THE USE OF ANTI-FIBRIN ANTIBODIES FOR THE DESTRUCTION OF TUMOR CELLS

by

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ABSTRACT

Antibodies specific for the unique antigenic determinants of guinea pig fibrin, which are distinct from the antigenic determinants shared by both fibrinogen and fibrin, were isolated with appropriate immunosorbents from antisera produced in rabbits and goats by immunization with fibrin. The specificity of the purified anti-guinea pig fibrin antibodies (AGFA) was demonstrated by immunelectrophoresis and by the double antibody precipitation method using ^{131}I-labelled fibrinogen and antibodies to rabbit anti-goat IgG. The ^{131}I-labelled AGFA were injected i.v. into inbred Sewall Wright strain 13 guinea pigs carrying the transplantable methylcholanthrene induced sarcoma (MC-D) growing within a fibrin matrix and were shown to be localized in the tumor tissue at considerably higher concentration than in other organs.

Next, the transplantable MC-D sarcoma in strain 13 guinea pigs was used to test the hypothesis that tumor cells growing within a fibrin matrix could be destroyed by an immunologically specific strategy involving an indirect cell-mediated immune reaction. The experimental design consisted of two steps: (i) in vivo fixation of AGFA on the fibrin matrix enmeshing the tumor cells and (ii) the reaction between AGFA fixed to the fibrin matrix and lymphoid cells from syngeneic animals which had been sensitized to xenogeneic immunoglobulins isotypic with AGFA. Indeed, using ^{51}Cr-labelled lymphoid cells, evidence was obtained for the localization of these sensitized lymphoid cells within the fibrin lattice when the latter was coated by AGFA. Moreover, significant tumor growth suppression ($P < 0.01$) was achieved in guinea pigs which had received intravenously rabbit or goat AGFA and subcutaneously lymphoid cells from syngeneic guinea pigs sensitized to a state of cell-mediated immunity to rabbit or goat IgG. On the other hand, the administration of the antibodies or of the sensitized cells alone did not affect the growth of the

tumor. Preliminary results suggest that peritoneal exudate cells may play an important role for the success of the strategy for tumor cell destruction.

Finally, the possibility of using AGFA as specific carriers for cytotoxic drugs to tumor nodules was tested. Daunomycin was coupled with the aid of glutaraldehyde to goat AGFA. The resulting daunomycin-antibody conjugates inhibited cellular RNA synthesis and induced cell death in vitro of a MC-D sarcoma of strain 13 guinea pigs. The cytotoxic capacity of the conjugate was not significantly different from that of free daunomycin. The specific localization of daunomycin-antibody conjugates within the fibrin matrix enmeshing the tumor tissue was demonstrated by indirect immunofluorescence with FITC-conjugated rabbit antibodies to goat γ-globulins. Multiple injections of daunomycin-antibody conjugates intratumorally in vivo, into well established MC-D tumors, led to significant tumor growth retardation and complete tumor rejection occurred in 50% of the guinea pigs. Moreover, systemic tumor immunity was induced in the guinea pigs so cured, as demonstrated by the fact that these animals were resistant to a further lethal dose of MC-D tumor cells.

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CHAPTER I

An Overview of Tumor Immunology

Chapter I

AN OVERVIEW OF TUMOR IMMUNOLOGY

TUMOR ANTIGENS

At the beginning of this century, experiments performed in outbred animals have shown that the growth of transplanted animal tumors could be prevented by immunization of recipients with the same tumors. However, it was soon realized that the rejection of tumor grafts in these experiments was due mainly to the sensitization of the recipients to alloantigens present in the original tumor inocula. Nevertheless, these studies led to the search for a unique antigen which can only be found in tumor cells and should not be present in normal tissues.

According to the cellular distribution, tumor antigens can be distinguished in two categories: those that form part of the cell surface and those that do not. There is ample evidence to indicate that only tumor antigens that belong to the former category can elicit humoral and/or cell-mediated immune responses, which, in many occasions, lead to tumor rejection. Tumor antigens have been detected on cells of tumors induced by either chemical carcinogens or oncogenic viruses.

Chemically-Induced Tumors

Using a methylcholanthrene (MCA)-induced sarcoma in mice, it was demonstrated that syngeneic mice immunized by intradermal inoculation of MCA-induced tumor cells rejected the subsequent grafts of the same tumor (Gross, 1943). Unfortunately, this result cannot exclude the possibility that the tumor may have mutated during repeated transplantation since the histocompatibility within the experimental mice had not been checked.

More critical demonstration of the specific antigenicity of tumor cells was performed by Foley (1953). Using the MCA-induced sarcoma in inbred C3H mice, he demonstrated that after excision of these tumors by ligation the host could be rendered resistant to subsequent challenge with the same tumors. Later, Prehn and Main (1957) substantiated this observation with additional controls, including immunization of the host with normal tissues. This did not render the host with immune resistance to the tumor grafts; moreover, skin grafts from primary donor were accepted by the immune host. This excluded the possibility that tumor rejection was due to either tissue entigens or isoantigens and was thus tumor specific.

Tumor antigens of different tumors, induced in the same strain of animals by identical carcinogens, were shown to be individually specific, since no crossreactions were observed among different tumors of similar morphology (Klein et al., 1960; Old et al., 1962). However, there is also evidence supporting the view that crossreacting tumor antigens exist in tumors induced by either similar or different carcinogens (Reiner and Southam, 1969; Takeda, 1969; Holmes et al., 1971). One of the proposed mechanisms for the appearance of these crossreacting antigens is that the chemical carcinogen activates the oncogenic viruses which in turn induce neoplastic transformation of cells, giving rise to virus-induced type specific crossreacting antigens on their surfaces. Another mechanism, which is based on the studies of Reiner and Southam (1969), invokes the possibility that chemical carcinogens may induce several sets of tumor antigens on the surface of tumor cells, some having a crossreacting determinants which elict only weak immune responses which are difficult to detect.

Virus-Induced Tumors

Tumor antigens have been demonstrated in tumor cells induced by DNA and RNA viruses. Thus, it has been shown that mice after infected with polyoma virus became resistant to a cell transplant of a tumor induced by DNA polyoma. Since attenuated virus or passively transferred antibodies to virus had no such effect, it was suggested that polyoma-specific antigens were induced by the virus in the infected cells of mice (Habel, 1961; Sjogren, 1961). Two classes of antigens are induced by DNA viruses: (i) cell surface antigens which elicit tumor transplantation immunity, demonstrable by the induction of resistance to tumor grafts following immunization with the homologous DNA virus-induced tumor cells; (ii) T- or neoantigens which are intra-cellular and specific to the induced viruses.

Tumor antigens of RNA virus-induced leukemia cells were demonstrated by the presence of specific anti-leukemia antibody from the sera of mice which had been immunized with the leukemia cells (Old et al., 1963).

Recently spontaneous tumors have been shown to carry tumor antigens by the demonstration of retardation of tumor growth in hosts which had been immunized with the same tumor cells. Antigens responsible for the inhibition of tumor growth in immunized hosts have been shown to be tumor specific and not related to the tumor virus (Morton et al., 1969).

It has been recognized for some time that tumors induced by one type of virus show common crossreacting antigens, even if the tumors are of different morphology or are induced in different species. However, recent evidence has been adduced to show that mammary tumor virus (MTV)-induced carcinomas have individually specific as well as common antigens (Morton et al., 1969; Vaage, 1968). This finding is similar to that observed in

chemically-induced tumors which may indicate that both types of tumor cells could have individually specific as well as crossreacting antigens.

Tumor Associated Embryonic Antigens

Interest in studying the relationship between embryonic tissues and neoplastic transformation was first raised by the report of Schöne (1906) who showed that mice after injection with embryonic but not adult tissue could reject a tumor transplant. Although the exact mechanism underlying this observation is still not clear, the presence of embryonic antigens in tumor tissues was unequivocally proved by the demonstration of α-fetoproteins in the serum of hepatoma bearing mice (Abelev, 1963) and carcinoembryonic antigens in adenocarcinomas of the human digestive tract (Gold and Freedman, 1965a, 1965b).

Embryonic antigens are usually referred to as macromolecules which are found in embryonic as well as tumor tissues, and are demonstrable to be immunogenic either in syngeneic or xenogeneic (after proper absorption) hosts. It is currently postulated that the production of this macromolecule during the neoplastic transformation is due to the activation of silent genes normally expressed only in the embryo.

Embryonic antigens have been shown in many tumors of experimental animals induced by either chemical carcinogens (Brawn, 1970) or viruses (Coggin et al., 1970). Immunization of the animals with embryonic tissues sometimes protects the hosts from subsequent tumor challenge (Coggin et al., 1971; Ting et al., 1973). However, the relationship of antigenic specificity between embryonic and tumor tissues is still unclear.

HUMAN TUMOR ANTIGENS

Tumor Specific Antigens

Using several sensitive *in vitro* methods such as immunofluorescence (Klein et al., 1966), complement fixation (Eilber and Morton, 1970) and colony inhibition (Hellstrom et al., 1970), antigens on tumor cells which induce specific immune responses have been demonstrated in a variety of human tumors. For example, a high percentage of patients with Burkitt's lymphoma (Klein et al., 1966) and melanoma (Lewis et al., 1969) have antibodies to the surface antigens of tumor cells, detectable by immunofluorescence after the tumor had regressed.

Tumor antigens in human tumors have been also detected with other methods such as lymphocyte transformation (Vanky et al., 1971) and skin tests (Oren and Herberman, 1971). However, the specificity of these reactions is still not established.

Using the techniques of mixed hemadsorption and immune adherence, autologous antibodies to the surface antigens on the patients' own melanoma cells have been demonstrated. It appears that there are at least three kinds of surface antigens on melanoma cells: a) unique melanoma-specific antigens which are only found on autologous melanoma cells; b) common melanoma-specific antigens detected on melanoma cells of different patients, and not on other kinds of tumor cells; c) some antigens which have also been found on normal human cells and nucleated cells of some animals (Shiku et al., 1976a;1977). The presence of these complex antigens on human tumor cells adds to difficulty of distinguishing which antigen is unique to the neoplastic transformation of cells.

Tumor Associated Embryonic Antigens

In the search for tumor specific antigens of human tumors, it was found that a component from embryonic tissues could crossreact with antiserum against tumor antigens. It is apparent from this finding that there is a common antigen expressed on both embryonic and tumor tissues. Currently two types of embryonic antigens, namely, carcinoembryonic antigens (CEA) and α-fetoprotein (AFP), are being studied widely.

(a) Carcinoembryonic Antigens

CEA has been detected in carcinomas of the human digestive system and of the embryonic digestive organs. Anti-CEA antisera, prepared in rabbits and absorbed with normal colon tissues, were shown to contain antibodies which specifically reacted with CEA extracts but not with normal colon tissue extracts (Gold and Freedman, 1965a, 1965b).

Sensitive radioimmunoassays were developed to detect the circulating CEA levels in the serum of cancer patients (Thomson et al., 1969; Hansen et al., 1971; Berczi et al., 1976). The levels of circulating CEA in patients with colonic carcinomas was persistently higher than in normal individuals. Therefore, radioimmunoassays for CEA are useful for the diagnosis and more importantly for the prognostic assessment of colonic cancer. However, it ought to be stressed that increased levels of CEA have been also detected in other types of cancer, in non-malignant diseases and in heavy smokers (Hansen et al., 1974). The exact causes for these findings are still unknown.

(b) α-Fetoprotein

This antigen was first demonstrated in the sera of mice bearing hepatomas and was since found in serum of normal embryos (Abelev, 1963). Subsequently, AFP was also found in serum of patients with malignant

hepatomas (Tatarinov, 1964). AFP does not appear to be immunogenic in the same species, but anti-AFP antibodies can be readily obtained by immunization of another species. Clinically, AFP has been used in diagnostic tests for hepatomas and hepatitis.

IMMUNE REACTIONS TO TUMORS

Evidence from *in vivo* and *in vitro* studies has demonstrated that a host is capable of mounting specific immune response to antigenic tumor cells. Thus, normal syngeneic mice could be immunized to tumor antigens by excision of growing tumors, so that subsequent tumor cell isografts were rejected (Prehn and Main, 1957). Also, sarcomas induced in adult mice by murine sarcoma virus (MSV) sometimes regress due to specific immune reactions (Fefer et al., 1968).

However, inspite of the host's ability to mount immune reactions against the tumor cells, they continuously grow and eventually kill the host. This fact suggests that effective immune defence reactions against tumor cells may not be operating in the tumor-bearing host. In the following sections, the nature of humoral and cell-mediated immune reactions to tumor antigens, as well as their possible roles in tumor rejection will be examined.

EFFECTS OF ANTIBODIES ON TUMOR GROWTH

Cytotoxic Effects on Tumor Cells

(a) Complement-Dependent Cytotoxic Antibodies

It has been demonstrated with different tumor systems that cytotoxic antibodies can be produced in syngeneic animals by immunization with tumor

cells. For example, mice immunized with homogenates from lymphoma cells produced anti-lymphoma antibodies detectable by the complement-dependent cytotoxic test (Klein and Klein, 1964). Cytotoxic antibodies against sarcoma cells have been demonstrated in the IgM fraction of an antiserum produced in syngeneic mice immunized with MCA-induced sarcomas (Bloom and Hildemann, 1970). Similarly, in studies carried out in this laboratory (Dalton et al., 1976) it has been shown that antibodies produced in response to immunization with lymphoma L1117 cells in syngeneic A/J mice belonged primarily to the IgM class.

Antibodies cytotoxic to autologous tumor cells have been detected in the sera of patients whose tumors were surgically removed (Lewis et al., 1969; Morton, 1971). Cytotoxic antibody activity resided mainly in the IgM fraction of the serum immunoglobulins. In some patients whose tumors were completely removed, this antibody activity could be elevated by the injection of irradiated autologous tumor cells. This observation indicated that hosts were capable of producing specific cytotoxic antibodies against tumor cells once the tumor load was reduced.

(b) Antibody-Dependent Cellular Cytotoxicity (ADCC)

Sera obtained from animals immunized with tumor cells (De Landazuri et al., 1974a) or from hosts after tumor regression (Harada et al., 1972) have been shown to confer specific cytotoxicity onto non-immune lymphocytes against the tumor cells which were used as targets in vitro. Similar antibody activity had been reported earlier in allogeneic and xenogeneic graft systems (McLennan et al., 1969).

It was shown that antibodies of either IgG or IgM class with an intact Fc fragment were able to mediate this type of cytotoxic reaction (Basten et al., 1972; Lamon et al., 1977). This reaction does not require complement. The effector cells which mediate ADCC reaction have Fc recep-

tors (Pape et al., 1977), and have characteristics of bone-marrow derived (B) cells. However, there is no evidence to suggest that these cells are antibody-forming cells; therefore, these cells may be neither thymus (T) nor B cells and may probably be 'null' cells (Allison, 1974). The specificity of the ADCC reaction seems dependent on the binding of antibody onto the target tumor cells and not onto the lymphocytes, since ADCC activity can be completely abolished by absorption of the sera with the specific tumor cells. Also, the attacking lymphocytes, obtained from xenogeneic donors have been shown to react with antibody-coated tumor cells (Wunderlich et al., 1975).

(c) Synergistic Cellular Cytotoxicity by Immune Serum

Immune sera obtained from animals which had been immunized with syngeneic tumor cells (De Landazuri et al., 1974b) or infected with MSV (Skurzak et al., 1972) were capable of enhancing the cytotoxic reactivity of immune lymphocytes to tumor cells in vitro (Hellstrom et al., 1971).

In a virus-induced tumor system, it was found that non-T lymphocytes were the effector cells and the cellular component of this synergistic effect was nonspecific so that lymphoid cells sensitized to a chemically-induced tumor were also effective (De Landazuri et al., 1974b).

The role of these factors in the in vivo suppression of tumor growth remains to be elucidated.

Interference with Cell-Mediated Immune Reactions to Tumors

Early in vivo experiments showed that transfer of small amounts of heterologous or isologous anti-tumor serum into tumor-bearing animals could enhance tumor growth (Kaliss, 1958). It has been demonstrated in vitro that sera from tumor-bearing animals or cancer patients could block

the specific cytotoxic reactions of lymphocytes to tumor cells. It was suggested that the cause of this blocking phenomena was due to a 'blocking' factor in the sera of tumor-bearing hosts which can block the immune reaction of cytotoxic lymphocytes against the tumor cells (Hellstrom et al., 1969).

The nature of the 'blocking' factor in sera of tumor-bearing hosts has not been clearly defined. In the case of the MSV-induced sarcoma in mice, this 'blocking' factor could be absorbed out with the corresponding sarcoma cells, or with goat anti-mouse IgG, and it was shown by gel filtration to possess characteristies of IgG. Therefore, it was assumed that this 'blocking' factor was IgG antibody. In subsequent studies, however, antigen-antibody complexes (Sjogren et al., 1971) or soluble antigen alone (Brawn, 1971) have been implicated as factors responsible for the observed blocking phenomena.

More recent data have further complicated this interpretation. Thus, 'blocking' factors can be absorbed and eluted out from immunosorbent columns made by coupling to Sepharose immunoglobulin fractions of sera from mice immunized with the tumor. These 'blocking' factors bind to ConA-Sepharose indicating that they may belong to some types of serum glycoproteins. They can be identified as polypeptides smaller than conventional immunoglobulin (M.W. = 56,000). All these findings forced the authors to revise their orginal claim that 'blocking' factors were 'blocking' antibodies. The concept that 'blocking' factors may represent a kind of immunosuppressive molecules produced by the T cells of tumor-bearing hosts has been recently proposed (Nepom et al., 1977). This view may thus confirm the earlier demonstration in this laboratory that specific immunosuppressor T cells were present in tumor-bearing mice and that soluble suppressor factors were isolated from these T cells (Fujimoto et al., 1976a, 1976b; Greene et al., 1977).

If the 'blocking' factor were an anti-tumor antibody, it would be rather difficult to explain why in other studies anti-tumor antibodies were shown to be cytotoxic for tumor cells (see previous section). However, it is possible that this is an anti-idiotypic antibody, i.e. and anti-receptor antibody (Rowley et al., 1973; Wight and Binz, 1977) which may block the receptor sites on immune lymphocytes thus preventing them from reacting with tumor cells. A preliminary study carried out in this laboratory showed that antisera raised against the receptor of the immune lymphocytes to 1509a sarcoma indeed affected the growth of this tumor in vivo (Lee et al. unpublished data).

In a recent model proposed by Gorczynski et al. (1974), it was suggested that the immune reaction of T lymphocytes can be blocked by antigen-antibody complexes both specifically and non-specifically. Thus, in the specific blocking, the antigen reacts with a T lymphocyte receptor and the antibody then binds to this antigen. In the non-specific blocking, the antigen-antibody complex binds to T lymphocyte via the Fc receptor of the latter. This model may provide one way to explain the 'blocking' mechanisms. However, it is difficult to visualize that the former reactions of T lymphocyte receptors with antigen resulted in blocking instead of proliferation of this sensitized T lymphocyte, which is a common finding in in vitro culture of sensitized lymphocytes with antigen.

It should be borne in mind that these blocking phenomena were observed by in vitro experiments, and there is still no definite proof of an equivalent situation existing in vivo. In addition, the loss of immune cytolytic function of T lymphocytes after interaction with antigen or antigen-antibody complex may be brought about by other mechanisms, such as the induction of receptor modulation or shedding from the cell surface.

Antigenic Modulation

Another phenomenon related to the effect of antibody on tumor cells is antigenic modulation, by which the density of tumor cell surface antigens can be altered after reaction with specific antibodies. A classical example is the loss of the TL(thymic leukemia)-antigen on lymphoma cells after exposure to anti-TL antibody (Old et al., 1968). This reduction in antigen distribution may enable tumor cells to escape immune destruction. Evidence has been obtained in this laboratory to the effect that ascites fluid or serum from tumor-bearing guinea pigs was capable of inducing resistance of tumor cells to cytolysis mediated by antibody and complement (Abe et al., 1977). To explain these results, it was proposed that antibody-antigen complexes in the ascites fluid or serum of tumor-bearing guinea pigs was responsible for inducing the change of antigen density on the surface ot tumor cells, which provided a route for the tumor cells to escape from the immune destruction mediated by the cytotoxic antibodies. Possibly this type of antigenic modulation could also cause structural changes of tumor antigens which induce the production of suppressor T cells (Kirkwood and Gershon, 1974).

CELL-MEDIATED IMMUNE REACTIONS TO TUMORS

In Vivo Demonstration of Immune Cells to Tumor Antigens

Animals can be made highly immune to antigens of some tumors, e.g. MCA-induced sarcoma in guinea pigs and mice, either by excision of the tumors following repeated challenges with tumor cells, or by multiple injections of irradiated or mitomycin C treated tumor cells in Freund's complete adjuvant (FCA). These immune animals will reject even a supra-lethal dose of tumor cells.

Cell-mediated immunity is generally considered to be the main factor in prevention of tumor growth in the animals immune to tumor antigens. In fact, this can be demonstrated by the Winn assay (1959), in which sensitized lymphocytes obtained from immune animals are mixed with tumor cells in vitro and then inoculated into syngeneic recipients. Using this assay, it had been shown that immune lymphocytes were able to protect the hosts against the Gross virus-induced lymphomas (Slettenmark and Klein, 1962) and the MSV-induced tumors (Fefer et al., 1967).

Cell-mediated immune reactions to tumors can also be detected by delayed hypersensitivity reactions by challenging the immune animals either with the corresponding intact tumor cells (Churchill et al., 1968) or with saline extracts of the tumor cells (Oettgen et al., 1968). These reactions were shown to be tumor specific since normal cells or unrelated tumor cells failed to induce positive skin reactions.

In Vitro Demonstration of Immune Cells to Tumor Antigens

Lymphoid cells obtained from animals immune to tumor antigens have been shown to react with target tumor cells in vitro. Interactions between immune lymphoid cells and tumor cells can be detected by several in vitro methods: (a) *^{51}Cr release test* (Brunner et al., 1968), which detects the release of ^{51}Cr from tumor target cells after they had been destroyed upon contact with immune lymphoid cells; (b) *Colony inhibition test* (Hellstrom, 1967), which measures the degree of inhibition of tumor cell growth by immune lymphoid cells; (c) *Microcytotoxicity test* (Takasugi and Klein, 1970), which is a modification of test (b), probably measures the damage to tumor cells caused by immune lymphoid cells; (d) *Lymphocyte transformation test* (Stjernsward et al., 1970), which measures the transformation of immune lymphoid cells to blast cells upon stimulation by tumor antigens;

(e) *macrophage migration inhibition test* (Bloom et al., 1969) and *leucocyte migration inhibition test* (Falk and Zabriskie, 1971; McCoy et al., 1974), which detect the release of lymphokines by immune lymphoid cells upon contact with tumor antigens; these immune mediators exert their effects on macrophages or leucocytes preventing thus these cells from migrating out of the capillary tube; (f) *Leucocyte adherence inhibition test* (Halliday and Miller, 1972; Grosser and Thomson, 1975) also detects the reactivity of immune lymphoid cells with tumor antigens, the lymphokines released by immune reactions inhibit the adherence of leucocytes to the glass wall.

Although results obtained by these in vitro methods are considered to reflect the expression of in vivo cell-mediated immune reactions, a positive correlation is still lacking in some cases. For example, it is usually difficult to detect cell-mediated immune reactions in a tumor-bearing host whose immune mechanism is probably being suppressed. Moreover, in cases where cell-mediated immune reactions were demonstrated with lymphocytes of tumor-bearing animals, the in vitro methods usually required prolonged incubation time of the order of 3-7 days between lymphocytes and tumor target cells (e.g. in colony inhibition assay). Hence, it is uncertain if the observed reactions were due to the in vitro sensitization of the lymphocytes which were unreactive in vivo.

