THE UNIVERSITY OF MANITOBA

THE EFFECT OF EARLY TUMOUR GROWTH ON T CELL FUNCTIONS

by

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LIST OF ABBREVIATIONS

Ag	=	antigen
ALS	=	anti-lymphocyte serum
B Cell	=	bone marrow derived lymphocyte
BCG	=	bacille Calmette - Guérin strain of *Mycobacterium tuberculosis*
BDB	=	bis-diazotized benzidine
BSA	=	bovine serum albumin
DBS	=	Dulbecco's balanced salt solution
DDW	=	deionized distilled water
dHGG	=	deaggregated human gamma globulin
DNA	=	deoxyribonucleic acid
EA	=	hen egg albumin
FCA	=	Fruend's complete adjuvant
FCS	=	foetal calf serum
Fib	=	human fibrinogen
γF	=	mouse $7S\gamma_1$ globulin
γG	=	mouse $7S\gamma_2$ globulin
H_2	=	mouse histocompatibility complex of the mouse
HBSS	=	Hank's balanced salt solution
HGG	=	human gamma globulin
hrs.	=	hours
HRBC	=	horse red blood cells
Ia	=	I region-associated antigens
IACF	=	immunoglobulin-antigen complexing factor
Ig	=	immunoglobulin
i.p.	=	intraperitoneal(ly)

List of Abbreviations Cont'd.

i.v.	=	intravenous(ly)
LPS	=	lipopolysaccharide from *E. Coli*
MCA	=	methylcholanthrene
MEM	=	minimum essential medium
NMS	=	normal mouse serum
NNA	=	nylon wool nonadherent
PBS	=	phosphate buffered saline
PFC	=	plaque forming cell
R	=	receptor
RICA	=	reverse immune cytoadherence
RNA	=	ribonucleic acid
S.D.	=	standard deviation
SRBC	=	sheep red blood cells
T Cell	=	thymus derived lymphocytes
TL	=	thymic leukemia antigen
6HS	=	serum collected six hours after i.p. injection of antigen

To Mom, Dad and András

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ABSTRACT

Cytophilic complexes of immunoglobulin and antigen (Ig-Ag) have been previously detected in serum six hours after immunization with a variety of immunogens. These complexes which have been shown to enhance antibody formation by 50-80 fold are formed through the mediation of a "complexing" factor, IACF, derived from T cells as a result of a macrophage-T cell interaction. An inoculum of 100 live PX2-16 mastocytoma cells into syngeneic DBA/2 mice abrogates the formation of cytophilic Ig-Ag complexes *in vivo* by all immunizing stimuli. The specific enhancing activity for the 7S antibody response, normally detected in serum six hours following antigenic stimulation, is also abrogated by an inoculum of 100 live PX2-16 cells. Nylon wool nonadherent T cells from normal animals reconstituted the ability of lethally x-irradiated animals to form cytophilic Ig-Ag complexes. However, nylon wool nonadherent T cells from animals injected with 100 live PX2-16 cells failed to do so. Similarly, T cells obtained from animals injected with a tolerogenic dose of a deaggregated preparation of human gamma globulin were also unable to reconstitute the formation of Ig-Ag complexes in lethally x-irradiated animals.

Experiments *in vitro* demonstrated that macrophages obtained from animals exposed to 100 viable PX2-16 cells could cooperate effectively with T cells from normal animals for the production of IACF. On the contrary, macrophages obtained from normal animals combined with T cells obtained from animals injected with 100 live PX2-16 cells were unable to produce IACF. Finally, nylon wool nonadherent T cells from DBA/2

mice injected with 100 viable PX2-16 mastocytoma cells stimulated *in vitro* with 2-mercaptoethanol did not generate IACF while the nylon wool nonadherent T cells from normal DBA/2 mice did.

These data suggest that a tumour in its early stages of development can induce a non-specific T cell defect which inhibits the formation of cytophilic Ig-Ag complexes to antigenic stimuli. The suppression of one of the immune system's mechanisms for specifically enhancing immune responses, the formation of Ig-Ag complexes, by small amounts of antigen may be one mechanism of tumour escape from immune elimination.

INTRODUCTION AND LITERATURE REVIEW

An important development in the study of malignant disease was the demonstration of specific immunity in inbred strains of mice to tumour-associated antigens on syngeneic tumours (Foley, 1953; Prehn and Main, 1957). This observation laid the foundation for the hypothesis that tumour cells may be subject to control by the host's immune system. However, even though a host is capable of mounting an immune response directed towards the control and eventual elimination of neoplastic cells, spontaneous tumours can grow progressively despite the combined rejection responses.

