal and other factors influence immunological reactivity. Because of the limited extent of this survey and the still rudimentary nature of knowledge of these topics, they have not been included in this survey, but relevant data are cited in appropriate sections.

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