Immune Unresponsiveness to Tumor Antigens in Tumor-Bearing Hosts

It has been frequently observed that cell-mediated immune reactions to tumors could not be detected in hosts with large tumors. Spleen cells from mice bearing chemically-induced sarcomas could not suppress tumor growth when they were mixed with tumor cells and inoculated into syngeneic hosts in the Winn assay. Only spleen cells obtained from hosts from which the tumor had been excised or from hosts immunized with tumor could suppress

tumor growth (Mikulska et al., 1966). In addition, it was recently demonstrated by a similar procedure that spleen cells from tumor-bearing mice could enhance, rather than suppress, tumor growth (Treves et al., 1974). Evidence obtained in this laboratory also showed that spleen cells from tumor-bearing mice could suppress the ongoing tumor immunity when they were transferred into immune mice (Fujimoto et al., 1976a, 1976b).

Many in vitro studies have also shown that lymphocytes obtained from tumor-bearing hosts are unreactive to the corresponding tumor target cells. Peritoneal lymphocytes obtained from mice bearing virus-induced sarcomas had no colony inhibitory activity whilst such activity had been detected after tumor removal (Barski and Youn, 1969).

During the course of growth and regression of MSV-induced sarcomas in mice, it was found that cell-mediated cytotoxic activity could be detected using the microcytotoxicity tests at the beginning of virus infection and tumor regression. Nearly no cytotoxic activity could be detected in mice with progressively growing tumors (Lamon et al., 1972, 1973); these results are in contrast with an earlier report (Hellstrom et al., 1971) which claimed that in a similar tumor system almost the same degree of cytotoxicity could be detected in all stages of tumor growth.

Results obtained from the migration inhibition test also indicated that peritoneal cells from mice bearing MSV-induced sarcomas have no migration inhibition activity, whereas after sarcoma removal or regression such inhibitory activity can be readily demonstrated. It is also interesting to note that mixing the peritoneal cells from tumor-bearing mice with those of mice from which the tumor had been removed resulted in a loss of reactivity in the migration inhibition test, presumably due to the release of a suppressor factor from the peritoneal cells of tumor-bearing mice

(Halliday, 1972).

The above findings that lymphoid cells from tumor-bearing hosts cannot react with the corresponding tumor cells in vitro strongly suggest that the immune reactivity against tumor antigens was severely suppressed in tumor-bearing hosts and especially in animals with large tumor loads.

Effector Cells in Cell-Mediated Immune Reactions to Tumors

Several cell types involved in the cytolytic action against tumor cells have been identified in vitro. In a ^{51}Cr release assay, the cytotoxic reactivity of effector cells from mice immunized to MSV could be abolished by anti-θ serum plus complement treatment (Leclerc et al., 1973). Similarly, the cytotoxicity of immune cells against a syngeneic lymphoma in rats could be abrogated by pretreatment the cells with anti-thymocyte serum, but this was unaffected by the removal of adherent cells and B cells (Djeu et al., 1974). These results implicate, therefore, T cells as the effector cells in this system.

Several subsets of T cells that participated in cytotoxic reactions against tumor cells have been distinguished with the aid of Ly phenotype markers on T cell surface. Thus, the Ly phenotype of the cytotoxic T cells responsible for the destruction of the allogeneic tumor cells is Ly $1^-2/3^+$ (Cantor and Boyse, 1975; Shiku et al., 1975; Huber et al., 1976) and of the syngeneic tumor cells is Ly $1^+2/3^+$ (Shiku et al., 1976b). On further analysis of the cytotoxicity to syngeneic tumor cells, it was shown that at least two subsets of T cells are involved, i.e. the Ly $1^+2/3^-$ T cell subset, although is by itself not cytotoxic, can augment the generation of the subset of Ly $1^-2/3^+$ cytotoxic T cells. Moreover, the full expression of cell-mediated cytotoxicity requires also the participation of Ly $1^+2/3^+$ T cell subset, which probably is the precursor of Ly $1^-2/3^+$ cytotoxic T cells (Stutman et al., 1977).

However, in other studies cytotoxic cells have been shown could be cells other than T lymphocytes. Thus, neonatally thymectomized, virus-infected rats have been shown to be able to generate cells which inhibit the colonization of virus-induced tumor cells in a colony inhibition assay (Borum and Jonsson, 1972). Also, it has been clearly demonstrated that B cells, but not T cells, obtained from tumor-regressing mice were cytotoxic to tumor target cells in a microcytotoxicity test (Lamon et al., 1973).

Macrophages were also shown to mediate cytotoxic effects on tumor cells. These cytotoxic effects which could be either immunologically specific (Kramer and Granger, 1972) or non-specific (Hibb et al., 1972), may be due to the release of a lymphotoxin-like substance from macrophages (Shacks et al., 1973) or to the 'bridging' of macrophages and tumor cells by cytophilic antibodies resulting in the destruction of the latter cells (den Otter et al., 1972).

Recently, a new type of cytotoxic cell which can specifically kill the MSV-induced leukemia cells has been found in certain strains of normal mice. These cytotoxic cells have been named NK (natural killer) cells. They have no known T or B cell markers on their cell surfaces as judged by their resistance to anti-θ serum plus complement and anti-mouse Ig treatments. Removal of macrophages and monocytes by adherence did not decrease their lytic activity (Kiessling et al., 1975). However, these NK cells may possess Fc receptors for IgG since after incubation of these cells with IgG coated sheep red blood cell monolayers, their cytotoxic activities were drastically reduced (Herberman et al., 1977). Possibly these are the cells responsible for immune surveillance or are the 'null' cells involved in ADCC activity.

Results obtained from all these studies indicate that several types

of immune cells can have cytotoxic effects on tumor cells. The optimal condition for the effective destruction of tumor cells may be brought about by the cooperation of many types of immune cells, or by the participation of specific anti-tumor antibodies, ADCC activity, and cytophilic antibodies.

REGULATORY CELLS IN IMMUNE REACTIONS TO TUMORS

It has been established that the humoral immune response to hapten-protein conjugates requires the cooperation of both T and B cells. Evidence has now accumulated that the interactions between two different subsets of T cells will decisively influence the outcome of an immune response. Currently T cells are classified, according to their cell surface markers and their functional behavior in the immune response, into four subsets, namely, amplifier cells, helper cells, suppressor cells and cytotoxic cells. In this section the properties of the first three subsets of T cells will be described since they are considered as regulatory cells in the immune response. The property of cytotoxic cells had been discussed in previous section (p.21).

It has not been firmly categorized whether the amplifier cells exert their influences on the humoral or cellular immune response. Nevertheless, we can consider them as a subset of T cells capable of enhancing the effects of other T cells in an immune response. These T cells belong to Ly phenotype of $Ly1^{+}2/3^{+}$ and are Ia positive (Feldmann et al., 1977). They are relatively insensitive to antilymphocyte serum treatment in vivo and are rapidly depleted after adult thymectomy (Cantor et al., 1975). It has been shown that these amplifier cells act synergistically to killer T cells against syngeneic tumor cells (Stutman et al., 1977). In non-tumor systems,

it has been shown that these T cells can amplify the cytotoxic activity of killer cells to allogeneic target cells (Cantor and Boyse, 1975). Also, the number of IgM plaque-forming cells to a hapten-protein conjugates was increased in the presence of amplifier cells (Feldmann et al., 1977).

Helper cells are a subset of T cells which assist the B cells to produce antibody. These T cells are Ly $1^+2/3^-$, Ia^+ and possess Fc receptor. There is evidence indicating that helper T cells can be subdivided according to the category of B cells which they help, e.g. there are helper cells which help B cells of the IgG or IgE antibody responses (Kimoto et al., 1977), or of a particular allotype (Herzenberg et al., 1976).

There is essentially no study on the function of helper cells in the humoral response to tumor antigens, probably due to the difficulty to detect any antibody production in most tumor systems. Perhaps the lack of helper cells causes the poor antibody response to the tumor antigens.

Suppressor T cells are a subset of T cells which can act to suppress specifically both humoral and cellular immune responses. These T cells are Ly $1^-2/3^+$ and Ig negative in most findings (Beverley et al., 1976; Cantor et al., 1976), although in some cases when they exhibited non-specific suppression to antibody production, they have been characterized as Ly $1^+2/3^+$ (Pickel and Hoffman, 1977). Suppressor T cells also carry Ia antigen which is coded for by the I-J subregion of H-2 complexes (Murphy et al., 1976).

It is believed that suppressor T cells are needed in order to exert a homeostatic control of the immune mechanism of individuals (Gershon, 1973). Thus, in the cases of tumor-bearing hosts where suppressor T cells are overactivated, the hosts immune defenses to tumors are thwarted; on the other hand, a failure in suppressor T cell function may explain the auto-

immune diseases in New Zealand black mice and man.

The study of regulatory mechanisms underlying the immune response to tumor antigens has only been started recently. It was shown in this laboratory (Fujimoto et al., 1974, 1975, 1976a, 1976b) that cells prepared from thymuses or spleens of tumor-bearing mice were able to exert a short-term suppression of the state of immunity to the specific tumor in mice preimmunized to the tumor. The suppressive activity was shown to be T cell dependent. A soluble factor extracted by freezing and thawing from these suppressor T cells was shown to be capable of exerting the same suppressive effects. The suppression was tumor specific since T cells from mice bearing other types of tumors did not produce the same effects. In further studies of the soluble factor(s)(ISF) it was shown not to be an immunoglobulin and its activity was destroyed by treatment with pronase but not with RNase. The ISF was found to share the antigenic determinants of the products of the K end of the major histocompatibility complex of the mouse, in the I-J subregion (Greene et al., 1977).

The existence of a subpopulation of T cells in the thymuses and spleens of tumor-bearing hosts, which can enhance tumor growth by suppressing the specific anti-tumor immunity, has also been shown by other investigators (Kirkwood and Gershon, 1974; Treves et al., 1974). However, some studies have shown that suppressor cells in thymuses and spleens of tumor-bearing animals were 'B-cell-like' and acted non-specifically to inhibit the T cell response to mitogen _in vitro_ (Kilburn et al., 1974; Kirchner et al., 1974; Gorczynski, 1974). Whether these two types of suppressor cells can act synergistically in enhancing tumor growth _in vivo_ has still to be resolved.

Factor(s) leading to the generation of these suppressor cells in

tumor-bearing host have been postulated. They involve the action of antigen-antibody complexes which modulate the molecular structure of the tumor antigens and thus signal the production of immune suppressor T cells (Kirkwood and Gershon, 1974). If this mechanism is indeed operating in tumor-bearing hosts, a pre-requisite will be the production of anti-tumor antibody by the host prior to induction of suppression through suppressor cells. In suppressor T cell mediated allotype immunosuppression it has been shown that anti-allotype antibody was needed in order to induce such immunosuppression (Herzenberg and Herzenberg, 1974). A recent study showed that suppressor T cells can be activated by specific antibody and tumor cells possibly acting as complexes in an animal tumor system (Gershon et al., 1974). Further evidence to support this view has been obtained, thus spleen cells obtained from mice neonatally infected with Moloney leukemia virus had no cytotoxic activity against the leukemia cells, whereas spleen cells from mice immunized with leukemia cells showed such reactivity. This cytotoxic reactivity could be abrogated by treatment with anti-θ serum and complement. Neither 'blocking' antibody nor tumor specific antibody had been detected in the sera, yet specific antibody to leukemia cells could be found in kidney eluates of neonatally infected mice. All these results suggest that a state of immunologic tolerance for T cells in neonatally virus-infected mice may induced by antigen-antibody complexes (Chieco-Bianchi et al., 1974).

Although at present there is no direct evidence to show that immunologic tolerance may be mediated by suppressor T cells in tumor-bearing host, it has been demonstrated that tolerance can be caused by activation of suppressor T cells in non-tumor systems (Basten et al., 1974; Rouse et al., 1974; Baker et al., 1974; Dorsch and Roser, 1977).

A type of cells belonging to the macrophage series has also been shown to play an important role in regulating an immune response. These cells were shown to be required for the differentiation of helper precursor cells to functioning helper cells in vitro (Erb and Feldmann, 1975), and for the specific immune recognition of T cells in order to elicit an immune response in guinea pigs (Rosenthal and Shevach, 1973). In further studies, it was shown that the Ia molecules on macrophages played an essential role in these cells' presentation of antigen to T cells for eliciting a specific immune response (Shevach et al., 1977).

However, except for the numerous demonstrations of the killing activities of macrophages to tumor cells (Levy and Wheelock, 1974), the role of macrophage in regulation of the immune response to tumor has still to be explored.

Hence, it is important to analyse the contribution of each of these cells in regulating an immune response against tumor antigens.

IMMUNE SURVEILLANCE AND ESCAPE MECHANISMS

Immune Surveillance

The concept of immune surveillance, initially postulated by Thomas (1959) assumed that during the lifespan of an individual there was a continual emergence of some aberrant cell clones such as tumor cells, and that as a result of the normal immune defense mechanism within the individual these cells were recognized and destroyed. Although most experimental results are in favor of the existence of an immune surveillance mechanism, definite proof is still required. Some of the evidence in favor of immune surveillance will be discussed in the following sections:

(i) Since the immune reaction is thought to eliminate malignant cells,

then according to surveillance concept, the suppression of the immune response should increase the incidence of malignancy. For virus-induced tumors such as polyoma virus-induced tumors, it has been shown that if the immunologic reactivity of the animals was suppressed by immunosuppressive drugs, or anti-lymphocyte serum, or neonatal thymectomy, an increased frequency of tumor growth in the hosts was observed (Law, 1966; Allison and Taylor, 1967). However, the effects of immunosuppression on tumor induction by chemical carcinogens are less certain and this subject remains controversial (Balner and Dersjant, 1966; Vagner and Haughton, 1971). Clinical experience has shown that an increased frequency of tumors occurs in kidney transplant patients who have been treated with immunosuppressive drugs (Penn and Starzl, 1973). Similarly, a high frequency of malignancy is observed in patients with various immune deficiency diseases (Gatti and Good, 1971).

(ii) Conversely, the stimulation of the immune response by specific immunization with tumor antigens or oncogenic virus (Girardi, 1965) or by non-specific immunization with Bacille Calmette-Guerin (BCG) (Weiss et al., 1961) decreases the incidence of primary tumors, and the latency period of spontaneous mammary carcinomas or of chemically-induced sarcomas is prolonged.

(iii) Other factors such as ageing, which accompanies decreased immunocompetence, is usually associated with high tumor incidence.

However, the validity of the concept of immune surveillance has been challenged recently (Schwartz, 1975; Möller and Möller, 1975; Prehn, 1976). One observation that has been frequently cited to disprove this concept is the relative low incidence of primary tumors in thymusless (nude) mice (Rygaard and Povlsen, 1974). According to the surveillance concept, since

these mice lack the T-cell function they would be expected to develop a high incidence of tumors. However, a careful analysis of this study reveals that the incidence of primary tumors in nude mice was evaluated on a population of animals that had a very short life, i.e. only an average of 4 months due to poor health. By contrast, in another study, nude mice maintained under germfree environment lived for as long as 2 years and developed during their lifespan many tumors (Outzen et al., 1975).

Another observation has also been cited against the surveillance concept, which was based on the observation that a state of immunity to tumor resulted in stimulation of tumor growth (Prehn, 1976). On the other hand, there is recent evidence, strongly suggesting that this immunostimulation phenomenon was either not immunologically specific or not immunologic in nature (Lamon, 1977).

There is also well-documented evidence that immune surveillance can be due to some mechanisms other than those involving T-cells. These include ADCC effector cells (K cells), natural killer (NK) cells and stimulated macrophages (Allison, 1977). In this regard, the concept of immune surveillance requires to be redefined and reviewed in the light of new experimental facts (Allison, 1977; Ioachim, 1977; Klein and Klein, 1977).

Escape Mechanisms

There is ample evidence that hosts bearing antigenic tumors have their immunological defence mechanisms activated and that some of these have the potential to destroy the tumor cells; yet, in the majority of cases, the tumors grow and kill the host. Various mechanisms which have been proposed to explain the escape of tumor cells from the host's immune destruction are briefly reviewed below:

(i) The first possible route is via the induction of immunologic

tolerance to tumor antigens in the tunor-bearing host. The actual mechanism remains to be solved. It is conceivable that tolerance may be induced by antigen-antibody complex and continuously maintained by the action of suppressor T cells (see previous section, p.26). If suppressor T cells are short-lived, the term 'partial tolerance' as suggested by Chieco-Bianchi et al. (1974) should be used, since there is a need for the continuous replenishment of suppressor T cells through the production of small amounts of antigen-antibody complex in order to maintain the state of tolerance. Indeed, suppressor cells in partially tolerant hosts have been described in a tumor system by the same authors, and in an allogeneic system by Elkins (1972).

(ii) The density of tumor cell surface antigens can be altered phenotypically by a reaction with specific antibody which prevents the full expression of tumor antigenicity, thus permitting the altered cells to escape from immune destruction. A typical example is the antigenic modulation phenomenon of the TL-antigen change in lymphoma cells of mice (see previous section, p. 17).

Alternatively, the antigenicity of certain tumor cells could be altered by genotypic selection of a variant tumor cell line which possesses the least amount of antigen on the cell surface (Fenyo et al., 1968). The 'strength' of antigenicity in certain tumor cell lines is lost after several in vivo transplantations in syngeneic hosts, which may be attributed to this mechanism.

(iii) Blocking of immune effector mechanisms against tumor cells by serum factors has also been proposed to aid the tumor cells in their escape from immune destruction. More recent data have shown that the 'blocking' factor could be antibody, antigen, antigen-antibody complex, or immunosuppressor factor.

IMMUNOTHERAPY OF CANCER

There is still no satisfactory therapeutic treatment of cancer. Currently chemotherapy, surgical removal and radiation therapy are the main clinical methods in the treatment of cancer. Although these methods are effective in some cases they all have inherent limitations.

In a surgical intervention, ideally, all the tumor cells should be removed. However, frequently, in practice, small amounts of tumor cells are left in the patient's body after operation, which lead to later matastases. Hence, surgical operations may have only a short term and local effect.

Radiation therapy is also local and the killing is non-specific, i.e., it can kill tumor cells as well as normal tissues. Each treatment can kill only limited number of tumor cells.

Chemotherapeutic agents have the advantage that they can exert both systemic and local effects. However, they are extremely toxic and non-specific and may therefore, destroy normal as well as tumor tissues.

By comparison to the above treatments, immunologic strategies offer the promise. First of all, it may be visualized that immunologic methods may provide a means of preventing cancer prior to its establishment. Like in most other diseases, prevention is preferred to the treatment of established cancers. It is, therefore, hoped that in the future cancer can be prevented by vaccination with attenuated virus or by immunization with modified tumor antigens. Some experimental results with animal systems are indeed encouraging. Thus, one may cite the report demonstrating that Marek's lymphomatosis in chicken which is caused by a herpes virus, can be prevented by vaccination with the attenuated virus (Churchill et al., 1969).

Secondly, immunologic methods are specific; tumor sensitized lymphoid cells or antibodies will directly hit the tumor target cells thus avoiding any side effects or toxicity as encountered in other forms of treatment.

In the following sections several studies attempting to use immunologic methods for the distruction of tumors in animals and man will be discussed.

Non-Specific Immunotherapy

Certain bacterial adjuvants, notably Bacillus-Calmette-Guerin (BCG) and Corynebacterium parvum (C. parvum), have been shown not only to increase immune responses to conventional antigens but also to influence the growth of certain tumors in animals and man. The action of these bacterial adjuvants on tumor growth is believed to be due to the non-specific stimulation of the immunological apparatus of the host.

(a) BCG Immunotherapy in Animal Tumors

At present BCG is widely used in tumor immunotherapy because encouraging results have been obtained from this type of treatment. In a prophylactic study, it was demonstrated that pretreatment with BCG can interfere with the growth of chemically-induced sarcomas in allogeneic but not syngeneic mice (Old et al., 1969). Subsequent studies in other animals have confirmed the positive effects of BCG in preventing tumor growth (Weiss, 1972; Keller and Hess, 1972).

Administration of BCG alone or in combination with other treatments can also suppress the growth of established tumors (Mathé et al., 1969; Parr, 1972). A typical study is represented by the experiments performed with diethylnitrosamine-induced hepatomas in strain 2 guinea pigs. Intratumoral injection of living BCG caused regression of tumor nodules and elimination of lymph node metastases (Rapp, 1973).

The success of BCG immunotherapy depends on the following conditions: *(a) The sizes of the tumor*. If the size of the tumor was larger than 95 mg in terms of weight, in the case of the chemically induced hapatoma of guinea pig, the growth of the tumor was usually unaffected. *(b) The immunocompetent state of tumor-bearing host*. If BCG was given to an immunosuppressed tumor-bearing host, the treatment was ineffective. *(c) The substrain, dose and viability of the BCG preparation*. In general, 10^8 viable BCG are required for effective suppression in guinea pigs. In an animal model for BCG immunoprophylaxis study (Davies and Sabbadini, 1978), it was found that the effectiveness of BCG was greatly enhanced with the use of appropriate sequences of doses. Thus, administration of BCG in decreasing doses provided better protection from tumor grafts. Viable BCG is preferred although the methanol-extractable residue (MER) of BCG has been shown to be effective (Yashphe, 1971; Wainberg et al., 1976). *(d) The route of administering BCG*. This is critical. For leukemia, systemic administration of BCG was effective (Mathè, 1973). For solid tumors, intratumoral injection of BCG has been found most effective (Baldwin and Pimm, 1971; Zbar et al., 1972), probably due to BCG sensitized lymphocytes reacting with the bacillus and producing lymphotoxins which directly inactivate the tumor cells in the vicinity.

Conflicting results have been obtained in the study of the effect of BCG on carcinogenesis (Larson et al., 1971; Piessens et al., 1971). This may be due to the different time schedules and variation of carcinogens used.

There are also reports that BCG may stimulate tumor growth which could be related to the immunogenicity of tumors, since this usually happened in the hosts bearing weakly antigenic tumors, e.g., mammary carcinomas (Piessens et al., 1970). Another possible mechanism for the stimu-

lation of tumor growth by BCG may be due to the enhancement of suppressor T cell activity in mice given high doses of BCG (Geffard and Orbach-Arbouys, 1976).

(b) BCG Immunotherapy in Human Tumors

Interest in the prophylactic value of BCG led several investigators to analyse available medical records to check whether vaccination with BCG in children could provide effective protection against leukemia. Thus, a study of the incidence of leukemia deaths in children in Quebec (Davignon et al., 1970) and in Chicago (Rosenthal et al., 1972) showed that children who died from leukemia had a lower frequency of BCG vaccination. Results from these two retroactive studies implied that BCG vaccination did reduce the incidence of leukemia in children. However, in other studies the same conclusion could not be reached (Berkeley, 1971; Comstock et al., 1976). Therefore, a more extensive study is required to prove the value of BCG immunoprophylaxis in children.

Treatment of established tumors, after the intralesional injection of live BCG in patients with malignant melanoma, resulted in regression of the injected tumor nodules. Moreover, some of the non-injected nodules also regressed (Morton et al., 1970). In another study, when patients with malignant melanoma were given oral BCG as in adjunct to standard treatment, it was found that BCG treatment increased the survival in patients with visceral metastases and inhibited the further development of metastases in those patients who had surgical resection (McGregor et al., 1977). Requirements for successful BCG treatment in melanoma patients are very similar to those defined in animal models. For example, immunocompetent patients as shown by dinitrochlorobenzene (DNCB) skin tests and with a small tumor load had good clinical responses.

The results reported by Mathé et al. (1969), using a combination of BCG immunotherapy and chemotherapy for treatment of acute leukemia, showed some encouraging findings. A significant improvement in the survival of patients who were treated with BCG was observed after complete remission had been induced by chemotherapy and radiotherapy. A follow-up study of these patients has been reported recently, which shows that 7 patients out of 20 that received this form of treatment were still alive after 13 years (Mathé et al., 1977). Unfortunately, two well controlled studies conducted by the British Medical Research Council and the other by the United States Children's Cancer Group, have failed to confirm Mathé's observations. A separate study, using a similar protocol to Mathé's, also failed to confirm his claims (Leventhal et al., 1973). The failure of these studies to reproduce Mathé's work may be due mainly to the difference in the patients' conditions when BCG immunotherapy was applied. In Mathé's trial, all patients had been treated for intensive cytoreduction, but this was not the case in the other studies (Gutterman, 1977). Besides, the use of different BCG substrains and routes of administration may also produce different results. Thus, the question of whether BCG immunotherapy is an effective regimen for treatment of acute lymphocytic leukemia is still open.