The host's initial immune responses to the early stages of tumour growth are examined in this thesis. The model of a tumour growing in mice from an inoculum of a small number of syngeneic viable tumour cells was employed as a reasonable analogy to the conditions of spontaneous tumour-genesis. A prerequisite for this study is a knowledge of the concept of immune surveillance and of the hypothesis for the escape of tumours from immune elimination. The following literature review will deal with both of these topics and with the properties of one of the earliest immune responses to antigenic stimulation.

I. IMMUNE SURVEILLANCE

(i) The Development of the Hypothesis of Immune Surveillance

The concept that immune mechanisms are involved not only with protection against microbes and other outside parasites but also against aberrant cells of the organism itself was first proposed by Paul Erlich in 1909. Thomas (1959) later rephrased the idea: "It is a universal requirement of multicellular organisms to preserve uniformity of cell type .... the phenomena of homograft rejection will turn out to represent a primary mechanism for natural defense against neoplasia." The concept of immune surveillance was subsequently elaborated on by Burnet (1963, 1964, 1967, 1970, 1971). He defined the concept of immune surveillance as follows: "In large long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character."

Burnet (1970) suggested that when aberrant cells arise in the body they will carry new antigenic determinants on their cell surface. Therefore, when a significant amount of new antigen has developed, a thymus-dependent immunological response will be elicited. The aberrant cells will be eliminated in essentially the same way as a homograft is destroyed. According to standard selective theory, the normal animal possesses T cells with such a large repertoire of immune specificities that there will be some immunocytes capable of reacting significantly with any probable

aberrant cell surface antigen. Stimulation of these cells will induce clones which will act cytotoxically against the aberrant cell. Numerous reviews detailing the evidence for and against the hypothesis of immune surveillance have been published (Burnet, 1970, 1971; Moller and Moller, 1976, 1979; Stutman, 1975; Klein, 1973; Prehn, 1971, 1976).

The concept of immune surveillance was developed on the basis of experimental evidence collected by numerous investigators. The existence of an immune response to spontaneous tumours was demonstrated by Foley (1953), Prehn and Main (1957) and Klein *et al.* (1960). Tumours induced by chemical carcinogens in pure line mice were shown to carry new antigens recognizable by the host. Each MCA-induced tumour in a mouse was shown to possess a specific tumour antigen differing from all other antigens induced by the same agent on the same host strain. Consequently, it is possible by using the appropriate experimental protocol to immunize the original host against the autochthonous tumour without concurrently immunizing it against any other MCA-induced sarcoma.

Similarly, Habel (1961) and Sjögren *et al.* (1961) demonstrated that virus-induced tumours also expressed tumour-specific antigens. Group specific transplantation antigens have been shown to exist in various virus-induced tumour systems (Klein, 1969). In addition to carrying a common group specific transplantation antigen, some induced tumours express individually distinct antigens capable of inducing rejection (Morton *et al.*, 1969).

The antigenic differences between malignant cells and normal cells can, under appropriate conditions, be recognized as foreign and elicit a thymus-dependent immune response equivalent to homograft rejection. Churchill *et al.* (1968) produced transplantable hepatomas in pure line

strain 2 guinea pigs by feeding them diethylnitrosamine. An intramuscular injection of viable cells of the tumour into a syngeneic host produced a degree of partial immunity which was associated with both a typical delayed hypersensitivity response to a tumour cell extract injected intradermally and a significant degree of immunity. Similarly, Holmes *et al.* (1970) isolated a relatively pure preparation of soluble tumour-specific transplantation antigens from chemically induced guinea pig tumours which could elicit in a specific manner delayed hypersensitivity in a suitably sensitized animal.

(ii) Evidence For Immunological Surveillance

1) Evidence From Clinical Sources

Burnet (1971) stated that if the concept of immunological surveillance is legitimate, a number of phenomena should be observed at the clinical level.

a. *Correlation of the incidence of malignant disease to immunological status.*

Burnet (1971) suggested that the age of malignant disease should reflect a greater emergence of malignant change initiated at ages of relative immunological inefficiency. Full immunological capacity of an individual develops only after birth. The synthesis of immunoglobulins increases to significant levels after approximately six weeks of life (Cooper and Lawton, 1974). Many childhood cancers, such as neuroblastoma, Wilm's tumour, retinoblastoma, Burkitt's lymphoma and acute leukemia, show an age incidence which suggests that the process of malignant change was initiated perinatally (Collins *et al.*, 1956; Knox and Pillers, 1958; Pollock *et al.*, 1960). The increasing incidence of malignancy with the advance of old age may be related to the decreased capacity to mount an

immunological attack against foreign antigens to which there was no previous exposure (Sabin *et al.*, 1947).

b. Correlation of the incidence of malignant disease with depression of the thymus dependent immune system.