It appears that the most promising results of BCG immunotherapy may be expected in patients with minimal residues of tumor either after surgical therapy and/or chemotherapy. Apart from the studies of Mathé in acute lymphocytic leukemia, this combined treatment has been shown to prolong remission and survival of patients with metastatic gastrointestinal cancer (Falk et al., 1977), acute myelogneous leukemia (Powles, 1973), lymphoma (Ziegler and Magnath, 1973) and melanoma (Bluming et al., 1972).

(c) C. Parvum Immunotherapy

C. parvum belongs to a group of corynebacteria which have been demonstrated to have anti-tumor activity similar to that of BCG. Thus, intratumoral injection of C. parvum into a mammary adenocarcinoma in rats was shown to lead to complete rejection of this tumor, and the surviving rats exhibited tumor specific protection to subsequent tumor cell challenge (Likhite, 1977). Likewise, when C. parvum was injected either intravenously or intraperitoneally into nude mice, the mice were protected to subsequent tumor transplants (Woodruff and Warner, 1977).

From the results of studies involving animal tumors it may be concluded that C. parvum exert its beneficial effect when the tumor load is small (Halpern et al., 1966; Woodruff et al., 1972), and that the degree of anti-tumor effect of C. parvum depends on both the immunogenicity of the tumor and the routes of administration of this bacteria (Scott, 1972a, 1972b). Many studies have indicated that macrophage-like peritoneal exudate cells were involved in tumor rejection in animals treated with C. parvum. It was shown that the anti-tumor effect was abolished following the inhibition of the macrophage function but not the T-lymphocyte activity (Woodruff et al., 1973; Scott, 1975; Likhite, 1975).

(d) Mechanisms of Adjuvant Immunotherapy

The exact mechanisms involved in adjuvant induced tumor regression are unclear. Most likely, intralesional injection of BCG elicits a delayed hypersensitivity reaction to the bacteria within the tumor foci, which causes the release of lymphotoxin or related toxic chemicals from sensitised lymphocytes, these toxins exerting in turn a cytopathic effect on adjacent tumor cells. In addition, some chemotactic factors may be released in the inflammatory sites, attracting to the sites large numbers of macrophages or

other phagocytic cells which have the capacity of killing or inactivating the adjacent tumor cells. It is also important to note that intralesional injection of bacterial adjuvants could produce a form of active specific immunization. Therefore, in adjuvant immunotherapy both specific and non-specific immune reactions are probably involved. More recently, BCG and chemically-induced hepatocarcinoma cells have been shown to have crossreactive antigens (Bucana and Hanna, 1974; Minden et al., 1974). This raises the question as to whether or not the crossreactive antigens on BCG organisms are tumor specific or a type of embryonic antigens. The favorable results of BCG immunotherapy may also be considered to be due to the crossreactive antigens on BCG which would terminate the host's tolerance to tumor antigens. This is similar to the classical studies of breaking the rabbit's tolerance to bovine serum albumin (BSA) by chemically altered BSA (Weigle, 1962).

Topical Immunotherapy with Chemicals

A form of non-specific immunotherapy (Klein, 1969), especially useful for skin cancers, is the repeated application of DNCB or triethylene-imino-benzoquinone (TEIB) to the lesions in patients who thus become sensitized to a state of delayed hypersensitivity to these chemicals. Tumor nodule regression has been observed in patients with skin carcinomas after this type of immunotherapy. The mechanism underlying this form of therapy is believed to involve the unleashing of delayed hypersensitive reactivity to these chemicals by the patient's lymphocytes in tumor sites thereby leading in an indirect manner to tumor cell destruction.

Active Specific Immunotherapy

In this form of immunotherapy, autologous or allogeneic tumor cells were injected into tumor-bearing hosts with the hope that specific immune

response to tumor antigens can be induced. However, since the immune response of tumor-bearing hosts is usually undermined by the tumor, this form of immunotherapy has met with little success.

It has been shown that the intradermal injection of suitable doses of viable tumor cells, either immediately after tumor inoculation or before the tumor is palpable, can prevent the growth of carcinogen-induced hepatomas (Kronman et al., 1970) or sarcomas (Eilber et al., 1971) in strain 2 guinea pigs. It appears that this type of immunotherapy can be successful only when the hosts have small tumor loads.

Many physical and chemical procedures have been utilized to modify tumor cells in attempts to increase the immunogenicity of tumor antigens on the cell surface. Tumor cells that had been treated either by physical manipulations such as freezing and thawing, lyophilization and homogenization or by chemical coupling to foreign proteins such as γ globulin have been used in vaccination and immunization. However, these techniques have not produced impressive results in the prevention of tumor growth.

Another method of augmenting tumor cell immunogenicity is by infection the tumor cells with virus, usually influenza virus, before injecting them into tumor-bearing hosts. This phenomenon is called 'heterogenization', where new antigenic determinants are acquired on the tumor cell surface. Lindenmann and Klein (1967) showed that mice injected with influenza virus oncolysate of Ehrlich's ascites tumors were protected against subsequent challenge with the same tumor cells. However, Beverley et al. (see Mitchison, 1974) showed that virus-infected tumor cells were not more immunogenic than irradiated tumor cells.

Increased immunogenicity of tumor cells has been reported by treating tumor cells with neuraminidase *in vitro* and *in vivo*, which does lead to

tumor regression (Simmons et al., 1971). The effect of neuraminidase on tumor cells is explained as being due to the removal of sialic acid from the tumor cell surface by the enzyme thereby exposing the antigenic determinants. However, this cannot explain the fact that an immune animal is also capable of rejecting the tumor cell grafts which have not been pretreated with neuraminidase.

In clinical trials of active immunotherapy, the results of Mathé et al. (1969) for the treatment of acute lymphocytic leukemia provide a promising basis for developing effective protocols for the active immunotherapy in the presence of BCG. In this form of treatment, patients were first treated with irradiated allogeneic leukemic lymphoblasts admixed with BCG. These treated patients remained in remission for about four years.

While the exact mechanism leading to the tumor suppression in the patients during immunotherapy is still not clear, it has been shown that some components of the immunological apparatus in these patients were being activated, e.g., the numbers of immunoblasts and null cells in the peripheral blood as well as the in vitro reactivities of the lymphocytes to phytohemagglutinin and pokeweed in these patients were significantly increased, as compared to the normal controls or the patients receiving chemotherapy (Mathé, 1976).

Adoptive Immunotherapy with Lymphoid Cells

In animal tumor systems, it has been reported that the transfer of large doses of sensitized syngeneic lymphoid cells obtained from donors immunized with tumors can cause either inhibition of primary tumor growth (Delorme and Alexander, 1964) or the regression of established tumors (Fass

and Fefer, 1972) in the recipients. These inhibitory effects on tumor growth were also observed with sensitized lymphoid cells from allogeneic and xenogeneic donors (Alexander et al., 1966; Borberg et al., 1972). Possibly this was due to a strong allogeneic effect which enhanced the specific anti-tumor immune reaction of the transferred immune lymphoid cells. Again, favorable results were observed when the hosts had small tumor loads.

In clinical trials, patients with various types of cancer were paired according to tumor type and blood type, and were cross-immunized to allogeneic tumors in pairs. Sensitized peripheral lymphocytes were then exchanged. Some patients treated by this method had remissions; however, there was no evidence that tumor specific immune reactions were involved (Nadler and Moore, 1969). It is important to emphasize that deaths have been attributed to this treatment. One severe problem with this form of immunotherapy is the graft-vs-host (GVH) reaction caused by histoincompatibility between tumor patients. This is difficult to control in man, since it is known that even an HL-A identical graft will still be rejected due to differences in other minor histocompatibility antigens.

One way to circumvent the GVH reaction is to sensitize the autochthonous lymphocytes to mitomycin C treated tumor cells in vitro prior to reinfusion into the patients. Several clinical trials, however, did not show any positive responses to this treatment (Nadler and Moore, 1966; McKhann and Jagarlamoody, 1971; Seigler et al., 1972).

In experimental animal system, several groups have reported that normal lymphocytes can be sensitized in vitro to tumor antigens on syngeneic tumors of mice or rats (Wagner and Rollinghoff, 1973; Treves et al., 1975; Kall and Hellstrom, 1975). This was demonstrated by in vitro

tests that the sensitized lymphocytes were capable of specially killing the tumor cells. In an adoptive immunotherapy trial for syngeneic plasmacytoma in mice, it was found that lymphocytes sensitized to this tumor effectively inhibited the tumor growth in the Winn assay in which the sensitized lymphocytes were inoculated admixed with the tumor cells. However, the same sensitized lymphocytes were not effective when they were injected intraveously and the tumor cells subcutaneously (Burton and Wagner, 1977).

Some serious problems, arising from the use of in vitro sensitized cells for tumor immunotherapy, have been revealed from the works of Cohen and his colleagues. It has been shown that the lymphocytes sensitized in vitro to tumor antigens could lead to autoimmune disease when reinjected into the host, probably these lymphocytes were also sensitized to autoantigens after in vitro culture with syngeneic tumor cells (Cohen, 1973; Orgad and Cohen, 1974). Moreover, the lymphocytes sensitized to tumor antigens in vitro have also been found capable of stimulating tumor growth in vivo (Ilfeld et al., 1973; Levo et al., 1974).

Passive Transfer of Immunological Mediators

During the past few years, the clinical value of the transfer factor has been confirmed in congenital immunodeficiency diseases (Wybran et al., 1973) and in infectious diseases caused by fungi (Graybill et al., 1973). Transfer factor is a cell-free extract from human peripheral leucocytes capable of conferring a specific cell-mediated immune response on the recipients. It is insensitive to nucleases and appears to be a low molecular weight (2,000 - 4,000) polypeptide. The use of transfer factor in tumor therapy has been reported (Morse et al., 1973). Particularly impres-

sive results were obtained in treating nasopharyngeal carcinoma (Goldenberg and Brandes, 1972) and melanoma (Smith et al., 1973). The precise mechanism by which this low molecular weight substance causes clinical improvement in cancer patients is unknown. The fact that immunity conferred by transfer factor is confined to the cell-mediated type has been considered to be an advantage in cancer therapy. However, the favorable results obtained so far are based on studies involving a small number of patients. More studies, concerning the nature and mode of action of transfer factor, are needed in order to assess its role in cancer immunotherapy.

RNA extracted from immune lymphocytes has been shown capable of transferring immunity to normal lymphocytes which then produce specific antibodies (Fishman, 1961). Using the migration inhibition tests, it was shown that in the presence of 'immune' RNA extracts from lymph nodes of mice that had rejected an allogeneic tumor cells, the migration of immune spleen cells was inhibited as compared to the normal spleen cells (Likhite et al., 1972). It was also shown (Alexander et al., 1968) that the 'immune' RNA which was extracted from lymphoid cells of rats immunized to chemically-induced sarcoma could transfer specific anti-tumor immunity to normal non-immune syngeneic spleen cells and that recipients of this RNA-treated spleen cells were able to resist the growth of tumor grafts. These results have been extended by using allogeneic and xenogeneic 'immune' RNAs in animal tumors (Paque and Dray, 1972; Pilch et al., 1974).

In a clinical trial conducted by Pilch et al. (1975,1976), xenogeneic 'immune' RNA, which was prepared from lymph nodes and spleens of sheep that had been immunized with human tumor tissues emulsified in FCA, was used to treat a group of patients with a variety of malignancy. Improvement in clinical course has been noted in some patients. However,

detailed results of this study have not yet been reported. Clearly more extensive studies are needed on the exact nature and mode of action of this 'immune' RNA in order to establish this form of immunotherapy in cancer.

Passive Immunotherapy with Antiserum

The expected effect of anti-tumor antisera to destroy the tumor cells after administration into tumor-bearing hosts has not been fully substantiated. In many cases, the administration of antiserum has been shown to have an adverse effect on the tumor-bearing hosts. Apart from the toxic effects due to the transfer of allogeneic or xenogeneic antisera, it has been shown that anti-tumor antisera have the potential for enhancing tumor growth. However, the recent demonstration that certain anti-tumor antisera or sera from hosts whose tumors had regressed contain antibodies which have either a specific cytotoxic effect on tumor cells or are able to induce normal lymphocyte cytotoxic to tumor cells, has prompted many investigators to re-examine this form of immunotherapy.

There are several reports that the growth of certain transplantable animal tumors can be suppressed by the transfer of specific anti-tumor antibodies (Old et al., 1964; Lindenmann and Klein, 1967). In a recent study, antibodies to lymphoma in mice have been shown to suppress tumor growth when admixed with an inoculum to normal mice. On further analysis of the mechanism underlying this observation, it was found that macrophages and lymphocytes obtained from peritoneal exudates of immune mice were the essential factors for successful tumor suppression (Shin et al., 1974). In another study it was shown that inoculation of lymphoma cells, after their treatment with a xenogeneic anti-lymphoma antiserum, into normal rats caused the rejection of lymphoma grafts (Hersey, 1973). Similarly,

even a lethal dose of leukemia cells pretreated with allogeneic or xenogeneic anti-leukemia antisera at high dilution (condition which favors the ADCC activity) were rejected on inoculation of mice. Furthermore, if the mice were treated with thioglycolate to increase the production of macrophages and subjected to the same serotherapy treatment, they were capable of rejecting a five times lethal dose of leukemia cells (Zighelboim et al., 1974). These results indicated that host factors (probably macrophages) are important for this type of tumor rejection and also strongly indicated that ADCC activity may be involved.

There were many attempts to use, presumably, anti-tumor antisera for immunotherapy of human cancer (for review see Rosenberg and Terry, 1977). Unfortunately most of these studies were poorly designed and the results obtained have no indication of any therapeutic effect from this type of treatment.

An improved form of passive immunotherapy was shown to consist in the administration to patients of anti-tumor antibodies mixed with toxic agents. The rationale behind this approach was that under this condition the toxic agents would augment the effect of anti-tumor antibodies. Indeed, an additive or synergistic effect of drugs and antibody against tumors has been reported (Ghose et al., 1972; Davies et al., 1974). One may visualize that a more effective treatment would be devised by the use of stable conjugates of antibodies with cytotoxic drugs subject to the drugs retaining their cytotoxicity after coupling to the antibody. Thus, diphtheria toxin has been coupled to antibodies specific to SV 40 transformed lymphoma by glutaraldehyde and the resulting conjugates displayed a certain degree of anti-tumor effects (Moolten et al., 1975). More recent approaches involve the linking of chemotherapeutic drugs with anti-tumor antibodies

(Linford et al., 1974). Thus, some beneficial effects have been shown with anti-tumor antibodies coupled to the alkylating agent, chlorambucil (Ghose et al., 1972), and to two anti-tumor drugs: p-phenylene-diamine mustard (Rowland et al., 1975) and daunomycin (Hurwitz et al., 1975).

As will be shown in Chapter IV, in view of the difficulty of producing anti-tumor antibodies in large amounts, an alternative procedure has been developed in the present study which consist of the administration of anti-fibrin antibodies coupled to daunomycin into strain 13 guinea pigs bearing the MC-D sarcoma which grows within a fibrin matrix. These antibodies were shown to be localized primarily within the tumor fibrin matrix and multiple intratumoral injections of these conjugates resulted in complete rejection of established tumors in 50% of the animals so treated.

Discussion

From the preceding cursory overview of the fast developing area of tumor immunology, it is clear that there is still no foolproof method that can be advocated for the treatment of cancer.

The non-specific stimulation of the immune responses of tumor-bearing hosts by adjuvants is currently receiving more attention than other forms of therapy because of the more consistent results obtained by this method, especially when it is combined with chemotherapy and surgery. However, there are still several important issues that must be clarified in the use of BCG immunotherapy with respect to the questions as to which substrain of BCG, dosage, time of administration would provide the more beneficial effects to the cancer patient. The basic mechanism underlying this form of immunotherapy must also be extensively studied since in addition to the beneficial effects of BCG, BCG treatment has been reported to

result in enhancement of tumor growth together with other undesirable side effects, such as hepatic granulomas and hypotension.

Immunoprophylaxis of cancer is a highly desirable method for preventing cancer. Recent progress in several fields makes this approach more realistic. The first is concerned with the viral etiology of cancer in man. It has been repeatedly reported that several types of virus are associated with certain types of human cancer, such as Epstein-Barr virus in Burkitts lymphomas (Klein, 1971). Although this issue has to be subjected to final proof, the fact that viruses could cause certain types of cancer in man is highly possible. If the viral etiology of cancer is proved, it is hoped that the success of viral vaccination for Marek's lymphomatosis in fowls could be equally well applied to man. Secondly, with the new techniques for isolation of tumor antigens, such as 3 M KCl extraction and enzymatic treatment of tumor cells, it is hoped that pure tumor antigens can be isolated in larger amounts and used for the production of tumor vaccines. In this context, one cannot overlook the observation in animal tumor systems that soluble antigen preparation may hehave either as immunogens or so-called 'blocking' factors. Obviously, the concentration, route of injection and the form of this antigen preparation, when administered to the host, may determine its effect on the immune response. The immunogenicity of soluble antigen preparations may be increased by coupling with either a highly immunogenic carrier such as sheep erythocytes or a protein molecule such as BSA. This method is well established for the hapten-protein conjugates in order to elicit immune response to the non-immunogenic hapten molecules. The recent demonstration that the coupling of a hapten to autologous lymphocytes can induce killer cells specific to the 'altered-self' antigens <u>in vitro</u> (Shearer, 1974),

which indicates that not only foreign proteins, the syngeneic lymphocytes as well can be used in the coupling strategy for enhancing the immunity to tumor antigens.

The demonstration of ADCC activity and of 'potentiating' antibodies in tumor systems deserves a re-examination of the value and nature of serotherapy for cancer. The activities of ADCC and 'potentiating' antibodies could be exerted by anti-idiotypic antibodies in the antisera, since it is now known that a minute quantity of these antibodies, which is also the condition favorable for the ADCC activity, were capable of sensitizing the T-helper as well as B-precursor cells (Eichman and Rajewsky, 1975). Methods for increasing immunogenicity of tumor antigens in several forms as described above should be tried. It is hoped that by employing these methods, highly cytotoxic antibodies to tumors could be obtained in large quantity.

Many difficulties are associated with the immunotherapy involving adoptive transfer of lymphocytes, i.e. the unavailability of effective immune lymphocytes from human donors, the large number of lymphocytes required, and the difficulty in controlling the GVH reaction.

The use of the transfer factor or 'immune' RNA are the two attractive alternatives, both of these factors not being associated with the risk of GVH. However, the effectiveness of transfer factor and 'immune' RNA in clinical immunotherapy has not been documented and the question remains if combination of transfer factor (or 'immune' RNA) with non-specific adjuvant stimulation immunotherapy would prove valuable in the control of tumors.

In summary, the prospect for eradication of tumors by immunological methods appears to be promising particularly for small tumors or when the bulk

of tumors had been removed by surgery, radiotherapy and chemotherapy. The prospect for immunoprophylaxis is equally good if the etiology as well as the nature of tumor antigens could be revealed in the foreseeable future. However, at the present time, attempts to use immunological methods in a classical, direct manner for the eradication of established tumors have met with many problems, some of which may have no ready solution. It is based on this conclusion that the model system described in this thesis was devised, which circumvents the problems mentioned earlier, by the use of indirect immunological methods for the distruction of solid tumors growing within a fibrin matrix.

SCOPE OF THE PRESENT INVESTIGATIONS

The general strategy employed in the present investigations involving the indirect, but immunologically specific manipulations which consist in essence of two approaches aimed at developing, by utilizing the unique specificity of the anti-fibrin antibodies, immunotherapeutic procedures for the destruction of a chemically-induced sarcoma growing within a fibrin matrix of strain 13 guinea pigs.

(A) The first approach involved the study of the possibility that destruction of tumor cells could be induced by the indirect cell-mediated immune mechanisms mounted not against tumor antigens, but against the antigenic determinants of antibodies combining specifically with the antigens of tissue components located within the tumor foci, e.g. the unique determinants of the fibrin matrix formed within the foci of many animal and human tumors (O'Meara, 1958; McCardle et al., 1966). It was thus envisaged that tumors growing within a supporting fibrin lattice could be destroyed by a two-step immunologically specific strategy (Figure 1), i.e. (a) injection of the tumor bearing host with heterologous anti-fibrin antibodies, and (b) administration of lymphoid cells from syngeneic animals which had been sensitized to a state of cell-mediated hypersensitivity against immunoglobulins isotypic with the anti-fibrin antibodies. It was assumed that these lymphoid cells would home onto the antibody-coated fibrin lattice and would lead, on contact with this lattice, to a local cellular inflammatory reaction which would be cytotoxic to the tumor cells.

The plausibility of this approach of tumor destruction by an indirect, cell-mediated immune process is supported by the following observations:

(a) The reported successful eradication of cutaneous malignancies in over 50 patients in whom cell-mediated hypersensitivity was induced actively or passively to simple contact-sensitizing chemicals, such as trenimon or dinitrochlorobenzene (William and Klein, 1970), provided that these substances could penetrate into the neoplastic area;

(b) The demonstration in this laboratory (Sin et al., 1971) that, in a xenogeneic in vitro system, sarcoma I cells coated with iso- or hetero-anti-serum were lysed when mixed with lymph node cells of guinea pigs which had been sensitized to the corresponding mouse or rabbit gamma globulins; this cytotoxic effect was immunologically specific and the cytotoxic capacity of lymph node cells was observed concomitantly with the appearance of delayed hypersensitivity in the sensitized animals to the corresponding antigens;

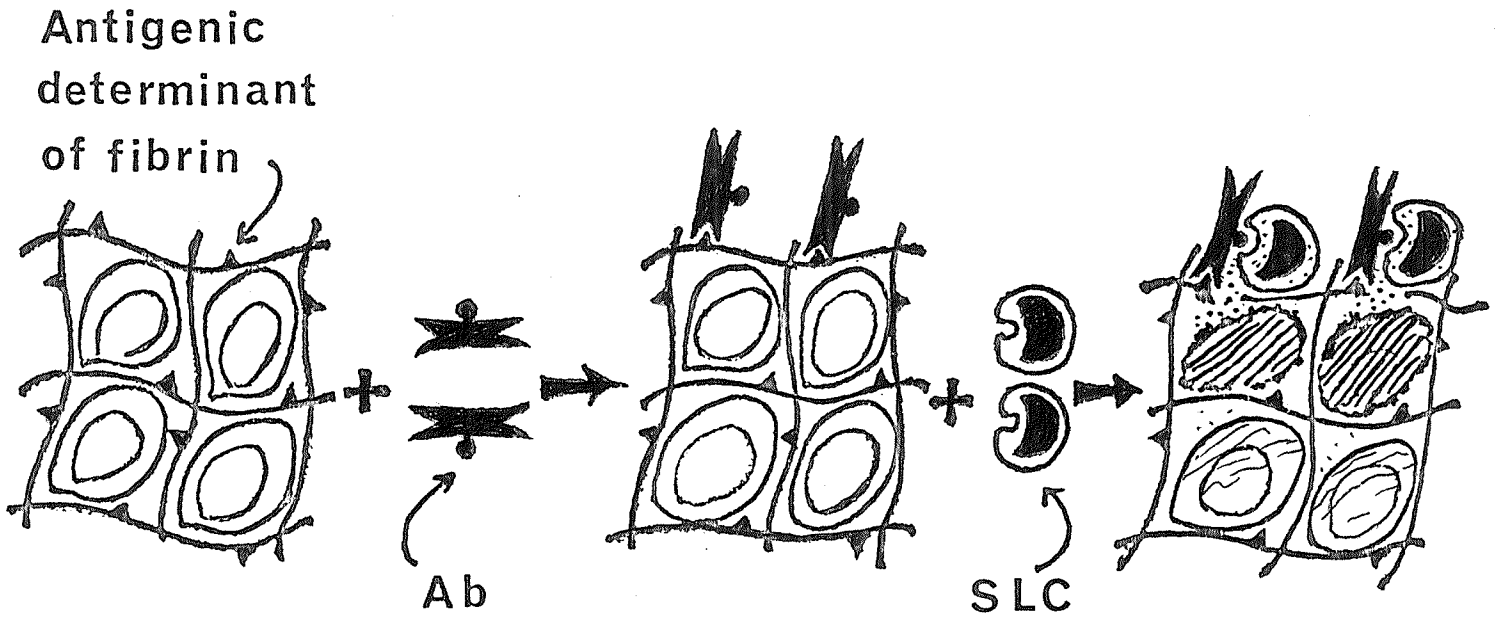
(c) The demonstration in this laboratory (Berczi et al., 1972) that the growth of a polyoma virus-induced murine tumor was significantly suppressed in vivo by lymphoid cells from mice sensitized to rabbit immunoglobulins isotypic with the specific rabbit anti-polyoma virus antibodies coating the tumor cells.

Results obtained from the present study clearly showed that the growth of the MC-D sarcoma in strain 13 guinea pigs was significantly retarded by the successive intravenous administration of (i) pure goat or rabbit antibodies to the distinct antigenic determinants of the fibrin enmeshing this tumor, and (ii) syngeneic lymphoid cells sensitized to a state of CMI to these xenogeneic immunoglobulins. These antibodies, as well as the sensitized cells, were localized predominantly within the tumor foci as demonstrated by immunohistochemical and ^{51}Cr-labelled techniques.

(B) In the second approach, the possibility of using anti-fibrin antibodies as specific carriers for cytotoxic drugs to the tumor sites was investigated. This study was based on: (i) the numerous observations that many anti-tumor drugs can be coupled to antibodies specific to tumor antigens (Rowland et al., 1975; Hurwitz et al., 1975) by means of chemical reagents, and that the drug-antibody conjugates can exert a preferential killing of tumor cells; and (ii) the results of a previous study (Lee et al., 1978a) that up to 70% of the anti-fibrin antibodies, injected into tumor-bearing guinea pigs, was localized within the fibrin matrix of tumor nodules. In this study, daunomycin was coupled to the anti-fibrin antibodies by means of glutaraldehyde, and the resulting drug-antibody conjugates were tested in vitro and in vivo for the cytotoxicity against MC-D sarcoma cells of strain 13 guinea pigs.

The results of this study showed that the drug-antibody conjugates retained both the pharmacological and antibody activity in vitro, and that multiple intratumoral injections of these conjugates induced in vivo rejection of established MC-D sarcoma in some guinea pigs and the guinea pigs so cured were resistant to a further injection of a supralethal dose of MC-D sarcoma cells.

DESTRUCTION OF TUMOR BY AN INDIRECT CELL-MEDIATED IMMUNE MECHANISM



CHAPTER II

Localization of Anti-Fibrin Antibodies in a Methylcholanthrene-Induced Sarcoma in Guinea Pigs

SUMMARY

Antibodies specific for the unique antigenic determinants of guinea pig fibrin (AGFA), which are distinct from the antigenic determinants shared by both fibrinogen and fibrin, were isolated with appropriate immunosorbents from antisera produced in rabbits and goats by immunization with fibrin. The specificity of the purified goat AGFA was demonstrated by immunoelectrophoresis and by the double antibody precipitation method using ^{131}I-labelled fibrinogen and antibodies to rabbit anti-goat IgG. The ^{131}I-labelled AGFA were injected i.v. into inbred Sewall Wright strain 13 guinea pigs carrying the transplantable methylcholanthrene induced sarcoma (MC-D) growing within a fibrin matrix and were shown to be localized in the tumor tissue at considerably higher concentration than in other organs.

INTRODUCTION

It is now generally accepted that malignant cells possess specific antigenic determinants which appear as new antigenic moieties on cells transformed by viruses (Stüch et al., 1964; Pasternak, 1967; Steeves, 1968) or chemical carcinogens (Foley, 1953; Prehn and Main, 1957; Klein et al., 1960). In spite of painstaking efforts to regulate the immune defense system of the tumor-bearing host (TBH) so as to increase the host's resistance to tumor, there is as yet no consistent method for prevention of tumor growth. Among the immunological approaches used one may cite the specific, active (Mathé et al, 1969; Nadler and Moore, 1970; Morton et al, 1970) and passive (Woodruff and Nolan, 1963; Symes et al., 1968; Moore and Gerner, 1970; Curtis, 1971; Alexander and Delorme, 1971) immunization procedures and the nonspecific procedures, such as immunization with BCG (Bast, 1974) or Corynebacterium parvum (Woodruff and Boak, 1966; Currie and Bagshawe, 1970), considered to result in the elevation of the total immune reactivity of the host. The failure to induce an effective anti-tumor immune response in TBH may be attributed to a "negative" regulatory mechanism due to suppressor cells and their soluble factors (Fujimoto et al., 1975, 1976a, 1976b; Greene et al., 1977) which underlies the immune response in TBH and/or to "blocking" of the cytotoxic cell-mediated response by circulating tumor antigens (TA) or antibody-antigen complexes (Baldwin et al., 1973; Currie, 1973; Hellström and Hellström, 1975).

In the hope of circumventing these unresolved tumor-host relationships, studies were devised in this laboratory with a view to evaluating the possibility of developing two model systems for the destruction of tumors by immunological mechanisms involving either (i) an indirect cell-mediated reaction (Berczi et al., 1972) or (ii) the use of conjugates of cytotoxic

drugs with antibodies directed to antigenic determinants of tissue constituents which are present in the tumor foci, rather than to TA per se because of the difficulty in inducing anti-TA antibodies in adequate amounts. In recent studies in this laboratory, advantage was taken of the observation that fibrin is deposited in a variety of animal and human solid tumors (O'Meara, 1958; Day et al., 1959; Spar et al., 1964) suggesting that fibrin may be an important supporting lattice for tumor growth by providing a source of protein and a barrier for free circulation of tissue fluids in the tumor foci, thereby impeding the contact of immunocompetent cells with tumor cells. The first model described in the accompanying paper (Lee et al., 1978a) consists in essence of two stages: (a) the injection of xenogeneic antibodies specific for the unique antigenic determinants of fibrin, and (b) the transfer of lymphocytes from donors syngeneic to TBH in whom a state of cell-mediated immunity to immunoglobulins isotypic with the anti-fibrin antibodies has been induced. Thus, it was visualized that (a) the anti-fibrin antibodies would become fixed to the fibrin matrix enmeshing the tumor, and (b) the sensitized lymphocytes would home onto the antibody-coated fibrin lattice and would liberate, on contact with these antibodies, factors leading locally to a cytopathic inflammatory response, which would culminate in the destruction of the tumor nodule. The second model, described in a separate paper (Lee et al., 1978b), consists in administering conjugates of goat anti-guinea pig fibrin antibodies with daunomycin, which is a potent toxic drug used in cancer chemotherapy.

In this article are described the procedures used for the preparation of pure antibodies specific for the unique antigenic determinants of guinea pig fibrin and for the in vivo localization of these antibodies in the methylcholanthrene induced sarcoma (MC-D) transplantable in strain 13 guinea pigs,

which grows within a fibrin lattice. The preliminary results of this study were communicated earlier (Fujimoto et al., 1973), but the present paper was not submitted for publication in anticipation of completing the two studies described in the succeeding papers (Lee et al., 1978a, 1978b), which demonstrate the usefulness of anti-fibrin antibodies for developing immunotherapeutic strategies for the destruction of tumors growing within a fibrin mesh. More recently, evidence has been presented (Schlager and Dray, 1975) that the subcutaneous injection of antibodies to the fibrin fragment E, after the intradermal implantation of a uniformly lethal dose of line-10 tumor cells in strain 2 guinea pigs, resulted in complete regression of the tumor and the animals so treated became resistant to subsequent tumor challenge.

MATERIALS AND METHODS

Tumor. The MC-D sarcoma, originally donated by Dr. H.F. Oettgen of the Sloan-Kettering Institute in New York, was used in this study. The tumor was maintained by subcutaneous implantation into adult, male and female inbred Sewall Wright strain 13 guinea pigs (Weizmann Institute for Science, Rehovot, Israel). The presence of fibrin in the tumor was demonstrated histologically by staining with phosphotungstic acid-hematoxylin.

Guinea Pig Fibrinogen and Fibrin. Guinea pig fibrinogen and fibrin were prepared according to the method of Day et al. (1959). Guinea pig fibrinogen was precipitated from fresh plasma (with 0.38% sodium citrate) by two volumes of a NaCl solution (277.5 gm NaCl/l). The precipitate was dissolved in distilled water and was reprecipitated with the NaCl solution. The fibrinogen was finally dissolved in phosphate buffered saline (PBS), pH 7.2, and was dialyzed extensively against the same buffer.

For conversion of fibrinogen to fibrin the dialyzed fibrinogen solution was diluted to 1 liter with PBS. To this solution was added 5 ml of 0.2 M

$CaCl_2$ solution and 1,000 NIH units of bovine thrombin (Sigma Chemical Co., St. Louis, Mo.). The fibrin was wound around a glass rod as it was being formed and was dispersed into a suspension of fine particles in PBS with a glass homogenizer. These particles were washed several times with PBS until the optical density of the supernatant was less than 0.01 at 280 mμ and the preparation was stored at -20°C.

Production of Anti-Guinea Pig Fibrin Serum. For the production of anti-guinea fibrin serum, goats were rendered tolerant to fibrinogen by injection of 10 mg N aggregate-free guinea pig fibrinogen prepared by centrifugation at 100,000 g for 1 hour. The goats were then immunized by four intramuscular injections of 2 mg N purified guinea pig fibrin suspension of PBS, emulsified with the same volume of Freund's complete adjuvant (FCA), at 2-week intervals. The goats were bled and the antisera were pooled, heated at 56°C for 30 minutes to inactivate complement and stored at -20°C. Anti-guinea pig fibrin serum was also produced in rabbits by the same procedure.

Isolation of Specific Anti-Guinea Pig Fibrin Antibodies (AGFA). Before isolation of specific antibodies to guinea pig fibrin, the pooled goat antiserum was first absorbed (i) with normal guinea pig serum to remove antibodies to constituents of the serum which had been occluded in the fibrin used for immunization of the goats, and (ii) with fibrinogen to remove antibodies directed against antigenic determinants shared by both fibrin and fibrinogen. The completeness of these absorption steps was further confirmed by the standard precipitin test and by immunoelectrophoresis.

For the final isolation of antibodies only to the unique antigenic determinants of fibrin, appropriate immunosorbents were used. First, the antibodies were absorbed onto fibrin. For this purpose the suspension of guinea pig fibrin was washed once with glycine-HCl buffer, pH 2.8, and then with PBS several times. The washed purified fibrin (20 mg N) was mixed with 100 ml of the above antibody preparation. The suspension of fibrin-antibody complexes was

incubated for 1 hour at room temperature and then at 4°C overnight with continuous gentle shaking. It was then washed with PBS until the optical density of the supernatant was less than 0.01 at 280 mμ. Anti-fibrin antibodies were then eluted off the fibrin immunosorbent with glycine-HCl buffer, pH 2.8, at 4°C. Immediately after elution, the antibody solution was neutralized by adding NaOH dropwisely and dialyzed against PBS, pH 7.2. Furthermore, to exclude the possibility that the purified antibodies might have still contained antibodies capable of crossreacting with fibrinogen, the preparation was passed again through an immunosorbent column consisting of purified guinea pig fibrinogen (15 mg) coupled to CNBr activated Sepharose 4B (20 ml) (Pharmacia Fine Chemicals, Uppsala, Sweden), according to the method of Axen et al. (1967).

In general, the nitrogen content of protein samples was established by the method of Koch and McMeekin (1924).

Preparation of Rabbit Anti-Guinea Pig Fibrinogen Antibodies. For production of anti-guinea pig fibrinogen antibodies, rabbits were given at one week intervals two series of subcutaneous injections, consisting of 1 mg N of purified guinea pig fibrinogen emulsified in 1 ml of FCA; the fibrinogen had been isolated as described previously. The rabbits were bled one week and two weeks after the second injection. The antisera were pooled, heated at 56°C for 30 minutes. The IgG fraction of this pool was isolated by chromatography on a Sephadex G-200 column (2.6 x 100 cm), which had been equilibrated with 0.005 M borate buffered saline, pH 8.0, and was concentrated by negative pressure dialysis after overnight dialysis against PBS.

^{131}I-labelled AGFA. One mg of AGFA was reacted with 500 μCi of ^{131}I at 4°C using the chloramine T method of McConahey and Dixon (1966) with slight modifications. After labelling, the antibodies were passed through a Sephadex G-25 column to remove any free radioactive iodine.

To eliminate any AGFA which may have been inactivated during radio-iodination, the labelled antibodies were repurified by treating them with the fibrin immunosorbent and the ^{131}I-labelled antibodies were eluted off with glycine-HCl buffer, pH 2.8. After neutralization with 1.0 N NaOH, the ^{131}I-labelled antibody preparation was extensively dialyzed against PBS before use.

In Vitro Binding of AGFA with Fibrin and Tumor Tissue Pellet (TTP). Constant amounts (0.1 µg N) of ^{131}I-labelled AGFA were added to a series of test tubes containing serially diluted purified guinea pig fibrin or a suspension of TTP, prepared by homogenization of the tumor and extensive washing with PBS until the supernatant had an O.D. less than 0.02 at 280 mµ. The TTP was used either when freshly prepared or after lyophilization. For control, ^{131}I-labelled normal goat IgG was used instead of AGFA in all tests. After 24 hour incubation on a shaker at 4°C, the residue in each test tube was washed five times with cold PBS and the bound radioactivity was determined in a well type γ-ray counter (Nuclear Chicago).

Double Precipitation Test for the Specificity of AGFA. To ascertain that the AGFA preparation was indeed free of antibodies to fibrinogen, which may have been present in too low a concentration for detection by the standard precipitin test, a double antibody precipitation procedure was resorted to. For this purpose constant amounts of ^{131}I-labelled guinea pig fibrinogen (0.1 µg N) were added to three sets of test tubes containing serial dilutions of (a) rabbit anti-guinea pig fibrinogen antibodies (positive control); (b) the goat AGFA preparation; (c) normal goat IgG (negative control). After thorough mixing, the test tubes were maintained at 4°C for 30 minutes and subsequently optimal amounts of sheep anti-rabbit IgG serum were added to the first set of tubes and of rabbit anti-goat IgG serum were added to the other two sets; the optimal amounts of these antisera (determined previously by the precipitin test), were

chosen so as to lead to complete precipitation of the rabbit and goat IgG present in the corresponding test tubes. After mixing, the test tubes were kept on a shaker at $4^{o}C$ for 24 hours. The precipitates were centrifuged down and washed 5 times with cold PBS and finally the residual radioactivity was determined.

Preparation of Fluorescent Antibodies. In vivo localization of goat anti-fibrin antibody within the tumor tissue was demonstrated by the indirect fluorescent antibody technique. The IgG fraction of rabbit anti-goat IgG serum, isolated by DEAE-cellulose chromatography with 0.01 M phosphate buffer, pH 8.0, was labelled with fluorescein isothiocyanate (FITC) using the method of Wood et al. (1965) with slight modifications. FITC was added to the IgG solution (1% w/v) which had been adjusted to pH 9.1 with 0.5 M carbonate buffer at $4^{o}C$ and the mixture was maintained with stirring at $4^{o}C$ for 6 hours. The unconjugated FITC was removed by gel filtration through a Sephadex G-25 column and the FITC-antibody conjugates were fractionated by DEAE-cellulose chromatography using stepwise elution with 0.01 M, 0.05 M, 0.1 M and 0.1 M phosphate buffer, pH 8.0; the last buffer contined 2 M NaCl. The fluorescein to protein molar ratio of the conjugated antibody was determined spectrophotometrically. The fraction of FITC-antibody conjugates possessing a fluorescein to protein ratio of 1.0 was used for the experiments.

Fluorescent Staining. The fresh tumor tissue was cut into blocks of about 125 mm^3 which were immediately frozen in hexane cooled to $-78^{o}C$ with the aid of a dry ice-acetone bath; after 10 minutes the frozen tissues were transferred into tightly capped vials and stored in a freezer at $-70^{o}C$.

Several 4 μ thick serial sections were sliced off the frozen tissue in a cryostat at $-20^{o}C$, dried on microscope slides at room temperature, fixed for 10 minutes in cold acetone and rinsed in cold PBS, pH 7.2, for 5 minutes. The

tissue sections were then treated with the purified goat AGFA preparation (concentration: 1 mg/ml) at room temperature in a humid chamber for 45 minutes. The slides were washed at least 3 times with cold PBS and then treated with FITC-labelled rabbit anti-goat IgG (concentration: 1 mg/ml) for 45 minutes at room temperature. The slides were finally rinsed again with cold PBS, mounted in buffered glycerol and examined under a Zeiss fluorescence microscope.

Two control slides were also included in this test, i.e., (i) without the addition of the goat AGFA, and (ii) substitution of normal goat IgG for the goat AGFA in the first step of this "sandwich" technique.

Immunoelectrophoresis. Immunoelectrophoresis was carried out according to the micromethod of Scheidegger (1955) in 0.85% agar in barbital buffer, pH 8.2, ionic strength = 0.05.

In Vivo Localization of ^{131}I-labelled AGFA in the MC-D Sarcoma. Since the immunotherapeutic protocols underlying the proposed models require the in vivo fixation of AGFA on the fibrin lattice in the tumor tissues, localization of these antibodies when administered in vivo was examined. For this purpose the MC-D tumor-bearing guinea pigs were divided into three groups, consisting of 3 guinea pigs per group. One group of guinea pigs served as controls and were injected i.v. with 20 µg N of ^{131}I-labelled normal goat IgG. The remaining two groups of guinea pigs were injected with the same amount of either ^{131}I-labelled rabbit AGFA or ^{131}I-labelled goat AGFA. Twenty-four to 48 hours after injection of radioactive antibodies, or normal IgG, two ml of blood was collected from each animal by heart puncture and each animal was then given 500 units of heparin. The animals were then killed gradually by extensive perfusion with 500 ml of PBS containing 500 units of heparin by cannulation of vena cava caudalis.

Whole tumor tissue, lungs, heart, liver, spleen and kidneys were excised from the perfused animals. Each tissue was weighed and the radioactivity

FIGURE 2 Immunoelectrophoretic analysis of the goat AGFA preparation.

Slide A and B demonstrate the specificity of goat AGFA which did not react with GP plasma or fibrinogen.

Slide C shows that goat AGFA belongs to the IgG class of antibody.

(GP refers to guinea pig)

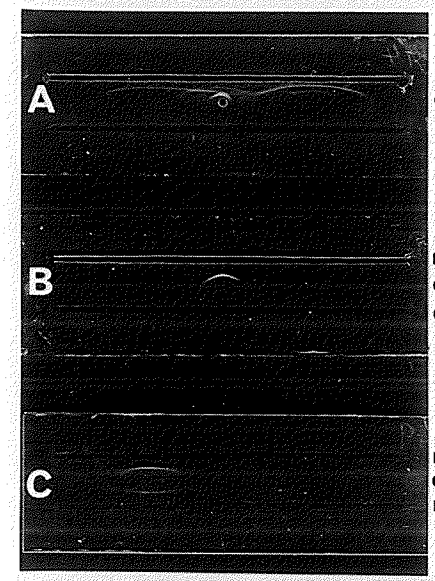
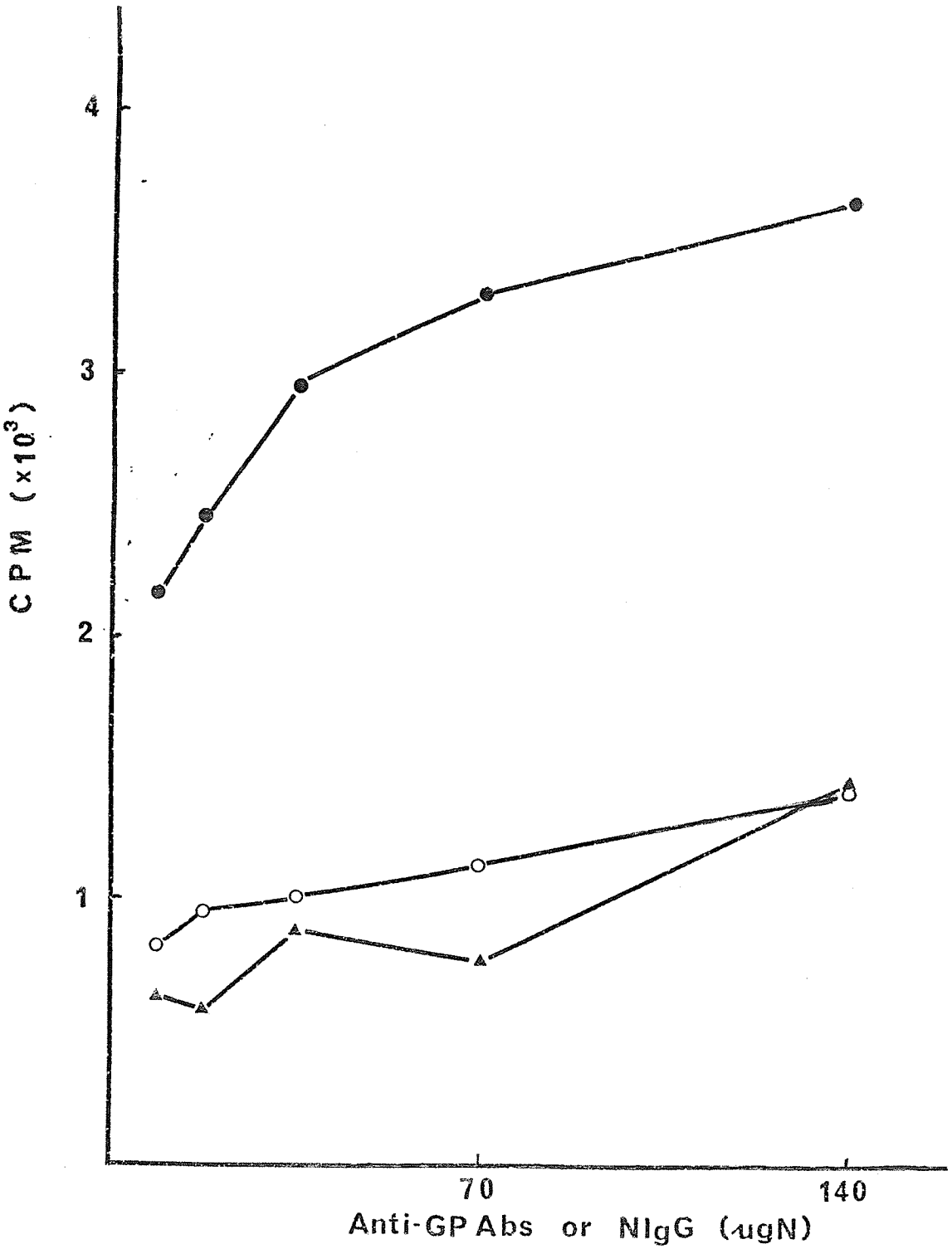

A
Rabbit Anti-GP Plasma
GP Plasma
Goat Anti-GP Fibrin (Absorbed)
B
Rabbit Anti-GP Plasma
GP Fibrinogen
Goat Anti-GP Fibrin (Absorbed)
C
Rabbit Anti-Goat Serum
Goat AGFA
Rabbit Anti-Goat IgG

FIGURE 3 Double antibody precipitation of ^{131}I-labelled guinea pig fibrinogen. The three different systems consisted of successive additions to ^{131}I-labelled fibrinogen of (a) rabbit anti-guinea pig fibrinogen and sheep anti-rabbit IgG serum (●——●), (b) goat AGFA and rabbit anti-goat IgG serum (o——o) and (c) normal goat IgG and rabbit anti-goat IgG serum (▲——▲).


4
3
2
1
CPM (x10³)
70
140
Anti-GP Abs or NIgG (ugN)

per gram of tissue and per ml of blood was determined in a well-type γ-ray counter (Nuclear Chicago). The total volume of blood was estimated as 8% of the weight of the animal. The percentage of the radioactive antibodies localized in each tissue was calculated using the expression:

$$\frac{\text{CPM/g x organ weight - background (CPM)}}{\text{Total CPM injected - background (CPM)}} \times 100$$

RESULTS

Specificity of AGFA. As would be expected, the pooled goat anti-fibrin serum reacted not only with fibrin, but also with fibrinogen and other serum components of guinea pigs. However, after absorption of this antiserum with fibrinogen and normal guinea pig serum, the antiserum was depleted of detectable antibodies reacting with any of the components of guinea pig plasma or fibrinogen, as shown in slides A and B of Fig. 2. The specific anti-fibrin antibody had the electrophoretic mobility of IgG as shown in slide C of Fig. 2. From these results, it may be concluded that the antibody isolated with the fibrin immunosorbent was directed against antigenic determinants of fibrin not shared by the other components of guinea pig plasma.