The immune surveillance concept predicts that conditions associated with depression of the thymus-dependent immune system should increase the incidence of malignant disease (Burnet, 1971).

The failure of skin sensitization with dinitrofluorobenzene in cancer patients suggests a general weakness of the thymus-dependent immune system (Levin *et al.*, 1964). Similarly, Harris and Copeland (1974) reported that some cancer patients have decreased cell-mediated immune response to a variety of antigens. Eilber and Morton (1970) suggested that patients with decreased cell-mediated immunity have more rapid tumour growth.

Good (1973) concluded that patients with primary immunodeficiency diseases, Bruton-type agammaglobulinemia, ataxia-telangiectasia, common variable immunodeficiency and Wiskott-Aldrich syndrome, developed cancer far more frequently than occurs at a comparable age in the general population. The increased incidence of *de novo* tumours in people with naturally occurring immunologic deficiency diseases has also been reported by other authors (Waldman *et al.*, 1972; Page *et al.*, 1963).

Immunosuppressive drugs are given in high dosage for extended periods of time with organ transplantation. Penn (1975) reported that there is a 5.6% incidence of *de novo* malignancies in organ transplant recipients, undergoing chronic immunosuppressive therapy. This incidence is approximately 100 times greater than that observed in the general population in the same age range. Starzl *et al.* (1971) described four patients who had received kidneys from cancer victims as donors, were placed on chronic

immunosuppression, and developed malignancies of the same histologic type. The local or distant metastases in two patients underwent involution and finally complete disappearance after discontinuance of immunosuppression and loss of the renal homografts.

c. Correlation of spontaneous regression of malignant disease with a detectable immune response.

Burnet (1971) suggested that under certain circumstances spontaneous regression of cancer can occur and the results of therapy may be better than expected. There should be detectable indications that immune responses are playing a part in these phenomena.

The spontaneous remissions of different human tumours have been recognized (Lewison, 1976). Everson (1964) presented 130 instances in which a clinically and histologically diagnosable malignant tumour underwent spontaneous regression.

When tumour therapy is successful, it is reasonable to suggest that destruction or excision of all neoplastic cells is incomplete, but an initially ineffective immune response is able to eliminate the remaining tumour cells. Neuroblastoma has been successfully treated with surgery (Pollock *et al.*, 1960). Cytotoxic drug therapy has been successful in both choriocarcinoma (Hertz *et al.*, 1964) and Burkitt's lymphoma (Burkitt, 1967). In both cases there are antigenic differences between the tumour cells and their host. Choriocarcinoma can be considered as a homograft of foetal tissue. Burkitt's lymphoma has a specific antigen associated with it (Klein, 1969).

d. Correlation of histologically diagnosable cancer with the expected clinical incidence.

Burnet (1971) suggested that large scale routine histology of common

sites of cancer should reveal a higher incidence of histologically diagnosable cancer than the incidence that emerges clinically. Beckworth and Perrin (1963) found in randomly selected autopsies of infants up to three months of age that small histologically typical neuroblastomas are 40 to 50 times more frequent than would be expected from the incidence of clinical adrenal neuroblastoma. Two other types of malignant tumours which are more frequently diagnosed in random pathological samples than found clinically, are thyroid carcinoma (Lancet, 1964) and carcinoma of the prostate (Asheley, 1965).

2) Experimental Evidence for Immune Surveillance

Burnet (1971) suggested four phenomena which could be expected in relation to laboratory studies on experimental cancer if the concept of immune surveillance is correct.

a. Correlation of neonatal thymectomy and tumour incidence.

Klein (1973) suggested that immune surveillance against virus-induced tumours is strongly supported by the reports of many investigators that neonatal thymectomy or treatment with anti-lymphocytic serum (ALS) increased the frequency of experimental tumours induced by DNA or RNA viruses in various laboratory rodents. Law (1966) demonstrated that C57BL mice are resistant to tumour development induced by polyoma virus if they were born from an uninfected mother and can be rendered susceptible by thymectomy at age three days. Allison *et al.* (1967) reported an increased incidence of tumours by adenovirus type 12 in mice which were thymectomized or treated with ALS. Furthermore, Allison and Taylor (1967) reported that reconstruction of the T cell system would prevent the tumours. Similarly, adult laboratory mice that were normally resistant to the oncogenic effect

of the virus became susceptible to tumour induction by room infection if x-irradiated (Law and Dawe, 1960) or treated with ALS (Gaugas *et al.*, 1973).

b. Correlation of immunological tolerance to oncogenesis.

Burnet (1971) suggested that foetal or perinatal tolerance should be able to annul the immune surveillance effect. In tumours associated with vertically transmitted viruses such as the Moloney leukemia virus (Klein and Klein, 1965), the Bittner milk agent (Attia *et al.*, 1965), and the Gross virus (Axelrad, 1963), it has been demonstrated that there is specific immunological tolerance to both the virus and the tumour cells. Attia *et al.* (1965) demonstrated that intrauterine infection with the Bittner virus allowed the development of tolerance not only to the antigens of the Bittner virus but also to any derived transplantation antigens. Similarly, Klein and Klein (1965) reported that in the case of the Moloney leukemia virus tolerance to the specific transplantation antigen was attributable to neonatal infection. Mice inoculated with either the Moloney leukemia virus or the Bittner virus as adults can develop leukemia or mammary carcinoma but at a lower frequency than neonatally infected mice.

c. Correlation of immunosuppressive agents with facilitation of experimental transfer of tumour cells.

Several authors have observed that carcinogenic hydrocarbons can depress immune responses. Immune responses that have been reported to be depressed by carcinogenic hydrocarbons are antibody titres (Malmgren *et al.*, 1952), plaque-forming cells (Stjernswärd, 1966a) and the rejection of skin homografts (Linder, 1962). Prehn (1963) demonstrated the facilitation of tumour graft acceptance in syngeneic mice by MCA treatment. Similarly, Stjernswärd (1966b) reported that MCA treatment increased tumour take of injections of 10^3 and 10^4 tumour cells as opposed to untreated

animals.

d. Correlation of immune paralysis with excess antigen from an active tumour.

If the concept of immune surveillance is valid, immune paralysis by excess antigen from an active tumour should be observed in studies on experimental cancer (Burnet, 1971). Soluble "blocking factors" in the serum of tumour bearing hosts have been widely studied (Hellström and Hellström, 1974; Baldwin, 1973). The factors have been reported to block target tumour cells from lymphocyte mediated cytotoxicity *in vitro* and to enhance tumour growth *in vivo*. The "blocking factors" have been found associated with chemically induced, virally induced, and spontaneous tumours in several species. Both soluble free tumour antigen and soluble tumour-antigen-antibody complexes can successfully block cytotoxic lymphocytes and have been implicated as two possible "blocking factors" (Baldwin, 1973).

(iii) Evidence Against Immune Surveillance

In the last few years the concept of immune surveillance has been challenged by several authors (Prehn, 1971, 1976; Moller and Moller, 1976, 1979; Stutman, 1975; Rygaard and Polvsen, 1976).

1) Evidence From Clinical Sources

An immunodeficient individual is predicted by the concept of immune surveillance to exhibit an increased frequency of polyclonal tumours. It is interesting to note that most benign and malignant tumours in man studied with cell markers have a monoclonal origin (Friedman and Fialkow, 1976; Fialkow, 1974).

In organ transplant patients undergoing chronic immunosuppressive therapy there is a remarkably high incidence (33%) of lymphomas as compared to 3% - 4% in the general population (Penn, 1979). The types of cancer

that are most frequently seen in the public at large are uncommon in transplant patients. Examples of this observation are carcinoma of the prostate (17% versus 2%), carcinoma of the female breast (26% versus 11%), and lung cancer in males (20% versus 8%) (Penn, 1979). The only non-lymphoid tumours which show a slight increase of incidence in transplant patients are skin, lip and cervical cancers (Stutman, 1975; Penn, 1979). Moller and Moller (1976) note that since tumours with increased incidence are suspected to be of viral origin, this supports their hypothesis that the function of the T cell network is to counteract virus infections and not to reject newly arising tumours.

Furthermore, there are several diseases such as leprosy, sarcoidosis and uremia with pronounced immunosuppression but no increase in tumour incidence (Stutman, 1975).

2) *Evidence from Experimental Sources*

The concept of immune surveillance predicts that tumours induced *in vitro* should possess generally stronger tumour-specific antigens than tumours arising spontaneously *in vivo*. Experiments by Prehn (1970) and Heidleberger (1973) do not agree with the prediction. Prehn (1970) demonstrated that *in vitro* tumours were on the average less antigenic than *in vivo* induced tumours. Heidleberger (1973) found that *in vitro* induced tumours ranged from no antigenicity to the expected antigenicity as compared to those induced *in vivo*.

Nude mice which are congenitally athymic and therefore lack T cell function do not develop spontaneous tumours. Rygaard and Povlsen (1976) did not detect one spontaneous malignant tumour in the observation of 15,700 nude mice for a total of 67,200 months of mouse life, corresponding to approximately 5,600 mouse years. The immune surveillance concept pre-

dicts that these mice would develop a high incidence of spontaneous tumours. Nude mice, however, are susceptible to virus induced tumours (Allison *et al.*, 1974).