The exclusive specificity of AGFA in their reaction with the unique determinants of fibrin was further demonstrated by the more sensitive double precipitation method using ^{131}I-labelled fibrinogen as antigen. As shown in Fig. 3, and as would be expected, the rabbit anti-guinea pig fibrinogen antibodies bound labelled fibrinogen, whereas the goat AGFA, which was isolated with the aid of fibrin used as an immunosorbent and further depleted of any antibodies to fibrinogen by passage through the fibrinogen-Sepharose immunosorbent, showed no significant binding activity toward fibrinogen. This and the previous

FIGURE 4 Binding curves for (a) ^{131}I-labelled goat AGFA with guinea pig fibrin (●——●) and the MC-D sarcoma pellet (TTP) (▲——▲), and (b) ^{131}I-labelled goat normal IgG with guinea pig fibrin (o——o) and TTP (Δ——Δ).

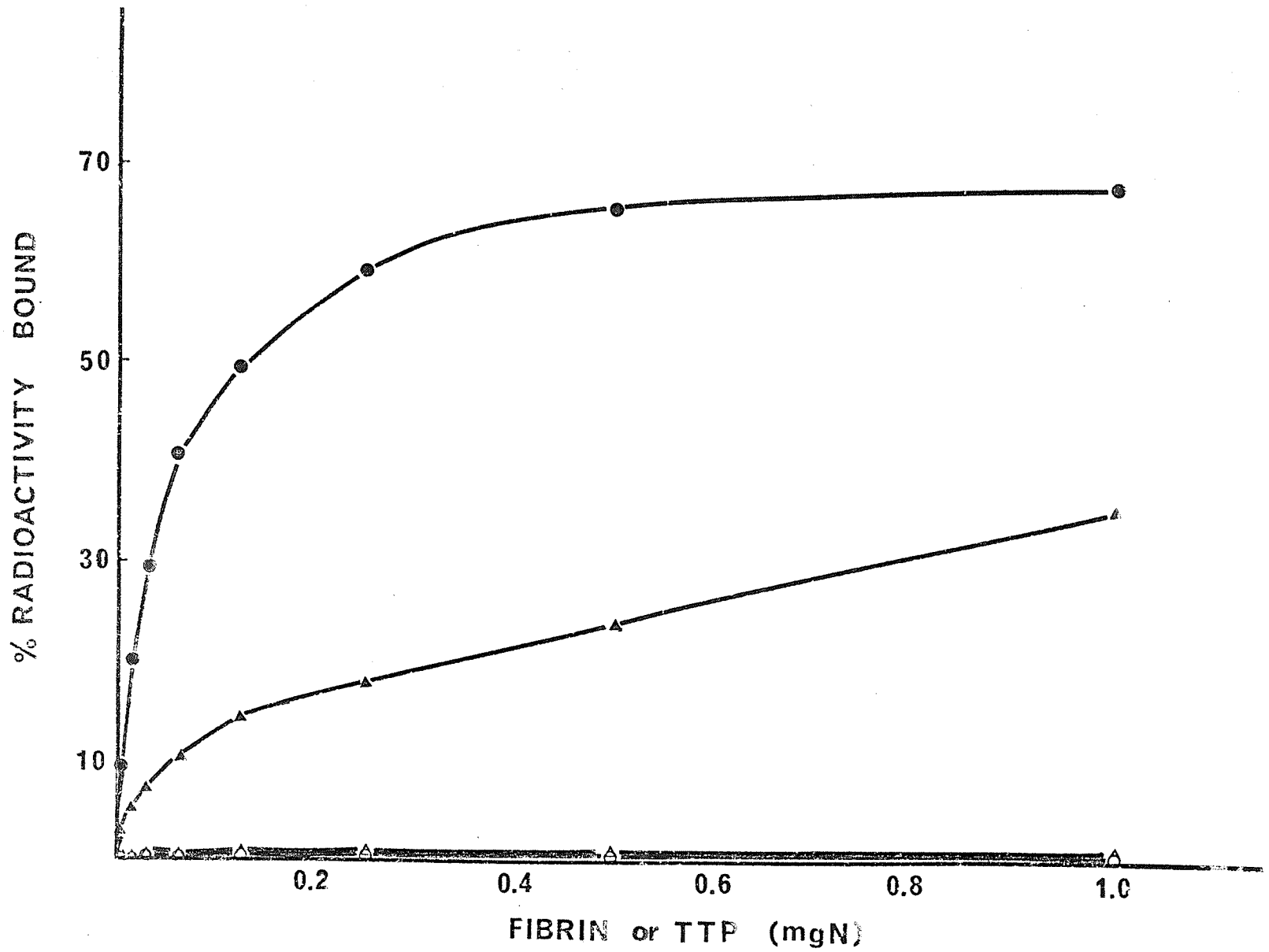
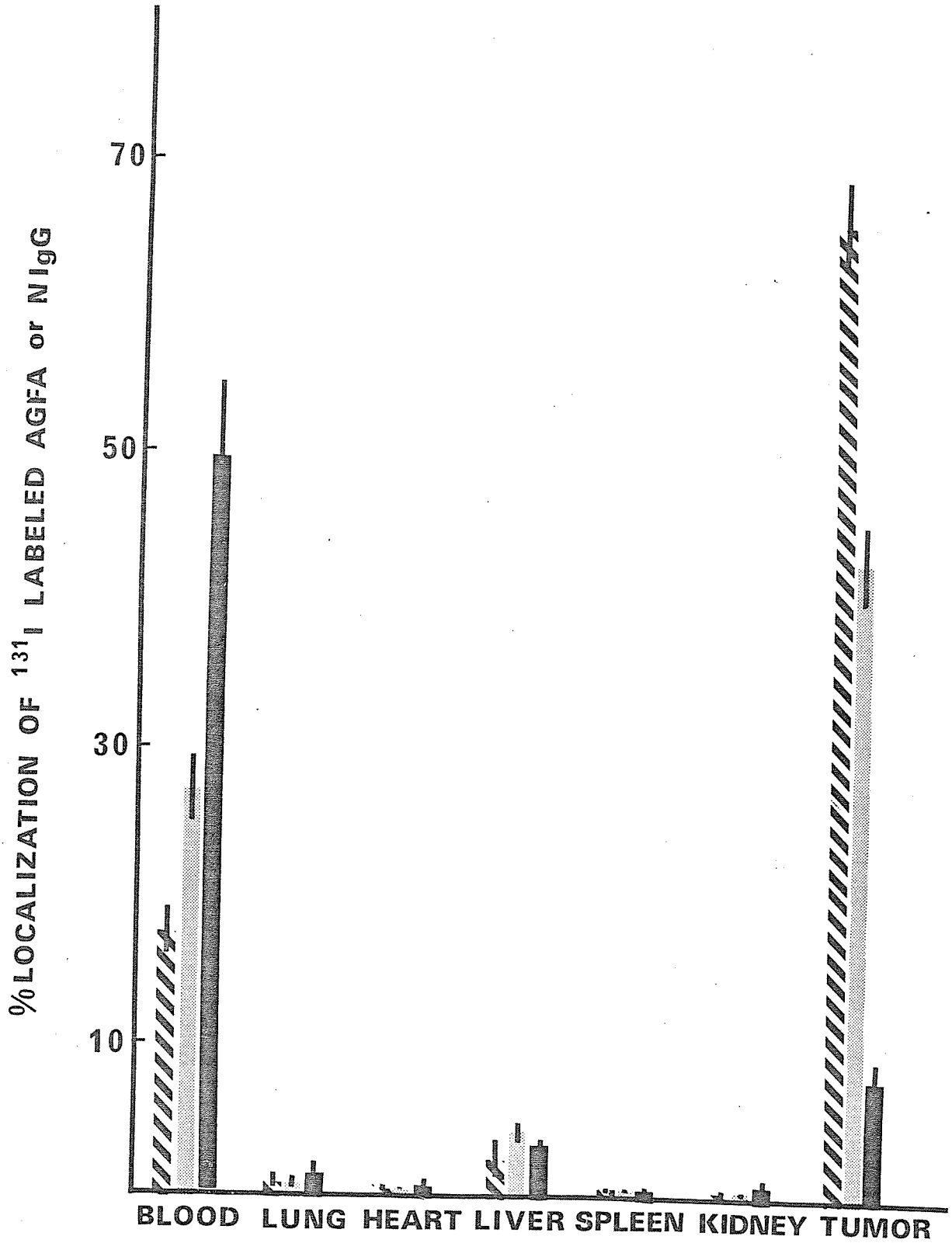

% RADIOACTIVITY BOUND
70
50
30
10
0.2
0.4
0.6
0.8
1.0
FIBRIN or TTP (mgN)

FIGURE 5 Distribution of ^{131}I-labelled rabbit (striped bars) and goat (shaded bars) AGFA and of ^{131}I-labelled goat normal (full bars) IgG in the MC-D sarcoma and other tissues 48 hours after intravenous injection of 20 µg N of these radioactive preparations.

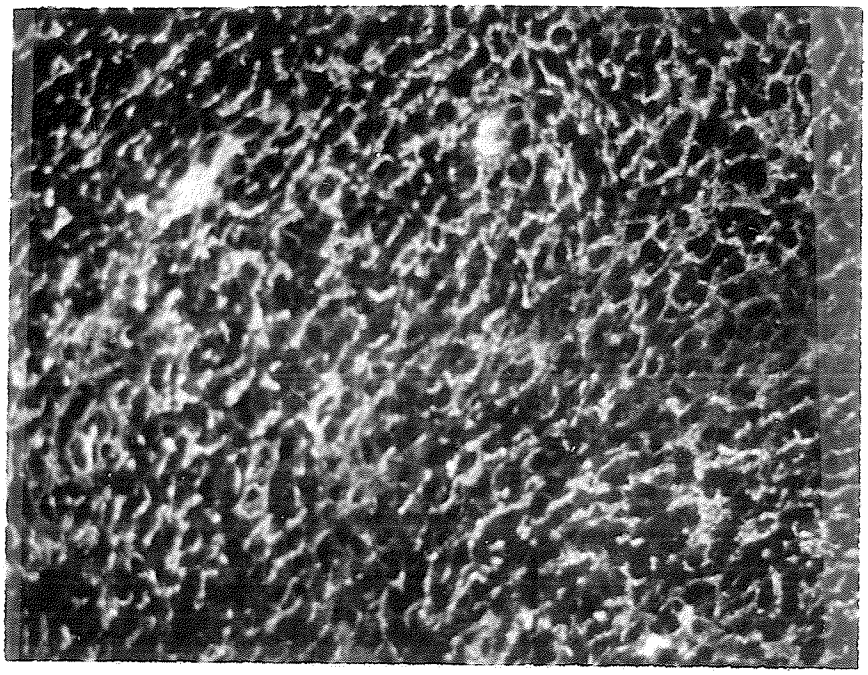

% LOCALIZATION OF ^{131}I LABELED AGFA or NIgG
70
50
30
10
BLOOD
LUNG
HEART
LIVER
SPLEEN
KIDNEY
TUMOR

experiments clearly demonstrate the immunochemical purity of the anti-fibrin antibodies prepared in this study, i.e., these antibodies reacted uniquely with the specific antigenic determinants of fibrin.

In Vitro Binding of AGFA to Fibrin. In Fig. 4 are shown the binding curves of the purified ^{131}I-labelled AGFA to fibrin and to the tumor tissue pellet (TTP). It is evident that the extent of binding of these antibodies to TTP was less than that to fibrin. Moreover, while the binding curve with fibrin reached a plateau at 70% of the radioactivity of the AGFA preparation, no plateau was reached with the TTP preparation at the same concentrations, which was not unexpected since TTP contained materials other than fibrin. On the other hand ^{131}I-labelled normal goat IgG showed no binding activity either to fibrin or to TTP, indicating thus that binding of the AGFA to fibrin or to TTP was immunologically specific.

In Vivo Localization of AGFA in Tumor-Bearing Guinea Pigs. The distribution of ^{131}I-labelled AGFA among different tissues in the sarcoma bearing guinea pigs, 48 hours after administration of 20 µg N antibody, is shown in Fig. 5. Radioiodinated normal goat IgG was used as control in lieu of AGFA. The mean tumor weight of these guinea pigs was 30 ± 10 gm. The localization data are reported here as percentages of the injected radioactivity dose found in the different tissues. From this graph, it can be clearly seen that AGFA was localized at a significantly higher level in the tumor tissue than in other organs and that normal goat IgG was localized only to a small extent. Thus, these results indicate that the localization of AGFA in tumor tissue was specific and substantial (≈70% of injected dose). Moreover, in a number of experiments it was shown that this amount of AGFA was localized in the tumor nodules when these pure antibody preparations were injected in limiting amounts, i.e. 10 or 20 µg N, respectively. Obviously, injection of AGFA in higher amounts resulted in the localization of

FIGURE 6 Immunofluorescence of section of MC-D sarcoma (x 125) treated successively with specific goat AGFA and FITC-labelled rabbit anti-goat IgG antibodies. The fibrin lattice is clearly visible within the tumor tissues.



a relatively lower fraction of these antibodies in the tumor foci. The difference, as observed in this graph, between rabbit and goat AGFA in their localization in the tumor tissue may probably reflect the different affinity of these two AGFA preparations.

Immunofluorescent Staining of Fibrin in Tumor Tissue. The localization of FITC-labelled rabbit anti-goat IgG antibodies illustrated in Fig. 6 demonstrates the presence of a typical linear pattern of fibrin within the sarcoma tissue. On the other hand, treatment of an identical tumor section only with FITC-labelled rabbit anti-goat IgG antibodies (i.e. without prior treatment with goat AGFA) was completely devoid of fluorescence (figure not shown).

DISCUSSION

As outlined in the Introduction, the hypothesis underlying the two proposed strategies for the destruction of tumor nodules is based on two mandatory conditions, i.e. (i) the fibrin matrix must exist in the tumor nodule and (ii) the administration of xenogeneic anti-fibrin antibodies into tumor-bearing animals must result in the predominant localization of these antibodies within the tumor site by fixation to the enmeshing fibrin matrix. The results of the *in vitro* experiments involving the binding of ^{131}I-labelled and of fluorescein-tagged AGFA to the sarcoma tissue, as well as the *in vivo* localization of AGFA, clearly demonstrate that the MC-D sarcoma provides an appropriate model fulfilling these requirements.

The success of this investigation is a reflection of the efforts made to remove any traces of antibodies that have affinity for the guinea pig fibrinogen from the AGFA preparation. The results of immunoelectrophoresis and double antibody precipitation tests presented here convincingly demonstrate that the crossreacting antibodies to fibrinogen had been eliminated from the

AGFA preparation. Since fibrin shares the major antigenic determinants of its precursor, i.e. the ubiquitous fibrinogen, most of the antibodies raised against either of these antigenic moieties will react with the other. The failure of earLier investigators (Day et al., 1959; Spar et al., 1964) to achieve high in vivo localization of anti-fibrin antibodies within a fibrin lattice may, therefore, be attributed to the presence of antibodies to fibrinogen in their preparation of anti-fibrin antibody. In an experiment not reported here, evidence has been obtained to show that the half-life of goat IgG in tumor-bearing guinea pigs was about 4 1/2 days. Therefore, had the crossreacting anti-fibrinogen antibodies not been removed from the anti-fibrin antibody preparation, most of the injected antibodies would have reacted with the fibrinogen in circulation leading to soluble antigen-antibody complexes in antigen excess, which may have remained in circulation for extended periods or which may have become deposited in various tissues nonspecifically.

The fact that only 70% of the ^{131}I-labelled goat AGFA preparation was bound to fibrin is interpreted as indicating that some of the antibodies had been denatured during the iodination procedure and probably also on elution from the fibrin immunosorbent with glycine-HCl buffer. It has been shown in another study in this laboratory (Kisil et al., 1974) that denaturation of antibodies occurs at low concentration and particularly readily at the low pH used for their elution from the immunosorbent and that this may be minimized by adding proteins such as serum albumin to act as stabilizers. Since, for testing in vivo the validity of the hypothesis underlying this study, it will not be necessary to label AGFA with a radioactive marker prior to injection into MC-D sarcoma bearing guinea pigs, the extent of denaturation of these antibodies, if any, will be obviously less than 30%.

The important conclusion which can be derived from this study is that it is possible to prepare and isolate antibodies specific to the unique

antigenic determinants of fibrin which are distinct from the antigenic determinants shared by fibrin and fibrinogen. A plausible explanation for the antigenic uniqueness of fibrin stems from a consideration of the chemical and physical differences between it and fibrinogen. Thus, during conversion of fibrinogen to fibrin in the presence of thrombin, the fibrinopeptides A and B are split off and the resulting polymer, i.e., fibrin, obviously differs in its primary and tertiary structures from fibrinogen; this is also reflected in the difference in their solubilities, the fibrin being an insoluble matrix of long chain polymers.

The succeeding manuscripts describe the utilization of this AGFA preparation for testing the strategies proposed in the Introduction for the destruction of the MC-D tumor in vivo by an indirect, cell-mediated and immunologically specific reaction (Lee et al., 1978a) or by conjugates of AGFA with daunomycin (Lee et al., 1978b). Moreover, it is obvious that, in addition to their therapeutic potential, anti-fibrin antibodies may prove to be valuable diagnostic tools for localization of tumor foci associated with fibrin.

ACKNOWLEDGMENT: This study was supported by a grant from the National Institutes of Health, Bethesda, Md. (CA-13192).

CHAPTER III

Indirect Cell-Mediated Immune Destruction of the Guinea Pig MC-D Sarcoma

SUMMARY

The transplantable methylcholanthrene-induced sarcoma (MC-D) in strain 13 guinea pigs was used to test the hypothesis that tumor cells growing within a fibrin matrix could be destroyed by an immunologically specific strategy involving an indirect cell-mediated immune reaction. The experimental design consisted of two steps: (i) in vivo fixation of anti-guinea pig fibrin antibodies (AGFA) on the fibrin matrix enmeshing the tumor cells and (ii) the reaction between AGFA fixed to the fibrin matrix and lymphoid cells from syngeneic animals which had been sensitized to xenogeneic immunoglobulins isotypic with AGFA. Indeed, using ^{51}Cr-labelled lymphoid cells, evidence was obtained for the localization of these sensitized lymphoid cells within the fibrin lattice when the latter was coated by AGFA. Moreover, significant tumor growth suppression ($P<0.01$) was achieved in guinea pigs which had received intravenously rabbit or goat AGFA and subcutaneously lymphoid cells from syngeneic guinea pigs sensitized to a state of cell-mediated immunity to rabbit or goat IgG. On the other hand, the administration of the antibodies or of the sensitized cells alone did not affect the growth of the tumor. Preliminary results suggest that peritoneal exudate cells may play an important role for the success of this strategy for tumor cell destruction.

INTRODUCTION

While it is now generally recognized that malignant cells possess specific tumor antigens (TA), painstaking attempts in many laboratories to isolate TA and to produce anti-TA antibodies in significant quantities for further immunochemical manipulations have met with little success. In a number of investigations (Spar et al., 1964; Day, 1965; McCardle et al., 1966) deposition of fibrin in a wide variety of animal and human tumors has been noted suggesting that fibrin was an important supporting lattice for tumor growth. On the basis of these observations has been proposed the working hypothesis underlying this investigation that tumor cells could be destroyed by an indirect cell-mediated immune response involving "killer" lymphocytes from syngeneic donors sensitized not against the specific tumor antigens, but against (i) antigens normally present on or artificially coupled to anti-tumor antibodies capable of coating the tumor cells, or (ii) antigens of xenogeneic antibodies directed against the unique antigenic determinants of the fibrin of the tumor-bearing host.

In previous studies (Fujimoto et al., 1973; Lee et al., 1978) pure rabbit and goat IgG antibodies specific for the antigenic determinants of guinea pig fibrin (i.e. freed of antibodies reacting with fibrinogen and other plasma components) were isolated with the aid of appropriate immunosorbents. On injection of these anti-guinea pig fibrin antibodies (AGFA) into strain 13 guinea pigs (Gp 13) bearing the transplantable methylcholanthrene induced sarcoma (MC-D), it was shown that these antibodies were localized in tumor tissue at a concentration considerably higher than in other organs. The experiments reported here represent the extension of this study designed to establish if the MC-D tumor, growing within a fibrin matrix, could be destroyed

in vivo with cells of syngeneic guinea pigs sensitized to a state of cell-mediated immunity (CMI) against immunoglobulins isotypic with the heterologous AGFA, i.e. to rabbit or goat IgG. Clearly, this experimental model is based on the assumption that (i) cells sensitized to immunoglobulins isotypic with AGFA would home onto the fibrin matrix which would have been coated in vivo with AGFA, and (ii) interaction between these sensitized cells and AGFA within the tumor site would lead to a local inflammatory reaction resulting in the release of cytotoxic and chemotactic factors which by themselves or in conjunction with other cells of the host, that would be recruited to the tumor site, would annihilate the malignant cells within the fibrin matrix.

MATERIALS AND METHODS

Animals, Tumor and AGFA. Details relating to these items were given in the preceding paper (Lee et al., 1978). The animals used in this experiment were adult Gp 13 of both sexes weighing 400-500 gm. Rabbit and goat AGFA were used in this study. The lethal dose of MC-D cells in Gp 13 was 10^5; in this study the animals received 10^6 MC-D cells and died 50-60 days after tumor implantation.

Elicitation of CMI in Guinea Pigs to Xenogeneic IgG. In order to induce sensitized cells in Gp 13 to xenogeneic IgG, 80 µg of rabbit or goat IgG emulsified in Freund's complete adjuvant (FCA) was injected into the footpads of guinea pigs. The IgGs were isolated from the corresponding normal sera by column chromatography on DEAE-cellulose using phosphate buffers, pH 8.0, (0.01 M for rabbit IgG and 0.02 M for goat IgG) for elution. The following in vivo and in vitro tests were used to establish the development of sensitized lymphoid cells to xenogeneic IgG.

Skin Tests. To ascertain if sensitized cells had been induced in immunized animals, they were challenged intradermally 2 weeks after sensitization with several doses of the immunizing antigen (10, 20 and 30 µg in 0.1 ml) and, 48 hours later, the diameters of the skin reactions were measured with a vernier caliper. Only skin reactions with diameters larger than 10 mm were considered positive.

Passive Transfer of Delayed Hypersensitivity. The capability of lymphoid cells from immune animals to transfer CMI to normal syngeneic animals was tested. Regional lymph nodes (popliteal, inguinal and axillary), spleens, peritoneal exudate cells and peripheral lymphocytes were collected from animals sensitized 14 days earlier.

The lymph nodes and spleens were cut into small pieces. The cells were then separated from the fibrous tissue by gentle pressing with a loosely fitted glass grinder, were passed through a platinum sieve (mesh #150), washed with chilled Hanks' solution, and the number of viable cells was determined by the trypan blue dye exclusion method. For harvesting peritoneal exudate cells, the animals received intraperitoneally (i.p.) 30 ml of paraffin oil 11 days after sensitization. Three days later, these animals were bled to death by heart puncture with a heparinized syringe and were then given 50 ml of cold Hanks' solution i.p. The middle of the abdomens were opened and the peritoneal fluids were carefully pipetted and transferred to a separatory funnel to separate the paraffin oil from the cell rich suspension which was washed 3 times with Hanks' solution and resuspended in Eagle's minimal essential medium (MEM; Grand Island Biological Co., Grand Island, N.Y.). For collection of peripheral lymphocytes, the blood of these animals was mixed with one-fifth of its volume of 6 % dextran (M.W. 250,000; Pharmacia, Uppsala, Sweden) and the mixture was allowed to stand for 60-90 minutes. The upper portion of

the cell suspension was transferred into glass culture bottles which were incubated for 90 minutes at 37°C in a CO_2-incubator by turning the bottle onto its other side after the first 45 minutes. The nonadherent blood lymphocytes were then removed from the culture bottles, washed 3 times with chilled Hanks' solution and finally resuspended in MEM medium.