Similarly, immunosuppressed mice do not develop spontaneous tumours. Simpson and Nehlsen (1971) reported that in anti-thymocyte serum treated CBA mice the very low frequency of spontaneous tumours was equivalent to that of untreated CBA mice. In one group in their studies a high incidence of tumours was induced by polyoma virus contamination of the anti-thymocyte serum. Doherty and Zinkernagel (1974) demonstrated that the T cell system specifically recognizes virus induced alterations of histocompatibility antigens.

Both immunosuppressed and nude mice are susceptible to viral and chemical oncogenesis (Rygaard and Polvsen, 1976). Moller and Moller (1976) suggest that the lack of spontaneous tumour appearance when T cell function is absent cannot be attributed to another surveillance mechanism peculiar to these mice.

The immune surveillance theory predicts that tumours should occur in immunologically privileged sites such as the anterior chamber of the eye, cheek pouch of the Syrian hamster, or the brain. While immune surveillance predicts that tumour incidence at these sites would be very high, this is not the case (Moller and Moller, 1976).

The appearance of multiclonal and polyclonal tumours once the T cell system has failed is predicted by the immune surveillance theory. Tumours are generally of monoclonal origin in animals (Potter *et al.*, 1973; Prehn, 1970) and occur at one site (Moller and Moller, 1976). The frequency of double tumours is that expected by chance from the frequencies of each tumour type (Moller and Moller, 1976).

Prehn (1971, 1976) has questioned the general efficiency of immunosurveillance. He suggests that there are three observations which argue against the possibility that levels of immunity too low to be detected by the standard transplantation tests might be effective as a surveillance mechanism. Firstly, it has been observed that a nascent tumour probably does not immunize the host appreciably. This observation has been demonstrated with both murine skin papillomas and murine hydrocarbon induced mammary tumours (Slemmer, 1972; Andrews, 1974). Secondly, inocula of very small numbers of tumour cells take preferentially over inocula of larger numbers of the tumour cells. This phenomenon known as "sneaking through" or "dilution escape" has been observed with highly immunogenic tumours transplanted into syngeneic mice and even into allogeneic mice (Humphreys *et al.*, 1962; Bonmassar *et al.*, 1974; Old *et al.*, 1966; Mengersen *et al.*, 1975). Thirdly, a very weak immune reaction, rather than inhibiting tumour growth, may stimulate it. Prehn (1971, 1976) suggests that the immune reaction to tumour-associated antigens under natural conditions and early in the development of transformed cells *in situ* mildly stimulates the growth of the tumour.

II. TUMOUR ESCAPE FROM IMMUNE ELIMINATION

Small numbers of tumour cells are able to escape immune elimination and proliferate, resulting in the death of the host. Prehn (1971) suggests that if the progressive growth of a tumour is a result of disruption of an immune surveillance mechanism, then malignancy may represent a failure of the host's immune system. The following is a list of mechanisms for the failure of the immune response in the tumour bearing host that have been demonstrated to operate in various tumour systems.

(i) Non-Antigenic Tumours

A tumour may escape destruction by an immune response by not expressing cell surface antigens that can be recognized by the autochthonous host as foreign. Baldwin (1973) demonstrated that a variety of histological types of rat tumours induced by acetylaminofluorene are not detectably antigenic in rejection tests. This has also been demonstrated with a number of spontaneous carcinomas and sarcomas (Baldwin, 1973; Prehn, 1969). Strouk *et al.* (1972) found a murine sarcoma virus induced but murine leukemia virus free line of cells. The cells lacked detectable surface antigenicity as tested by a number of sensitive humoral antibody tests. These tests can readily detect surface antigens associated with murine leukemia virus carrying lymphomas or sarcomas.

(ii) Antigenic Modulation

Tumour cells under immunological attack may circumvent the immune system by not expressing the tumour-specific antigen to which the immune response is directed. Boyse and Old (1969) originally detected in the study of the thymus leukemia antigen in mice that when some cell types are exposed *in vivo* to an ongoing immune response against an antigenic determinant the determinant will no longer be expressed. The phenotypic change is re-

versible since the determinant is re-expressed after *in vitro* culture of the cells. Fenyo *et al.* (1968) produced a stable subline of a Moloney lymphoma immunoresistant to Moloney-specific cytotoxic antiserum by incubating Moloney lymphoma cells in the cytotoxic antiserum and complement, and injecting the treated cells into mice preimmunized with the Moloney lymphoma. The loss of H_2 antigens of one parental strain from a tumour arising in an F_1 hybrid animal can be accomplished by passaging the tumour in the other parental strain (Bjaring and Klein, 1968). The evidence suggests that significant antigenic changes coupled with selective outgrowth of the variant cell is a possible mechanism for tumour escape from immune elimination.

(iii) Specific Immune Tolerance

Specific tolerance has been demonstrated in the case of several tumours induced by vertically transmitted virus - Moloney leukemia virus (Klein and Klein, 1965), Bittner milk agent (Attia *et al.*, 1965), and the Gross virus (Axelrad, 1963). The specific immune tolerance is directed against both the virus antigen and the virus derived transplantation antigen.

(iv) Immunodeficiency of the Host

In the literature there are numerous examples in both experimental and human tumour systems where increased tumour incidence has been attributed to a deficiency of the immune system. As previously discussed, neonatal thymectomy, anti-lymphocyte serum treatment and x-irradiation all increased tumour incidence in experimental situations (Allison *et al.*, 1967; Law and Dawe, 1960; Gaugas *et al.*, 1973). Chronic immunosuppressive therapy in organ transplant patients increased the incidence of several types of tumours but not a general increase in tumour incidence (Penn, 1979). Patients with certain primary immunodeficiency diseases such as Wiskott-

Aldrich syndrome, ataxia telangiectasia and common variable immunodeficiency exhibited an increased incidence of malignant disease (Good, 1973; Waldman *et al.*, 1972).

(v) Immunologically Privileged Sites

Certain sites in experimental animals and in humans — such as the syrian hamster cheek pouch and the brain — have been reported to be immunologically sheltered (Barker and Billingham, 1977). It is possible that tumour initiation could occur at these sites hidden from surveillance by the host's immune system. Since tumours are relatively rare at these sites (Moller and Moller, 1976), it is unlikely that immunologically privileged sites are a major escape mechanism.

(vi) Immune Enhancement

Kaliss (1958, 1970) described immune enhancement as the successful establishment of normally rejected tumour homografts in recipients who were pretreated with either antiserum directed against the tumour or with repeated injections of a tumour antigen preparation which induced humoral antibody formation. The enhancement of tumour growth *in vivo* was attributed to soluble "blocking antibodies" (Kaliss, 1958; Hellström and Hellström, 1970). Numerous studies have also demonstrated that serum from tumour bearing individuals can inhibit all cell mediated cytotoxicity *in vitro* (Hellström and Hellström, 1974; Baldwin, 1973; Bowen *et al.*, 1975). The experimental evidence indicates that the most significant "blocking factors" are free tumour antigen or immune antigen-antibody complexes or antibody (Bowen *et al.*, 1975; Tamerius *et al.*, 1976). The factors may function by blocking the target cells thereby preventing recognition by sensitized lymphocytes or by directly inhibiting the effector cells (Baldwin, 1973). The interference of humoral factors with cellular immunity may contribute to the

escape of tumours from immunological control.

The "blocking" activity of serum from tumour bearing animals has been reported to be inhibited by serum from tumour regressor animals (Hellström and Hellström, 1974; Bowen *et al.*, 1975). This has been interpreted to result from the conversion of blocking complexes containing antigen in excess to complexes of antibody excess.

(vii) Suppressor Cells

Recently, it has been proposed that host suppressor cells and their factors facilitate tumour growth by inhibiting the immune reactivity of the host. Numerous investigators have reported direct experimental evidence indicating the effect of specific and nonspecific suppressor cells on the interactions between the host's immune system and the tumour (Broder and Waldman, 1967; Noar, 1979; Kamo and Friedman, 1977; Gorczynski, 1974; Fujimoto *et al.*, 1976a, 1976b).

Specific suppressor cells inhibit immune responses directed towards the tumour antigens which had originally specifically induced the appearance of the suppressor cells. Unrelated immune responses are not affected. Fujimoto *et al.* (1976a, 1976b) demonstrated the presence of specific suppressor T cells which enhanced tumour growth in mice immunized against the syngeneic tumour. An intravenous transfer of 10^7 to 10^8 thymus or spleen cells from A/Jax mice bearing the MCA-induced S109a syngeneic tumour to S109a immunized A/Jax mice significantly inhibited rejection of the tumour (Fujimoto *et al.*, 1976a). The transfer of 10^7 to 10^8 thymus or spleen cells from normal A/Jax mice or from A/Jax mice bearing an unrelated tumour did not have a suppressive effect. The suppressor cells were cortisone resistant but sensitive to treatment with anti-theta antisera and complement (Fujimoto *et al.*, 1976b).