Different doses of the sensitized cells (10^5, 10^6, 10^7, 10^8, 10^9) were transferred intravenously into normal Gp 13 and 72 hours later the animals were challenged by intradermal injection of 20 µg of rabbit or goat IgG and the skin reactions were read as described above.

Antigen-Induced Stimulation of ^{3}H-Thymidine Uptake by Sensitized Lymphoid Cells. The in vitro stimulation of ^{3}H-thymidine incorporation into the DNA of lymphoid cells of sensitized Gp 13 in presence of the immunizing antigen was used to establish the degree of CMI. For this purpose, 2 x 10^6 lymphoid cells (from axillary, inguinal and popliteal lymph nodes) of guinea pig immune to goat IgG in 0.5 ml RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.), containing 10% fetal calf serum (FCS), were incubated in culture tubes with 0.5 ml of goat IgG at several concentrations (1, 10 or 100 µg/ml) at 37°C in a CO_2-incubator. After 72 hours, 1 µCi of ^{3}H-thymidine (6.7 Ci/mmole, New England Nuclear, Boston) was added to each culture tube and incubation was continued for 8 more hours. To remove free ^{3}H-thymidine, the cells were washed 3 times with cold phosphate buffered saline (PBS), pH 7.2. The DNA and proteins were precipitated with 5% trichloroacetic acid (TCA) after the addition of one drop of normal guinea pig serum (this served as carrier proteins) and the precipitate was washed twice with 5% TCA and once with cold ethanol, and was then dissolved in 1 ml of Protosol (New England Nuclear, Boston) by incubating in a waterbath for 2 hours at 50°C. The solution was transferred to scintillation vials with 10 ml of scintillator

cocktail (6.0 gm of PPO and 0.2 gm of POPOP in 1 liter of toluene) and the radioactivity of each vial was determined in a Mark II Liquid Scintillation Counter (Nuclear Chicago) at 4°C. The experiment was performed in triplicate and the results were corrected for efficiency and quenching. The results of these three tests were used to establish both the optimal time for harvesting sensitized cells from immune animals and the suitable doses of sensitized cells for use in subsequent experiments.

Localization of ^{51}Cr-labelled Sensitized Lymphoid Cells in Tumor-Bearing Gp 13.

In order to test if indeed lymphoid cells of Gp 13, which had been sensitized to goat IgG, would 'home' to the MC-D sarcoma in tumor-bearing animals (after these animals had received intravenously the goat AGFA), these cells were labelled with ^{51}Cr. The localization of these cells, after adoptive transfer, was established in the different organs of the tumor-bearing animals. For this purpose, lymphoid cells (pools of lymph nodes, spleens and peritoneal exudates) of both sensitized and normal Gp 13 were labelled with ^{51}Cr by the addition of 1 mCi of ^{51}Cr (Sodium chromate, Atomic Energy of Canada Ltd., Ottawa) to 5×10^9 cells in RPMI 1640 medium containing 10% FCS. The mixture was maintained for 30 minutes at 37°C with occasional shaking. The labelled cells were then washed 5 times with Hanks' solution and their suspension was adjusted to 2×10^8 cells per ml.

Two weeks after implantation of the MC-D cells, i.e. at a time when the tumor nodules were 1-2 cm in diameter, each of the test animals received intravenously 20 µg N of the purified goat AGFA and the control animals received the same amount of goat IgG; this dose of AGFA was chosen since it had been previously shown that intravenous administration of 20 µg N of AGFA resulted in the localization of 70% of these antibodies within the tumor matrix (Lee

et al., 1978). In order to determine the most efficient route for localization of ^{51}Cr-labelled lymphoid cells in the tumor site, each guinea pig received, 48 hours after administration of AGFA, 2×10^8 of the labelled sensitized lymphoid cells intravenously, or intraperitoneally, or subcutaneously. The recipients were sacrificed 24 hours later by ether anesthesia and their blood, lungs, hearts, livers, spleens, kidneys and tumors were tested for ^{51}Cr content in a Nuclear Chicago γ-ray counter; one gm of each tissue was used for determination of the radioactivity and the total radioactivity was then calculated for the whole tissue.

Experimental Design. To test the hypothesis underlying this project, the following experimental protocol was designed. Five groups of 3 to 4 adult, male and female, guinea pigs (weighing 400-500 gm), received subcutaneously in their backs 10^6 tumor cells per animal 7 days prior to being subjected to immunological manipulation as described below. At this time, the tumor became palpable and its diameter was measured daily with a vernier caliper. Group I served as the control and was not subjected to any additional treatment; Group II received only the sensitized lymphoid cells to the rabbit or goat IgG; Group III received the rabbit or goat AGFA only; Group IV received both the rabbit or goat AGFA and sensitized lymphoid cells to the corresponding xenogeneic IgG; Group V received normal rabbit or goat IgG and sensitized lymphocytes to the corresponding IgG. The cells were injected subcutaneously within a a few cm distance from the tumor sites since, as had been shown, this route was the most effective for localization of the cells within the tumor. The time intervals between injections of xenogeneic IgG and the adoptive transfer of cells, as well as the type of cells transferred, will be indicated later.

The statistical significance of the differences in tumor size among different groups of guinea pigs was established by the Student's t-test.

TABLE 1

EVIDENCE FOR THE PRESENCE OF CMI TO XENOGENEIC IgG IN SENSITIZED GUINEA PIGS

Skin Reactions

Treatment of Guinea Pigs	Skin Lesions (average diameter, mm) Challenge Doses of Rabbit IgG		
	10 µg	20 µg	30 µg
Rabbit IgG + FCA			
Gp No. 1	15.0	20.0	22.5
Gp No. 2	14.0	19.5	25.0
FCA Only			
Gp No. 3	0.0	0.0	0.0

Passive Transfer of CMI

Number of Sensitized Cells Transferred	Skin Lesions (average diameter, mm)*
10^6	8.0
10^7	10.0
10^8	14.0
10^9	14.0

Antigen-Induced Stimulation of ^{3}H-Thymidine Uptake by 2 x 10^6 Sensitized Cells

Lymphocytes of Donors Sensitized to:	Goat IgG (µg/ml)	Mean CPM ± S.D.
Goat IgG + FCA	0	6,448 ± 2
	1	21,798 ± 936
	10	28,746 ± 593
	100	22,864 ± 4,177
FCA Only	0	677 ± 51
	1	585 ± 4
	10	645 ± 11
	100	501 ± 2

* Each animal was challenged with 20 µg of rabbit or goat IgG.

TABLE 2

Distribution of Radioactivity in Tissues of Tumor Bearing Gp 13 After Transfer of ^{51}Cr-Labelled Sensitized Lymphoid Cells*

Treatment of Recipients before transfer of Sensitized Cells	Route of Injection of cells	% of Radioactivity in Individual Organs†						
		Blood	Lung	Heart	Liver	Spleen	Kidney	Tumor
AGFA	s.c.	20.10	1.51	0.35	28.46	1.27	20.32	27.93
AGFA	i.p.	49.23	1.02	0.40	13.64	3.40	27.75	4.51
AGFA	i.v.	39.90	13.76	0.16	35.02	7.52	0.80	2.81
Normal IgG	i.v.	46.10	15.77	0.15	28.76	7.67	0.94	0.58

* The tumor bearing Gp 13 received first an i.v. injection of 20 µg N of purified goat AGFA and 48 hours later 2×10^8 lymphoid cells from guinea pigs exhibiting CMI. The recipients were sacrificed 24 hours after cell transfer.

† % of Radioactivity in Individual Organ = $\frac{\text{CPM of Individual Organ}}{\text{CPM of All Counted Organs}} \times 100$

RESULTS

CMI in Gp 13 to Xenogeneic IgG. The results listed in the first section of Table 1 demonstrate that marked delayed hypersensitivity skin reactions were produced in guinea pigs, which had been sensitized to xenogeneic IgG, on challenge with the immunizing antigen. Moreover, the data presented in the second section of this Table indicate that doses larger than 10^8 lymphoid cells of immune animals were required for passive transfer of marked skin reactions and, therefore, this was the minimal dose used in subsequent experiments. From the result given in the bottom section of this Table it is evident that increased ^{3}H-thymidine uptake by lymphoid cells from immune animals occurred upon culture with the immunizing antigen, thus corroborating the *in vivo* findings that immunization of Gp 13 with a single dose (80 µg) of rabbit or goat IgG in presence of FCA was sufficient to induce a strong CMI, which was transferable with lymphoid cells.

Localization of ^{51}Cr-labelled Sensitized Lymphoid Cells in Gp 13 Bearing the MC-D Tumor. Forty-eight hours after i.v. injection of purified goat AGFA into tumor-bearing Gp 13, ^{51}Cr-labelled sensitized lymphoid cells were transferred into these animals by one of the 3 routes, i.e. intravenously, intraperitoneally, or subcutaneously. Twenty-four hours later, the distribution of the ^{51}Cr-labelled cells in the blood, lungs, hearts, livers, spleens, kidneys and tumor sites was established. The results listed in Table 2 are expressed as the percentage of radioactivity residing in each tissue in terms of the total radioactivity determined for all tissues measured. From these results it is evident that the homing pattern of the injected ^{51}Cr-labelled cells depended on the route of injection and that the most effective localization

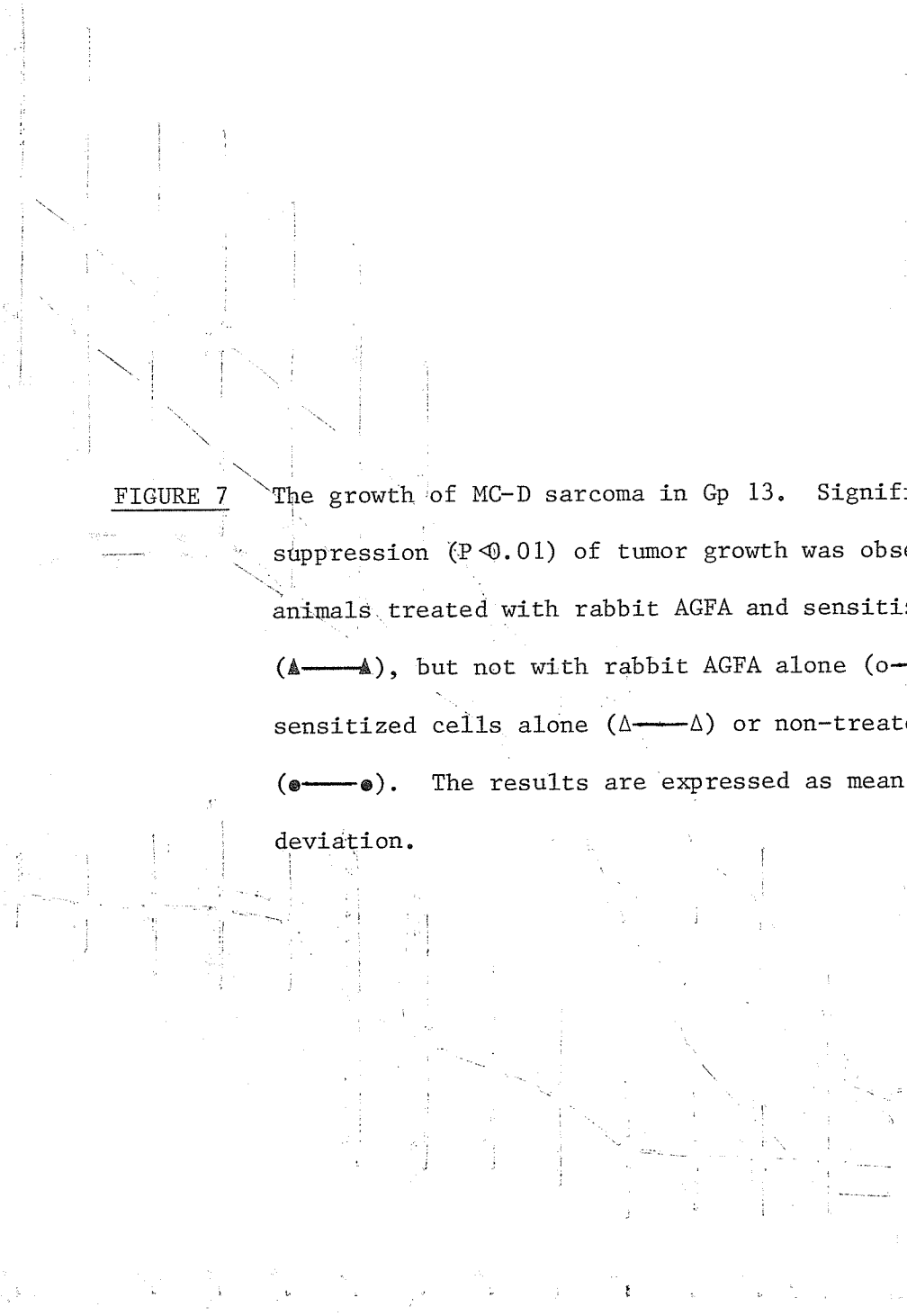
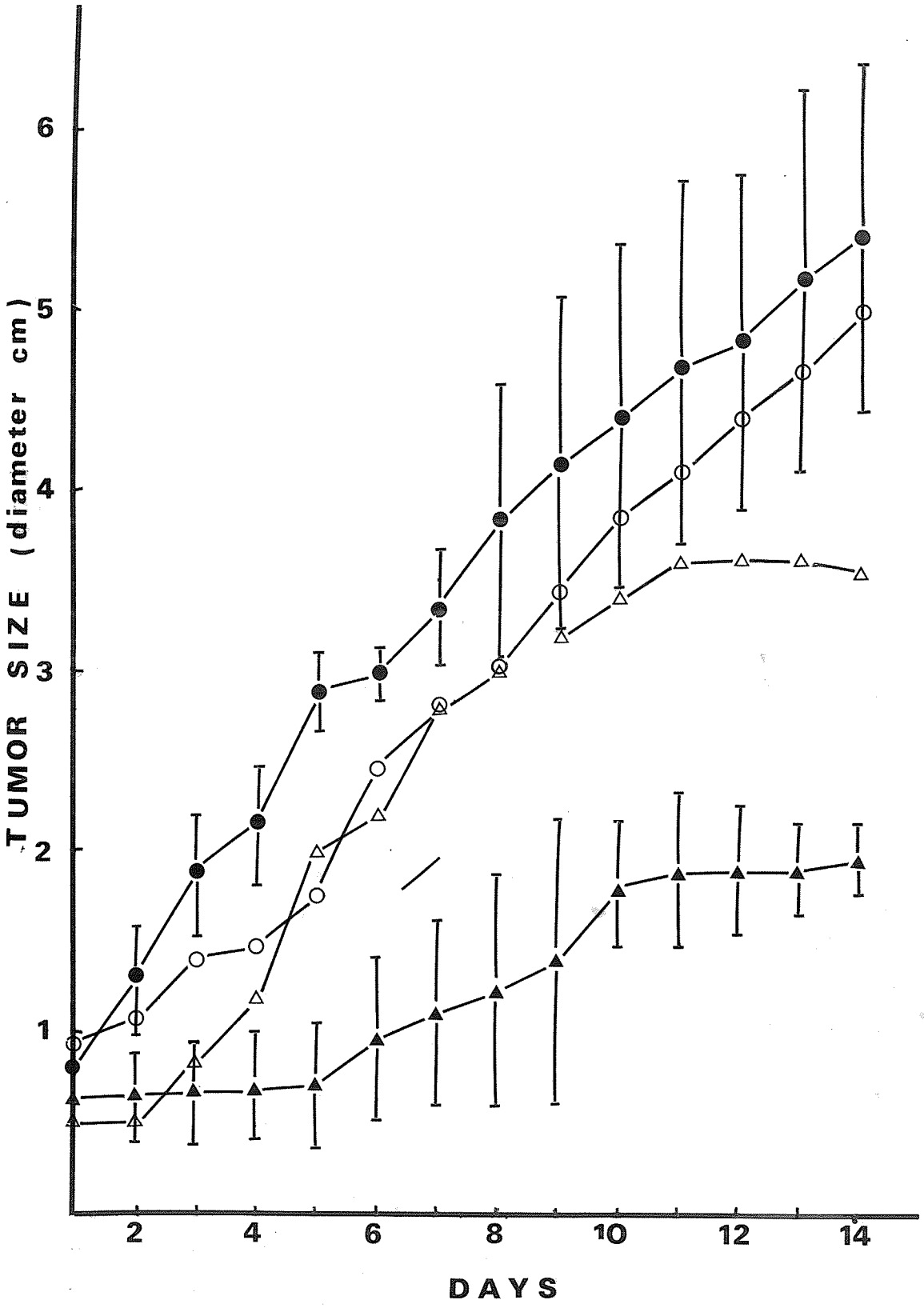


FIGURE 7 The growth of MC-D sarcoma in Gp 13. Significant suppression ($P < 0.01$) of tumor growth was observed in animals treated with rabbit AGFA and sensitized cells (▲——▲), but not with rabbit AGFA alone (o——o), sensitized cells alone (Δ——Δ) or non-treated control (●——●). The results are expressed as mean ± standard deviation.


TUMOR SIZE (diameter cm)
1
2
3
4
5
6
2
4
6
8
10
12
14
DAYS

in tumor sites occurred when the cells were administered subcutaneously. Therefore, in subsequent experiments, this route of injection was chosen for the transfer of sensitized lymphoid cells. Moreover, although these experiments were not exhaustive because of the limited availability of inbred guinea pigs, it is clear from the results given in this Table that a significantly larger number (by a factor of 5) of sensitized cells, transferred by the intravenous route, was localized in the tumor sites of animals which had received previously AGFA, as compared to the localization in animals which had been injected with normal goat IgG.

Testing of the Hypothesis for the Indirect Cell-Mediated Tumor Destruction.

In a series of experiments, each of the tumor-bearing Gp 13 received only a single injection of 10 μg N of rabbit AGFA, which was followed 2 days later by a single dose (8×10^8) of sensitized lymphoid cells consisting of a pool of cells from lymph nodes, peripheral blood and peritoneal exudates of the immune animals. As is evident from the results plotted in Fig. 7, significant suppression of tumor growth ($P<0.01$) was achieved in the animals which had received both AGFA and the sensitized cells. On the other hand, the tumor growth of animals which received only AGFA or only sensitized cells did not differ markedly from that of control animals which had not received any treatment after implantation of the tumor. Similarly, the growth of the tumor was not affected by administration of normal xenogeneic IgG and cells from guinea pigs which had been sensitized to a state of CMI to corresponding IgG (data not plotted).

This experiment was terminated after 14 days of observation, when the animals were killed using ether anesthesia. The animals were then subjected to autopsy and several organs were examined histologically. The tumor diameter

of control tumor-bearing animals at this time was larger than 5.5 cm and there was no detectable change in any of their organs. The size of the tumors of the animals treated with AGFA or sensitized cells approached that of controls; these two groups of animals did not show a significant change in their organs other than a somewhat enlarged spleen. In contrast, the size of the tumors of the animals which had received both AGFA and sensitized cells was about half that of the control guinea pigs and the tumors of the animals so treated were infiltrated with polymorphonuclear and mononuclear cells. Moreover, diffuse infiltration of tumor cells was found in the livers, spleens and lymph nodes of these animals and diffuse miliary necrotic lesions were detected in their organs and in the kidneys.

In another series of experiments, removal of adherent cells from pooled populations of spleen and lymph node cells of sensitized guinea pigs and exclusion of the peritoneal exudate cells in the cell transfers appeared to result in the abrogation of the retardation effect of sensitized lymphoid cells on tumor growth. All animals succumbed to the tumor growing locally and no tumor cells were found in other organs.

In a third series of experiments the effects of multiple injections of AGFA and of the peritoneal exudate cells of sensitized animals was evaluated. For this purpose all animals received three intravenous injections of 20 µg N of purified AGFA (i.e. total 60 µg N) on days 7, 12 and 16 after implantation of tumor cells. One group of animals received on each of days 8, 13 and 17 5×10^8 lymphocytes from a pool of cells obtained from spleens, lymph nodes and peripheral blood of sensitized guinea pigs. Another group received the same number of cells on the same days, except that the pool of cells contained also peritoneal exudate cells.

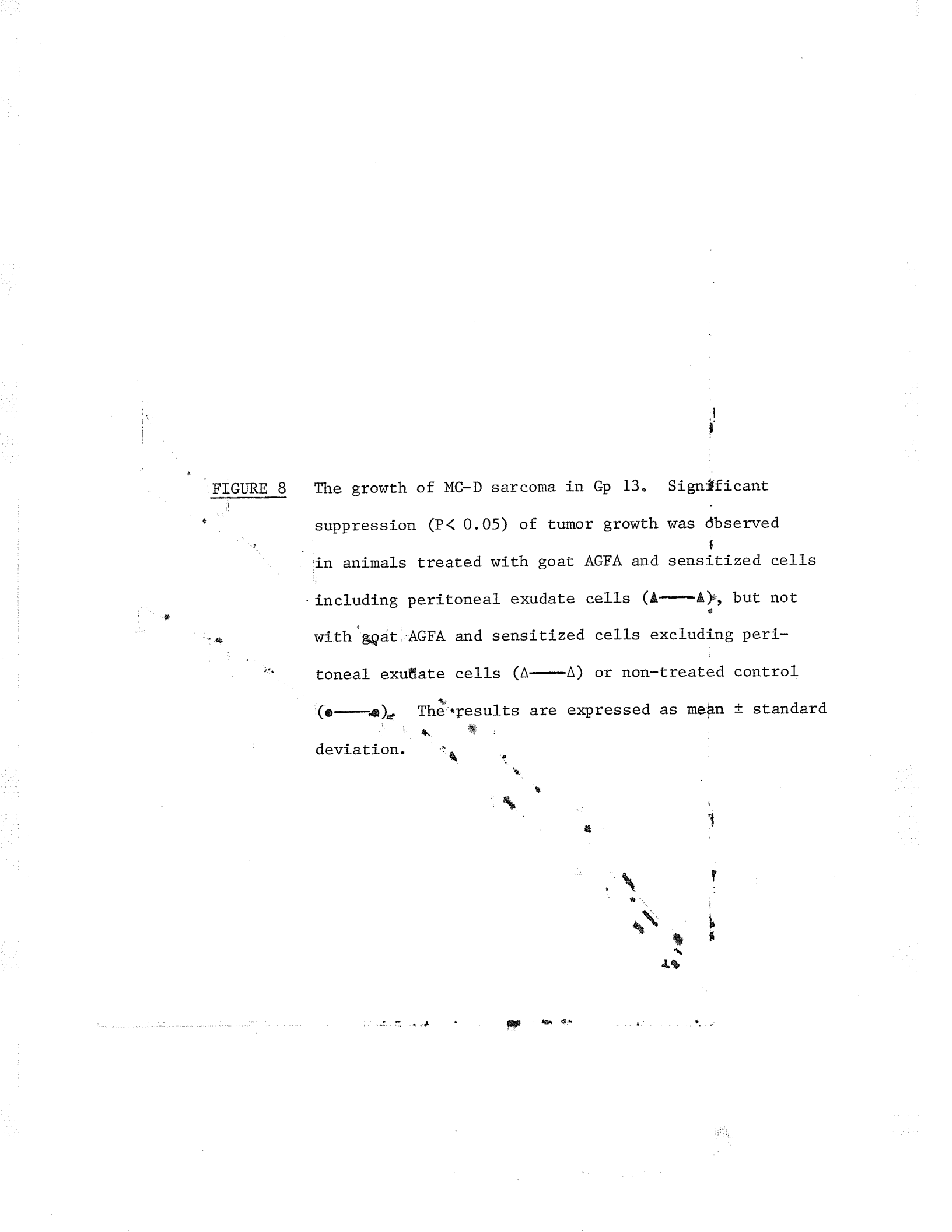
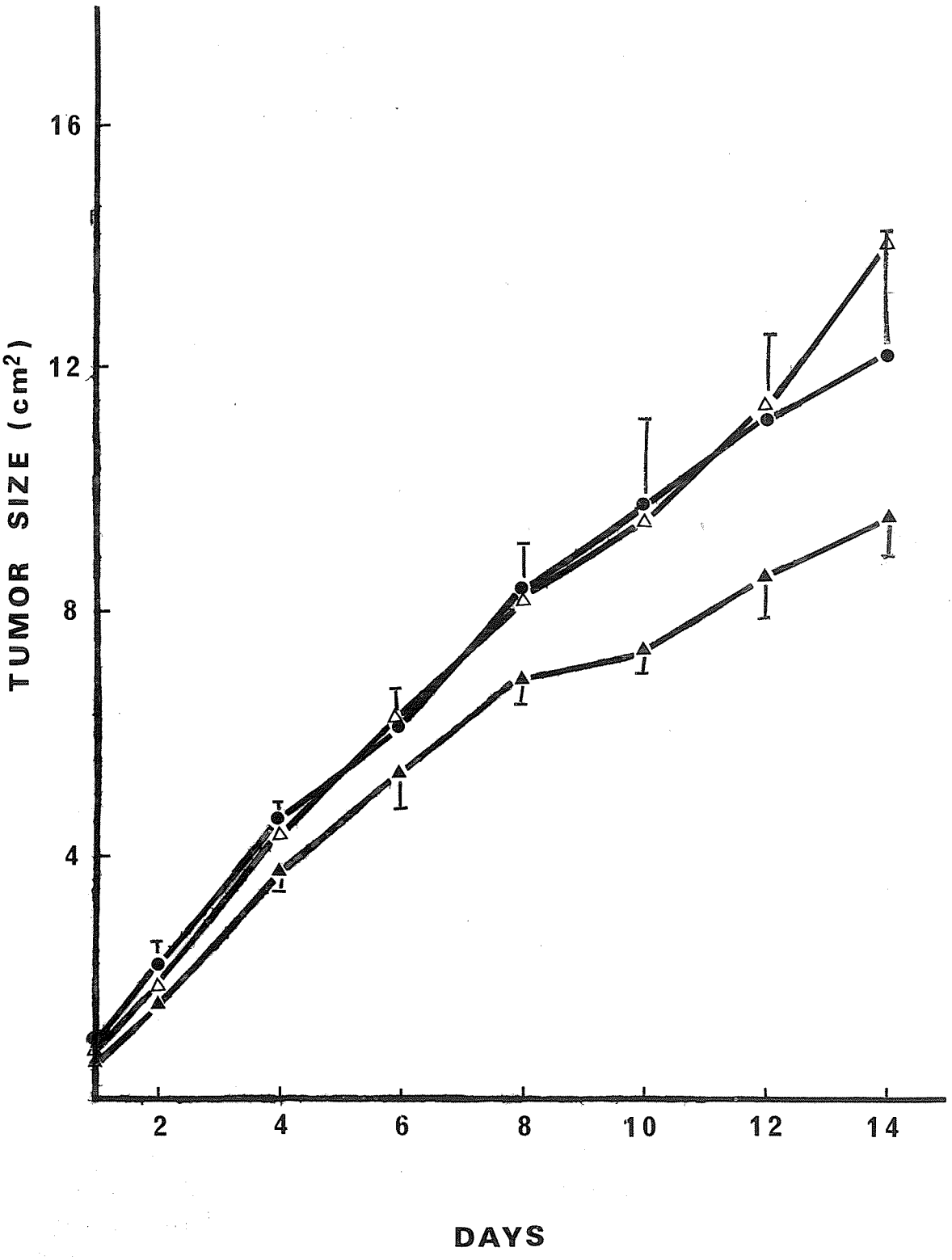