The immunosuppressive T cells were shown to produce soluble factors with similar suppressive activity (Greene *et al.*, 1977). The factor was immunologically specific since absorption of the factor with the syngeneic tumour but not with unrelated syngeneic tumours resulted in the complete loss of suppressive activity. The suppressive factor of the tumour bearing host suppressor cells carries $I\text{-}J^k$ and antigen specificities (Greene *et al.*, 1978). Fujimoto *et al.* (1978) found that spleen cells from tumour-bearing A/J mice which have been treated with anti-$I\text{-}J^k$ antiserum lost their suppressive activity. These experiments suggest that anti-I-J antiserum can selectively eliminate suppressor cells so that an immunological balance favouring tumour resistance is established.

The anti-tumour immune response is regulated by specific suppressor T cells in a number of different tumour-host systems (Fujimoto *et al.*, 1978; Takei *et al.*, 1976, 1977).

Nonspecific suppressor cells of normal or tumour-bearing mice inhibit immune responses which are not specifically directed toward the relevant tumour antigens (Noar, 1979). Nonspecific suppression could affect the immune responses directed toward the relevant tumour antigens, thereby promoting tumour growth. Gorczynski (1974) reported that the depressed immune responses of the Moloney sarcoma virus infected spleen cells was a result of the presence of suppressor cells. The suppressor cells were thought to be B cells, since they were sensitive to treatment with anti-mouse immunoglobulin antisera and complement but were resistant to treatment with anti-theta antisera and complement.

Treves (1974) demonstrated that splenocytes of tumour-bearing mice contained suppressor T cells which antagonized anti-tumour immunity, thereby promoting tumour growth. The suppressive effect was nonspecific and was

mediated by soluble factors (Treves *et al.*, 1976).

Igenito and Calkins (1981) demonstrated that inoculation of 10^6 L5178Y tumour cells into their syngeneic host activates cells capable of inducing nonspecific suppression of antibody responses *in vitro*. Nylon wool nonadherent Ly-1^+ T cells from the spleens of tumour-bearing mice apparently mediated this activity.

Nonspecific suppressor T cells have also been found in humans. The blood of patients with Hodgkin's disease contains mitomycin c resistant suppressor cells which could significantly inhibit the proliferative response of autologous cells to allogeneic stimulator cells in mixed lymphocyte culture (Hillinger and Herzig, 1977). Thorley-Lawson *et al.* (1977) found suppressor T cells that inhibited the transformation and proliferative response of B cells after *in vitro* Epstein-Barr virus infection.

(viii) "Sneaking Through" and "Dilution Escape"

In several different tumour-host systems very low doses of neoplastic cells have been observed to grow progressively in host animals while larger doses were either rejected or grew very slowly. The preferential take of small numbers of tumour cells has been described for both syngeneic and allogeneic tumours (Old *et al.*, 1962; Humphreys *et al.*, 1975; Bonmassar *et al.*, 1971, 1973, 1974; Kolsch *et al.*, 1973; Mengersen *et al.*, 1975; Cihak *et al.*, 1981). The terms "sneaking through" (Humphreys *et al.*, 1962) and "dilution escape" (Bonmassar *et al.*, 1971) were coined to describe this phenomenon.

The tumour-host system investigated by Bonmassar *et al.* (1971, 1973, 1974) was the LAF-17 lymphoma carried in B10.A mice with allogeneic B10.A(5R) mice as the hosts. They observed a 63% incidence of mortality after injection of 10^4 tumour cells as compared to a 3% incidence of mortality

following injection of 10^7 tumour cells. However, if the injection was preceded three days by an injection of 10^4 irradiated tumour cells, the inocula of 10^7 tumour cells were no longer rejected. The authors suggested that a diffusable factor associated with lymphomas and other tumours non-specifically depressed the anti-lymphoma reactivity of the mice.

Kolsch *et al.* (1973) reported an increased incidence of tumours after intraperitoneal injection of 10^2 to 5×10^2 BM3 mastocytoma cells into syngeneic Balb/c mice than after injection of 10^3 to 10^4 BM3 tumour cells. Pretreatment of Balb/c mice with 10^2 to 5×10^2 irradiated BM3 cells, the same dose range at which sneaking through was observed with live tumour cells, facilitates tumour take after injection of 10^5 living tumour cells (Mengersen *et al.*, 1975). Determinant-specific suppression of immune reactivity after injection of 10^2 living tumour cells in generally unsuppressed mice was also detected. The authors suggested that the inoculum of a small number of tumour cells induces low zone tolerance which is mediated by suppressor cells.

Similarly, Cihak *et al.* (1981) reported that specific suppression of the immune system was induced by injection of a dose of ADJ-PC-5 plasmacytoma cells into syngeneic Balb/c mice 10^3 times lower than that which could induce protective immunity. In both of the tumour systems discussed, suppression of the immune system was induced by a low tumour cell load.