FIGURE 8 The growth of MC-D sarcoma in Gp 13. Significant suppression ($P < 0.05$) of tumor growth was observed in animals treated with goat AGFA and sensitized cells including peritoneal exudate cells (▲——▲), but not with goat AGFA and sensitized cells excluding peritoneal exudate cells (Δ——Δ) or non-treated control (●——●). The results are expressed as mean ± standard deviation.


TUMOR SIZE (cm²)
16
12
8
4
2
4
6
8
10
12
14
DAYS

As is evident from the data plotted in Fig. 8, in the absence of peritoneal exudate cells there was no significant retardation of tumor growth. However, the inclusion of peritoneal cells among the lymphoid cell population in the adoptive transfer resulted in a considerable slowing down of tumor growth ($P<0.05$). These results thus confirm the findings illustrated in Fig. 7. Nevertheless, in spite of the lower rate of tumor growth in animals which had received both AGFA and sensitized cells, these animals succumbed to the tumor and died at a time not significantly different from control animals, probably due to enhanced metastasis which was caused by tumor cells emigrating from the tumor foci to the other organs.

DISCUSSION

It is obvious that this in vivo study is by no means complete and that the data obtained to date are based on a relatively small number of experiments. One of the main limitations of this model is the unavailability of inbred guinea pigs in sufficient numbers which are necessary for the relatively large number of lymphoid cells required in these experiments; thus, 8-10 sensitized guinea pigs provided the lymphoid cells for 3-4 experimental tumor-bearing hosts.

The observation that the growth of the MC-D sarcoma was significantly retarded in animals which had received both AGFA and lymphoid cells from syngeneic guinea pigs, which had been sensitized to immunoglobulins isotypic with AGFA, but not with either AGFA or lymphoid cells alone, is interpreted as being due to the local inflammatory reaction triggered by the sensitized cells on interaction with the AGFA-coated fibrin matrix present in the tumor

foci. Moreover, the fact that deletion of peritoneal exudate cells from the pool of sensitized lymphocytes resulted in loss of the capacity of these cells to suppress tumor growth suggests that peritoneal cells may be essential for the success of this type of immunological manoeuvre which leads to an indirect cell-mediated destruction of malignant cells. Since peritoneal exudate cells are usually rich in macrophages, it may be speculated that these phagocytic cells contributed substantially to the observed suppression of tumor growth. In support of this inference one may cite the evidence that macrophages have been shown to home to the site of delayed hypersensitivity reaction (Boughton and Spector, 1963).

Before any definite conclusion can be drawn as to the nature of the cells involved in tumor rejection in this model system, it will be necessary to repeat these experiments with well-defined subpopulations of lymphoid cells from guinea pigs sensitized to the antigenic determinants of AGFA. Nevertheless, these results lend further support to the concept that tumor cells may be destroyed as a result of CMI to antigenic determinants of constituents present in tumor foci other than tumor antigens per se. In this respect one may also cite the results of Williams and Klein (1970) who reported successful eradication of cutaneous malignancies in over 50 patients in whom cell-mediated hypersensitivity was induced actively or passively to simple contact-sensitizing chemicals, such as trenimon or dinitrochlorobenzene, provided that these substances could penetrate into the neoplastic area. Moreover, Zbar et al. (1970) had demonstrated that proliferation of a transplantable tumor in inbred guinea pigs was suppressed at the sites of delayed hypersensitivity skin reactions to another antigenically different transplantable tumor, as well as at the sites of delayed hypersensitivity skin reactions to tubercoloproteins, and that the effectiveness of the

immunologically nonspecific killing of tumor cells was directly related to the intensity of the delayed hypersensitivity reaction. The same group of workers (Zbar and Tanaka, 1971) also showed that tumor nodules in guinea pigs regressed at the site of inflammatory reactions provoked by living Mycobacterium bovis.

The finding in this study that the tumor size of the animals treated with both AGFA and sensitized lymphocytes was smaller than that of the controls, and that the organs of the former animals were infiltrated with tumor cells, is interpreted as being due to the local inflammatory reaction resulting not only in tumor cell destruction, but also in disorganization of the fibrin lattice allowing thus for the escape of tumor cells to other sites. However, it is important to point out that whereas in the present study injection of AGFA by itself did not affect the growth of the tumor, Schlager and Dray (1975) reported recently that complete tumor regression was achieved by subcutaneous injection of antibodies to fibrin fragment E into guinea pigs bearing a line-10 hepatoma which had been implanted intradermally into strain II guinea pigs. Since the tumors and the experimental conditions used in these two studies are different, it is not possible to draw any conclusion as to the reason for the diverging effects of antibodies to the constituents of fibrin.

In spite of the limited number of results, it may be stated that the findings presented in this paper support the validity of the working hypothesis underlying this study. It would, therefore, appear that the principle of indirect cell-mediated destruction of tumor cell represents a promising immunotherapeutic strategy, which involves the accumulation of sensitized

CHAPTER IV

Complete Regression of MC-D Sarcoma in Guinea Pigs by Conjugates of Daunomycin with Anti-Fibrin Antibodies

lymphocytes in the tumor foci, which are thus converted into battlegrounds unfavourable for supporting further growth of tumor cells.

ACKNOWLEDGMENT: This study was supported by a grant from the National Institutes of Health, Bethesda, Md. (CA-13192).

SUMMARY

Daunomycin was coupled with the aid of glutaraldehyde to goat antibodies specific to guinea pig fibrin. The resulting daunomycin-antibody conjugates inhibited cellular RNA synthesis and induced cell death *in vitro* of a methylcholanthrene-induced sarcoma (MC-D) of strain-13 guinea pigs. The cytotoxic capacity of the conjugate was not significantly different from that of free daunomycin. The specific localization of daunomycin-antibody conjugates within the fibrin matrix enmeshing the tumor tissue was demonstrated by indirect immunofluorescence with FITC-conjugated rabbit antibodies to goat γ-globulins. Multiple injections of daunomycin-antibody conjugates intratumorally *in vivo*, into well established MC-D tumors, led to significant tumor growth retardation and complete tumor rejection occurred in 50% of the guinea pigs. Moreover, systemic tumor immunity was induced in the guinea pigs so cured, as demonstrated by the fact that these animals were resistant to a further lethal dose of MC-D tumor cells.

INTRODUCTION

So far no therapeutic agents capable of eradicating completely established tumors have been discovered. Although chemotherapeutic agents are highly toxic to tumor cells, their disadvantage is that they are also toxic to normal tissues. An ideal therapeutic agent should possess high toxicity and specificity for the tumor. Such agents may, in principle, be prepared by linking cytotoxic drugs to tumor-specific antibodies by chemical methods. However, one of the main difficulties is to produce adequate amounts of specific anti-tumor antibodies for coupling to drugs.

Several groups of investigators have linked cytotoxic agents to anti-tumor antibodies in an attempt to use these preparations for the destruction of tumor cells. For instance, in an *in vitro* model system, conjugates consisting of diphtheria toxin coupled to anti-dinitrophenyl (DNP) antibodies were shown to display a potent cytotoxic effect towards tumor cells to which the DNP determinants had been previously attached (Moolten *et al.*, 1972). Others have linked alkylating agents either covalently (Linford *et al.*, 1974) or non-covalently (Ghose *et al.*, 1972; Davies and O'Neill, 1973) directly to anti-tumor antibodies. Trenimon, nitrogen mustard, adriamycin and daunomycin have all been conjugated covalently by chemical methods to anti-tumor antibodies. Such conjugates were shown to retain both pharmacological and antibody activity, and were able to exert preferential cytotoxicity against tumor cells *in vitro* (Linford *et al.*, 1974; Hurwitz *et al.*, 1975; Levy *et al.*, 1975).

As reported previously (Fujimoto *et al.*, 1973; Lee *et al.*, 1978), up to 70% of anti-guinea pig fibrin antibodies (AGFA), injected into strain 13 guinea pigs bearing the 3-methylcholanthrene-induced sarcoma (MC-D) was localized within the fibrin matrix enmeshing this tumor. Hence, in the present study we

prepared AGFA-daunomycin conjugates by crosslinking with glutaraldehyde in anticipation that these conjugates would home to the tumor foci and destroy the tumor. Indeed, daunomycin-AGFA conjugates were shown to retain both their drug and antibody activities. Moreover, multiple intratumoral injections of these conjugates induced in vivo rejection of established MC-D tumors in some guinea pigs and the guinea pigs so cured were resistant to a further injection of a supralethal dose of MC-D cells.

MATERIALS AND METHODS

Guinea Pigs and Tumor

Inbred Sewall Wright strain-13 guinea pigs, weighing 300-400 gm, were purchased from the Weizmann Institute for Science, Rehovot, Israel and were also generously donated by the Frederick Cancer Research Center, Fort Dietrich, Md., U.S.A.

The MC-D sarcoma was donated by Dr. H.F. Oettgen of the Sloan-Kettering Institute, New York, and was maintained by subcutaneous passage in strain-13 guinea pigs.

Daunomycin and Goat AGFA

Daunomycin hydrochloride was obtained either as the commercial product, Cerubidine, or as the pure compound, a gift from the manufacturers (Poulenc, Montreal, Quebec). We found no substantial differences in the reactivity of these two products during coupling experiments with AGFA.

Goat AGFA was prepared and purified as described in the preceding paper (Lee et al., 1978).

Conjugation of Daunomycin with AGFA

The linking of daunomycin to AGFA was achieved according to the method of Hurwitz et al. (1975), using glutaraldehyde (Baker, Phillipsburg, N.J.) as cross-linking agent. A mixture of daunomycin (0.5 ml, 1 mg/ml) and AGFA (0.5 ml, 3.0 mg) in 0.01 M phosphate-buffered saline (PBS), pH 7.2, was continuously stirred in a test tube. To this mixture, 100 µl of glutaraldehyde (0.25% solution in water) was added dropwise during a period of 5 minutes. The reaction was allowed to proceed for an additional 10 minutes, and then 50 µl of 1 M L-lysine was added to stop the reaction. Free and bound daunomycin were separated by gel filtration through a Sepharose 6B column (25 x 1.5 cm) equilibrated with PBS. The fractions containing daunomycin-AGFA conjugates (D-AGFA) were dialysed at 4°C for 24 hours against 2 changes of PBS. After concentrating the D-AGFA conjugates to a desired volume (usually 5.0 ml) with negative pressure dialysis, the preparations were centrifuged once at 15,000 rpm for 1 hour and stored at 4°C.

The average number of daunomycin molecules conjugated with one molecule of antibody was calculated from spectrophotometric measurements at 495 nm [$E_{1\,cm}^{1\%}$ for daunomycin = 196 (Bernard et al., 1969)] and protein concentrations determined according to Lowry's method (Lowry et al., 1951).

Pharmacological Activity of D-AGFA

The pharmacological activity of D-AGFA was measured in vitro by its inhibition of ^{3}H-uridine incorporation into cellular RNA of tumor cells, and by its direct cytotoxic action on MC-D cells as revealed by dye exclusion (0.2% trypan blue).

The assay for the inhibition of tumor cell RNA synthesis was performed in microtiter plates with V bottom wells (Cooke Engineering Co., Alexandria, Va.). The MC-D tumor cells were freshly prepared by digestion of tumor tissue with trypsin. The tumor cells were resuspended to contain 2 x 10^7 cells per ml in Dulbecco's modified Eagle's medium (DMEM: Grand Island Biological Co., New York, N.Y.) and 50 µl of this cell suspension (i.e., 10^6 cells) was dispensed into each well. Daunomycin either free or conjugated with AGFA was adjusted to contain 5 µg per ml in DMEM and added to the wells in 50 µl aliquots. The protein concentration of unconjugated AGFA was adjusted to the same level as that of D-AGFA. The plates were first incubated for 2 hours at 37°C in a CO_2 incubator; 1 µCi of [5-^{3}H]-uridine (New England Nuclear, Dorval, Quebec) in DMEM was then added to each well and the plates were incubated for another hour. The reaction was stopped by adding 25 µl of 25% trichloroacetic acid (TCA) per well and the plates were kept at 4°C overnight. The TCA precipitates were washed twice with cold 5% TCA. They were solubilized in 0.1 ml of 0.2 N NaOH and transferred to vials containing a toluene-based scintillation solution (PPO, 6 gm; POPOP, 0.2 gm in one liter of toluene) for counting of the incorporated ^{3}H-uridine. All samples were assayed in triplicate.

Since it could be envisaged that the inhibition of cellular RNA synthesis by D-AGFA was due only to a temporary suppression of template activity without causing cell death, the trypan blue dye exclusion method, which directly evaluates the number of dead cells, was used to assess the toxic effect of D-AGFA on tumor cells. The experimental conditions such as tumor cell number, D-AGFA concentration and temperature of incubation were identical to the previous experiment.

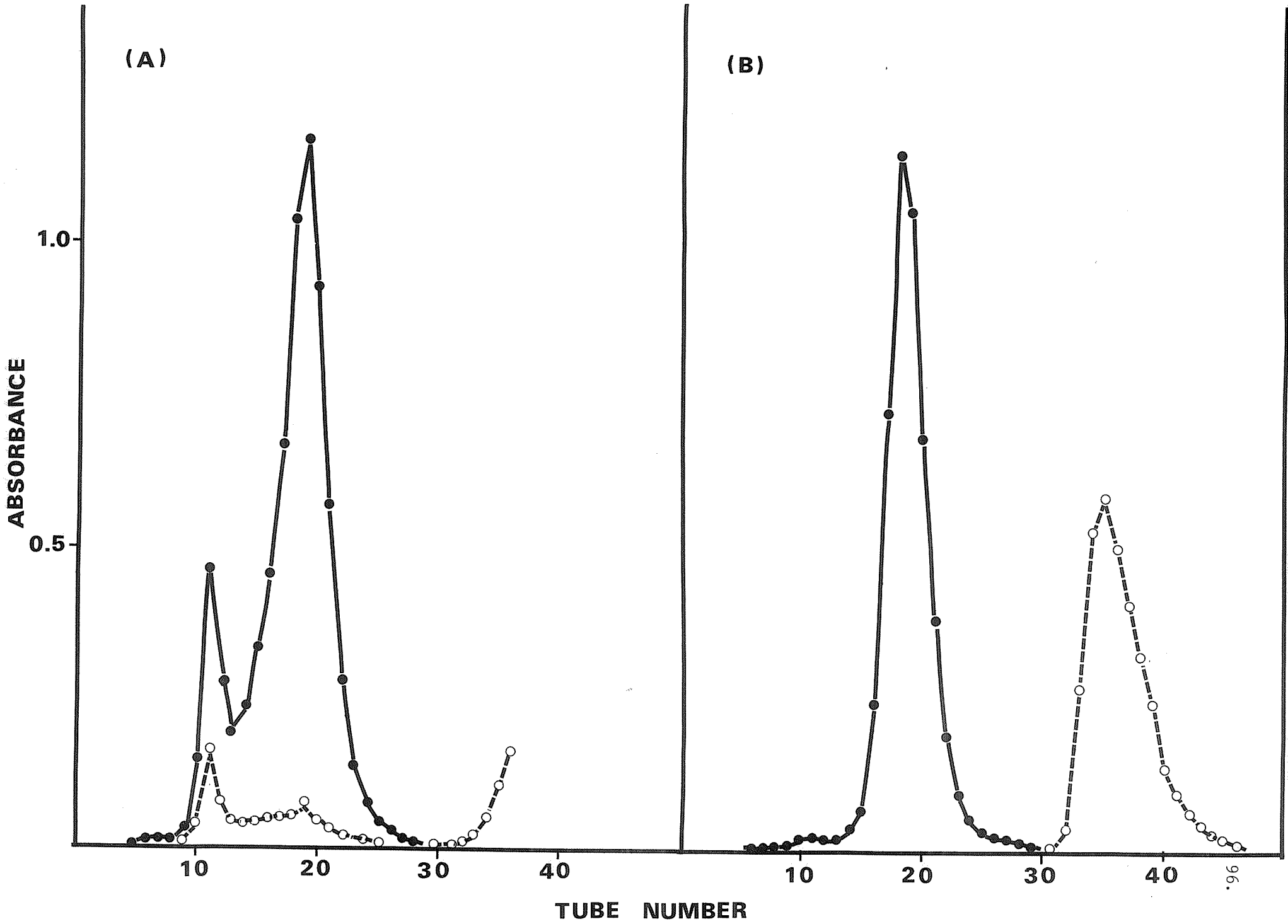
Antibody Activity of D-AGFA

The antibody activity of D-AGFA was demonstrated by the method of indirect immunofluorescence. Frozen MC-D tumor tissue was cut into approx. 4 μ thick sections, which were thawed at room temperature and were then fixed with cold acetone on a microscopic slide. The fixed tissue sections were first treated with D-AGFA (1 mg/ml) for 45 minutes in a humid chamber, washed three times with cold PBS and then treated with FITC-conjugated rabbit anti-goat IgG antibodies (1 mg/ml) for 45 minutes. Finally, the unreacted FITC-conjugated antibodies were washed out and the sections were examined under a Zeiss fluorescence microscope. The control sections were treated with normal goat IgG instead of D-AGFA; the rest of the procedure was as described above.

In Vivo Anti-Tumor Effect of D-AGFA

In order to test the effect of D-AGFA on MC-D tumor in vivo, strain-13 guinea pigs were inoculated subcutaneously on their flanks with a supralethal dose of MC-D tumor cells (10^6 per animal). On day 9, when the tumors had reached measurable sizes (0.5 - 0.9 cm diameter), the animals were divided into 4 groups, each consisting of 6 tumor bearing animals. One group of animals served as control and did not receive any additional treatment. The other three groups received a total of 7 intratumoral injections (0.5 ml/injection), given every other day, each injection for the respective groups consisting of (i) D-AGFA (2.5 μg in terms of daunomycin concentration), (ii) free daunomycin (2.5 μg), (iii) unconjugated AGFA (0.5 mg); all these materials had been dissolved in PBS. The tumor growth was followed by measuring the two perpendicular diameters at two day intervals.

FIGURE 9 Profile for the separation of free and bound daunomycin by gel filtration chromatography on Sepharose 6B; (A) daunomycin conjugated with AGFA by glutaraldehyde; (B) a mixture of daunomycin and AGFA; (●——●): absorbance at 280nm; (o——o): absorbance at 495 nm.


(A)
(B)
1.0
0.5
ABSORBANCE
10
20
30
40
10
20
30
40
TUBE NUMBER

For the detection of tumor-specific immunity in animals in which the tumor had regressed, a second dose of 10^6 tumor cells was injected subcutaneously on the contralateral flank 35 days after the treatment had been stopped. Normal animals received the same number of tumor cells to serve as controls.

RESULTS

Conjugation of Daunomycin with AGFA

The reaction of daunomycin with AGFA in the presence of glutaraldehyde produced not only D-AGFA, but also some protein-protein aggregates. As is evident from Fig. 9(A), two fractions were obtained after the products were separated by gel filtration on Sepharose 6B. The first fraction contained mainly aggregates of D-AGFA since, after dialysis and concentration, more than 90% of the material became insoluble and could be removed by centrifugation. On the other hand, the second fraction, emerging in a volume similar to that of unconjugated AGFA (compare O.D. profiles A and B in Fig. 9) remained soluble under the same conditions. Therefore, only the second fraction was used in this study.

The degree of coupling of daunomycin to AGFA by glutaraldehyde in the active D-AGFA fraction (i.e., the second fraction isolated by gel filtration) varied for different preparations being in the range of 1-2 moles of daunomycin per mole of antibody. The experimental conditions described here for the production of active D-AGFA were found to be optimal, since varying any one of the reaction parameters, such as increasing glutaraldehyde concentration, prolonging the reaction time, etc., produced a larger fraction of D-AGFA aggregates.

Table 3

Inhibition of RNA Synthesis by D-AGFA

Incubated with MC-D cells (10^6)	Inhibition of ^{3}H-Uridine Incorporation %
D-AGFA (0.25 µg/ml)*	50
AGFA (0.04 mg/ml)	11
Daunomycin (0.25 µg/ml)	52
Daunomycin + AGFA (0.25 µg/ml) (0.04 mg/ml)	49

* In terms of daunomycin concentration.

Table 4

Non-Complement-Dependent Cytotoxicity of D-AGFA

Treatment	% Dead cells after incubation for	
	2 hours	4 hours
D-AGFA (0.25 µg/ml)*	46	57
AGFA (0.04 mg/ml)	22	25
Daunomycin (0.25 µg/ml)	41	50
Daunomycin + AGFA (0.25µg/ml) (0.04 mg/ml)	39	44

* In terms of daunomycin concentration.

D-AGFA was stable for 2 weeks when stored at 4°C; beyond this time daunomycin appeared to be gradually released from the conjugates.

Pharmacological Activity of D-AGFA

Since an important biological effect of daunomycin is the inhibition of the DNA template activity, the ability of D-AGFA to block the ^{3}H-uridine incorporation into the RNA of MC-D tumor cells was used to measure its pharmacological activity. The results listed in Table 3 clearly show that there was no significant difference in the inhibitory effects of D-AGFA and free daunomycin on ^{3}H-uridine incorporation and it is, therefore, concluded that daunomycin in D-AGFA retained practically all of its pharmacological activity. As expected, AGFA alone showed a negligible toxic effect on tumor cells.

The results obtained in the trypan blue dye exclusion experiments, which are given in Table 4, indicate also that D-AGFA possessed pharmacological activity essentially identical to that of free daunomycin. As is shown in Table 4, a substantial amount of tumor cells died after incubation for 2 hours with free or bound daunomycin, the number of dead cells increasing slightly after incubation for an additional 2 hours. These results also show that the toxic effect of daunomycin resulted actually in the death of tumor cells and did not only inhibit temporarily their DNA template activities. It is to be pointed out that clumping of tumor cells was observed in the samples treated with AGFA alone, which was unexpected since there is no obvious reason why AGFA would combine with MC-D tumor cells; this effect may have been due to the presence of fibrin fragments on the surface of freshly prepared tumor cells which would provide a source of antigen for reaction with AGFA.

FIGURE 10 Demonstration of specific binding of D-AGFA to fibrin in MC-D tumor foci (x 400). The sarcoma section was treated with goat D-AGFA and rabbit FITC-anti-goat IgG.

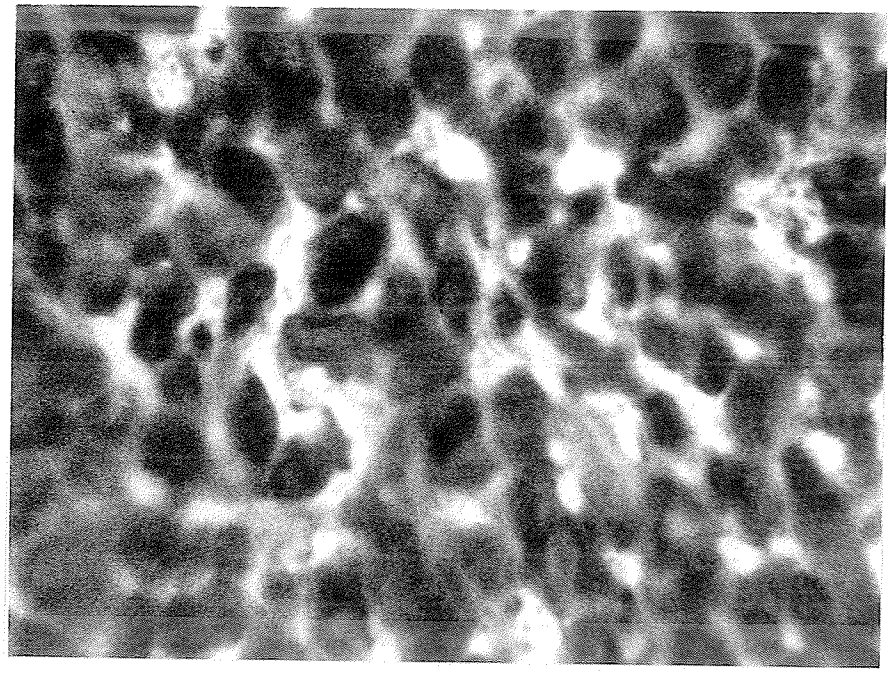
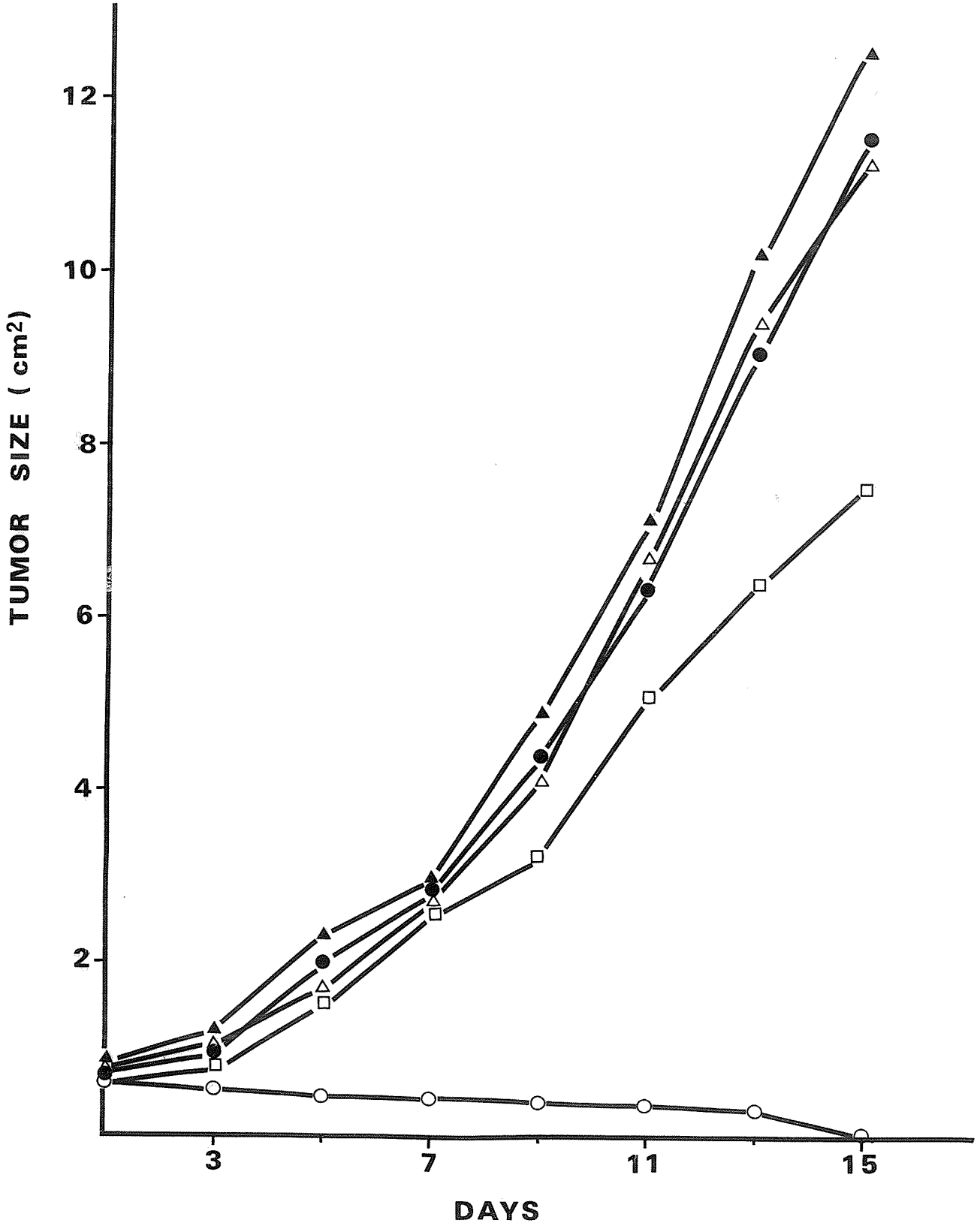


FIGURE 11 Anti-tumor effects of D-AGFA. Guinea pigs with established sarcomas received intratumorally the following substances: Free daunomycin (Δ); unconjugated AGFA (▲); D-AGFA (regressed tumors: o; non-regressed tumors: □). Control animals (●) did not receive any treatment.


TUMOR SIZE (cm2)
12
10
8
6
4
2
3
7
11
15
DAYS

Antibody Activity of D-AGFA

The antibody activity of D-AGFA was demonstrated by indirect immunofluorescence. In Fig. 10 is illustrated the specific staining of the fibrin matrix in the tumor tissue with D-AGFA and FITC conjugated rabbit anti-goat IgG, which reveals the typical linear pattern of the fibrin matrix enmeshing the tumor mass. On the other hand, when the tumor tissue was treated with normal goat IgG instead of D-AGFA, no staining of fibrin could be observed (figure not shown). These results clearly demonstrate that D-AGFA bound to the fibrin present in tumor foci and that coupling of daunomycin to AGFA did not impair their antibody activity.

In Vivo Anti-Tumor Effects of D-AGFA

For the evaluation of the *in vivo* anti-tumor effects of D-AGFA, one group of six tumor-bearing animals received 7 intratumoral injections of D-AGFA every other day (a total of 17.5 μg daunomycin was given). The tumors regressed completely in three of these animals. The tumor growth in the other three guinea pigs was somewhat suppressed initially, but all these animals succumbed eventually to the tumors. Free daunomycin or unconjugated AGFA failed to affect the tumor growth in two other groups of guinea pigs which had received 10^6 tumor cells. All these findings are represented in Fig. 11.

The three surviving guinea pigs were rechallenged with the supralethal dose of 10^6 MC-D tumor cells 35 days after the treatment had been stopped, but no tumor growth could be detected. On the other hand, normal guinea pigs receiving the identical dose of tumor cells at the same time developed, as usually, tumors and died eventually at 50-60 days after implantation of the MC-D cells.

DISCUSSION

The use of drug-antibody conjugates as anti-tumor agents represents a practical approach for immunotherapy of tumors, provided that the drug-antibody conjugates retain both their antibody activity and the pharmacological cytotoxicity of the bound drug, and that the conjugates are capable of selectively destroying tumor cells without affecting normal tissues. The results of this study, although not extensive because of the difficulty in obtaining a sufficient number of inbred guinea pigs, support the conclusion that D-AGFA satisfies these criteria and that similar antibody conjugates may prove to be effective therapeutic agents for the destruction of other tumors which are lodged within a fibrin matrix or which have fibrin attached to their membranes.

From the standpoint of cancer therapy, the most important demonstration in this study is that D-AGFA could induce complete eradication of established MC-D tumors in strain-13 guinea pigs. The tumors (with average diameter of 0.7 cm at the time when treatment was begun) in 50% of the animals had completely regressed after they had been injected with a total dose of 17.5 μg of daunomycin in the form of D-AGFA. These animals were free of any detectable tumor for over a year (as of the time of writing this paper). Tumor growth in the other 50% of the animals that received the same treatment was considerably retarded, although they eventually died of big tumors. On the other hand, the tumors in all other animals treated with free daunomycin or unconjugated AGFA, as well as in the untreated controls, grew progressively and all these animals died 50-60 days after tumor cell inoculation.

The anti-tumor activity of D-AGFA is attributed to the action of daunomycin which has been repeatedly shown to be strongly toxic for tumor cells. The basic mechanism of the toxic effect of daunomycin has been ascribed to its

ability to form complexes with DNA (Zunino et al., 1972; DiMarco, 1973), thereby altering the stereochemical structure of the DNA molecule which leads, in turn, to the inhibition of the enzymes involved in replication. It is therefore, essential for the daunomycin to penetrate into the cell nucleus in order to exert its cytotoxic effect.

The exact mechanism by which the covalently bound daunomycin residues exert their toxic effect on tumor cells is still not clear. However, at least three possibilities can be envisaged. The first possibility is that D-AGFA, once on the surface of the cell membrane, releases daunomycin, which can penetrate the tumor cell and thus becomes available for interaction with nuclear DNA. However, the anti-tumor effect of D-AGFA cannot be simply explained by the dissociation of daunomycin from it, since the intratumoral injection of daunomycin alone did not produce the same effect. Failure of the latter treatment to suppress tumor growth could be attributed to the rapid plasma clearance of daunomycin (DiFronzo et al., 1971; Yesair et al., 1972) from the tumor site, resulting in insufficient amount of the drug remaining at the site for effective killing of the tumor cells. If this explanation is plausible, one may suggest that the cytotoxic effectiveness of D-AGFA vis-à-vis tumor cells is due to the fact that the clearance rate of the conjugate, particularly after binding to the fibrin matrix within the tumor mass, is much slower than that of the free drug. Thus, AGFA would function as a 'concentrating agent' for daunomycin within the tumor site.

Another possible mechanism is that D-AGFA conjugates enter the tumor cells by pinocytosis and that active daunomycin molecules are released gradually after intracellular digestion of the conjugates by enzymes.

The third possibility is that tumor regression was caused by tumor-specific

immunity, which was induced as a result of the drug action on tumor cells. This interpretation is supported by the fact that, after rejection of a lethal dose of MC-D cells induced by D-AGFA, the surviving animals could reject the second tumor cell inoculum a long time after the expected clearance of D-AGFA. However, it is not possible to state on the basis of these limited data whether or not tumor immunity had been induced within the first few days after the intratumoral inoculation of D-AGFA, and if it contributed to the rejection of the first tumor graft. In relation to these findings, it is to be pointed out that several investigators have reported that tumor specific immunity can be detected in the hosts surviving chemotherapy (Potter and Walters, 1973; Bast et al., 1976). However, whereas injection of unconjugated AGFA did not affect the tumor growth, it is interesting to note that the intratumoral administration of antibodies to the fibrin fragment E resulted in complete regression of a line-10 hepatoma growing intradermally in strain II guinea pigs (Schlager and Dray, 1975).

In conclusion, the results of this study demonstrate that it is possible to induce tumor destruction with drugs conjugated to antibodies, which are directed against antigenic determinants present in the vicinity of tumor cells rather than against tumor antigens themselves. In the present system, advantage was taken of the fact that the MC-D tumor was growing within a fibrin lattice and of the possibility to produce specific antifibrin antibodies in large amounts. Moreover, the previous observation (Lee et al., 1973) that 70% of the intravenously injected AGFA (as distinct from the intratumoral route used in this study) was localized in the tumor site indicates that AGFA had a strong affinity for the fibrin specific antigens and that consequently most, if not all, of the cytotoxicity associated with D-AGFA was present in the tumor foci

rather than being distributed in other organs. By contrast, the preparation of tumor-specific antibodies in adequate amounts for the preparation of the corresponding cytotoxic conjugates is fraught with enormous difficulties and this is, at least in part, the reason for the lack of success reported in other studies in using this approach for eradication of tumors.

In view of the fact that fibrin has been conclusively demonstrated to be associated with many tumors (Lee et al., 1978; O'Meara, 1958; Day et al., 1959), including early metastatic tumor cells (Chew and Wallace, 1976), and since we had previously shown that AGFA can localize in the fibrin matrix of tumor foci after i.v. injection (Lee et al., 1978), we believe that the intravenous injection of conjugates consisting of cytotoxic drugs with purified anti-fibrin antibodies may provide an effective therapeutic procedure for the total eradication of the tumor cells remaining after surgical removal of the bulk of the tumor or after massive chemotherapy and radiation treatment, and thus prevent metastasis or recurrence of the tumor. Obviously, in order to confirm these conclusions, further studies are required with larger numbers of inbred guinea pigs and with other tumor systems growing within a fibrin matrix or in association with fibrin.

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CHAPTER V

General Discussion of the Results

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GENERAL DISCUSSION OF THE RESULTS

(I) Indirect Cell-Mediated Immune Destruction of MC-D Sarcoma

Several factors which would influence the outcome of this study are: (a) the specific localization of xenogeneic AGFA onto the fibrin matrix of tumor tissues in Gp 13; (b) the subsequent introduction of syngeneic lymphoid cells which had been sensitized to the xenogeneic IgG and would specifically home onto the antibody-coated tumor fibrin matrix; (c) the release of chemical mediators by the sensitized lymphoid cells upon reaction with the coated AGFA in the tumor fibrin matrix.

As has been demonstrated in Chapter II and III, the xenogeneic AGFA were specifically localized onto the tumor fibrin matrix. About 40-70% of the injected dose (10 - 20 µg N), i.e., approximately 50 - 80 µg antibody protein was found localized in the tumor sites. In terms of the numbers of antibody molecules, it was estimated that this would represent approximately 10^{14} antibody molecules. If all these coated antibody molecules could react with the sensitized lymphoid cells, it is expected that the amounts of chemical mediators released from this reaction would be sufficient to draw enough quantity of phagocytic cells (mainly macrophages) migrate to the tumor sites and deliver their cytotoxic effects to tumor cells. Results from one series of experiments (see Chapter III) indicated this was the case.

Many factors affect the maximal 'homing' of sensitized lymphoid cells onto the tumor fibrin matrix. Firstly, whether the lymphoid cells have been sensitized to the antigens (rabbit or goat IgG) is important. The results from the skin tests clearly showed that Gp 13 had been sensitized to a state of delayed hypersensitivity to these antigens and

that this hypersensitive state can be passively transferred to normal Gp 13. The lymphocyte transformation results also indicated that the lymphoid cells obtained from these antigen-sensitized Gp 13 were reactive to the antigens. Secondly, results were also affected by the choice of route of administration of the sensitized cells into the anti-fibrin antibody pretreated tumor-bearing Gp 13. Our data showed that the subcutaneous route was better than the intraperitoneal or intraveneous routes for the specific localization of sensitized cells in the tumor sites. A possible explanation for this observation is that the sensitized lymphoid cells were injected subcutaneously adjacent to the tumors which may provide a 'short-cut' for these sensitized cells to reach the tumor fibrin matrix. It is interesting to note that in the studies of active immunotherapy for sarcoma and lymphoma in mice, Borberg et al. (1972) and den Otter et al. (1974) have also found that the subcutaneous route for the injection of tumor cells as immunogen was more effective to induce tumor regression. Finally, the time for the introduction of the sensitized cells into the tumor-bearing hosts was also important. Sensitized lymphoid cells were administered to Gp 13 24 - 48 hours after the antibody injection. At this time, we showed that the anti-fibrin antibody was mainly localized in the tumor fibrin matrix. This should provide an optimal condition for the maximum interaction between sensitized cells and anti-fibrin antibodies within the tumors.

The present model requires the specific interaction of sensitized lymphoid cells with AGFA which were attached to the fibrin matrix in the tumor mass. This interaction would lead to the release of chemical mediators which might either have a direct cytotoxic effect on the tumor cells or, more likely, would attract large numbers of macrophages or other phagocytic cells which would exert a non-specific cytopathic effect on the nearby tumor cells.

In the tumor system, this phenomenon of non-specific destruction or suppression has been well documented. It has been shown that topical application of DNCB on tumor sites of sensitized hosts would induce a delayed hypersensitivity reaction which would lead to tumor destruction (Klein, 1969). The data of Zbar et al. (1970) also indicated that tumor cells can be killed at the site of delayed hypersensitivity reactions. Chemical mediators involved in this type of non-specific tumor destruction were postulated. Anti-tumor activity of BCG recently demonstrated in experimental animals and man is also thought to involve a mechanism of non-specific destruction of tumor cells in immunocompetent hosts (Bast et al., 1974).

In this study, the observation of suppression of tumor growth in Gp 13 that had received both anti-GP fibrin antibodies and sensitized lymphoid cells, clearly demonstrated the induction of local inflammatory reactions, which were elicited by the interactions of sensitized cells and anti-GP fibrin antibodies within the tumor foci. Although the exact mechanism leading to tumor death has not been studied, it is believed that it is analogous to those experiments which involve the non-specific destruction of tumors that have been mentioned previously. Our results also indicate that peritoneal exudate cells might be necessary for the success of this type of tumor suppression. It is speculated that macrophages may be the cells involved since peritoneal exudate is usually rich in macrophages. The arrival of macrophages at the site of a delayed hypersensitivity reaction elicited by tuberculin had been shown by Boughton and Spector (1963). More recent studies have shown that macrophages activated <u>in vivo</u> by the intraperitoneal injection of double stranded RNA (Alexander and Evans, 1971) or infected with protozoa (Krahenbuhl and Remington, 1974) displayed cytopathic effects to tumor cells <u>in vitro</u>. In addition, it has also been suggested that tumor regression produced

by BCG treatment may be due to BCG-induced macrophage activation which would mediate a cytostatic, growth inhibitory action on the tumor (Alexander, 1973).

Regression of tumor nodules in the Gp 13 which had received antibody and sensitized cells has been observed. Also, tumor cells have been found in several organs such as liver and lymph nodes in this group of animals. Since the spread of MC-D sarcoma to other organs in Gp 13 is rare, this finding was interpreted as an indication of the immune reaction between AGFA and sensitized cells occurring in the fibrin matrix, leading to the dissolution of the fibrin lattice structure, thus allowed some of the tumor cells to escape from the fibrin network. Some of these tumor cells may eventually infiltrate to other organs.

This study thus demonstrates that an inflammatory reaction can be induced in tumor foci by the interactions of sensitized cells and and anti-fibrin antibodies which have been pre-coated onto the fibrin matrix of tumor mass, and that the creation of this toxic environment to the tumor cells would deny their further growth. Several experimental alterations may be made in order to improve the outcome for this type of study. For example, in order to increase the cytotoxic activity of sensitized lymphoid cells, they may be induced by sensitization to xenogeneic dinitrophenol (DNP) coupled IgG instead of IgG alone since the former could be more immunogenic. The use of peritoneal exudate cells as the only source of sensitized cells can be tried because of the large amounts of macrophages and K cells. Hopefully by these modifications, the intensity of the inflammatory reaction triggered by the interaction of sensitized cells and AGFA would be increased and thus more

tumor cells would be killed so that the escape of tumor cells into the blood stream could be prevented. The intratumoral injection of AGFA can also be tried since this route of injection may increase the quantity of localized antibody on tumor fibrin matrix. Moreover, it may decrease the chance of antibodies going into circulation and thus minimizes the possibility of inducing antigen-antibody reaction in other organs.

(II) Complete Regression of MC-D Sarcoma in Guinea Pigs by Daunomycin-AGFA Conjugates

Results obtained from the treatment of guinea pigs bearing the MC-D sarcoma with daunomycin-AGFA conjugates are encouraging, since 50% of the tumor-bearing guinea pigs were freed of tumors after receiving multiple intratumoral injections of daunomycin-AGFA conjugates. Obviously, the ultimate goal for this kind of treatment is 100% cure in the treated animals. This may be achieved by increasing the numbers of daunomycin molecules bound to AGFA, so that cytotoxicity of the conjugate is enhanced. Unfortunately, several attempts for this purpose, such as increasing the concentration of glutaraldehyde or prolongation of coupling time, have failed to produce active conjugate with higher ratio of daunomycin to AGFA. The use of other coupling reagents such as periodate or ethyl-3-(3-dimethylaminopropyl) carbodiimide were also unsuccessful. On the other hand, other anti-tumor drugs such as trenimon, p-phenylenediamine mustard and 6-mercaptopurine may be tried for coupling on AGFA. All these drugs have been shown cytotoxic to a variety of tumors and the procedures for conjugation of these drugs to proteins have been described (Linford et al., 1974; Rowland et al., 1975; Wagner et al., 1974). However, one should be cautious that the conditions for conjugating these drugs onto proteins are more drastic as compared to the procedure of using glutaraldehyde as coupling reagent.

On the whole, this study clearly demonstrates that the unique specificity of AGFA to tumor fibrin may be utilized in cancer studies for the following purposes:

(a) to detect the possible location of tumor.

(b) to react with the lymphoid cells sensitized to the antigenic determinants of AGFA, in order to induce an inflammatory reaction within the tumor site, which would lead to the destruction of tumor cells.

(c) to serve as specific carriers for cytotoxic drugs to the tumor nodules.

CLAIMS TO ORIGINALITY

(1) The preparation of rabbit and goat antibodies which reacted specifically to the unique antigenic determinants of guinea pig fibrin and did not crossreact with fibrinogen.

(2) The demonstration that the growth of MC-D sarcoma in strain 13 guinea pigs could be significantly suppressed by an indirect cell-mediated immune mechanism which was induced by the successive administration of: (i) rabbit or goat AGFA; and (ii) lymphoid cells from syngeneic animals which had been sensitized to a state of CMI to rabbit or goat IgG.

(3) Daunomycin was coupled to AGFA by means of glutaraldehyde and the resulting daunomycin-AGFA conjugates retained both the pharmacological and antibody activity. Complete regression of MC-D sarcoma had been achieved after multiple intratumoral injections of these conjugates into tumor-bearing guinea pigs.

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