Grossman and Berke (1980) proposed a simple kinetic model for the interaction between a tumour and the immune system, paying special attention to the phenomenon of sneaking through. They proposed that the combination of two factors is essential for the explanation of this phenomenon: the dependence of the immune response on antigen dose and the negative intervention of immunosuppressors. The first feature enhances the chance of

survival of an inoculum of a small number of tumour cells. Combination of the two features enables a small tumour to grow progressively without eliciting a strong immune response.

III. CYTOPHILIC IMMUNOGLOBULIN-ANTIGEN COMPLEXES

Within six hours after immunization the sera of mice contain antigen-complexed immunoglobulin cytophilic for T cells. Both T cells which have taken up the cytophilic complexes and sera containing the cytophilic complexes can specifically and significantly enhance antibody formation. The following section will describe not only the physical, chemical and biological properties of the cytophilic complexes, but also the effect of an inocula of a small number of syngeneic tumour cells on the generation of the complexes.

(i) Detection of Cytophilic Immunoglobulin in Serum Within Six Hours of Antigenic Stimulation

Paraskevas *et al.* (1972) demonstrated that within six hours of intraperitoneal injection of soluble protein antigens in FCA or of particulate antigens there is a substantial and statistically significant increase of γ-globulin carrying cells. This increase was 8% to 10% of the spleen cells or 20% to 25% of γ-globulin carrying cells before immunization. The increase was followed by a decrease at 24 hours reaching a minimum two weeks after antigenic stimulation and returning to normal by the fourth week. They also found that treatment of normal spleen cells *in vitro* with sera collected six hours after immunization was capable of increasing the number of γ-globulin carrying cells to that observed *in vivo* in spleen cells from animals immunized with the same dose of antigen.

The changes in the number of γ-globulin carrying spleen cells after antigenic stimulation were studied using the reverse immunocytoadherence (RICA) technique (Paraskevas *et al.*, 1971a, 1971b). Briefly, this technique involves a 5S hybrid antibody with one anti-mouse immunoglobulin site and one anti-BSA site. Through the former site the hybrid antibody combines

with immunoglobulin carrying lymphocytes and through the latter site with BSA-coated sheep erythrocytes. A rosette of sheep erythrocytes is formed around the larger lymphocyte. The number of rosette forming cells per thousand splenic lymphocytes is determined. It has been found that RICA detects surface associated γ-globulin on lymphocytes but not "soluble" γ-globulin in the process of secretion as in normal (Paraskevas *et al.*, 1971b) or neoplastic plasma cells (Paraskevas *et al.*, 1970).

Lee and Paraskevas (1972) demonstrated using the RICA technique that the increase of γ-globulin cells six hours after antigenic stimulation occurs from the acquisition of a γG-globulin by a cell previously carrying no γ-globulin. The cell that takes up the cytophilic γG-globulin is a Thy-1 positive lymphocyte. Lethally x-irradiated mice reconstituted with thymocytes, which home to the spleen, show the ability to produce an increased number of γ-globulin carrying cells after challenge with antigen. The data suggests that antigen affects a much larger population of cells than just the antigen sensitive cells.

(ii) Detection of Cytophilic Ig-Ag Complexes in Serum Within Six Hours of Antigenic Stimulation

Orr and Paraskevas (1973) demonstrated that in the case of soluble proteins injected with Freund's complete adjuvant the cytophilic Ig detected in the serum within six hours of immunization was Ig-Ag complexes. Experiments involving the incubation of fractions from G-200 Sephadex column chromatography of sera collected six hours after intraperitoneal injection of BSA or human fibrinogen (Fib) in FCA, with normal spleen cells suggest that the activities which are detected by RICA are not associated with antigen alone or otherwise altered 7SγG globulin. Only the void volume fraction contained the activity of the 6HS induced by human Fib, while only the 7S fraction con-

tained the activity of the 6HS induced by BSA (6HS-BSA). By the Ouchterlony technique the 7S fraction of 6HS-BSA gave a strong precipitin line with anti-mouse Ig and a weak line with anti-BSA. Autoradiography of the gels demonstrated that both precipitin lines contained radioactivity indicating that the BSA in the 7S fraction of the 6HS-BSA is complexed with γ-globulin. It is highly unlikely that the complexes represent conventional antibody-antigen complexes since they appear too early after immunization.

Previously, complexes of Ig and Ag were detected in the serum of rabbits five hours after immunization by Yuan *et al.* (1970). The immunoglobulin that complexed with Ag in the circulation appeared to be primarily of the IgG class.

Indirect evidence (Paraskevas and Lee, 1976) strongly suggested that the cytophilic Ig taken up by T cells six hours after immunization with SRBC are also cytophilic Ig-Ag complexes.

(iii) Formation of Ig-Ag Complexes

A factor, detected in the 4S fraction of sera from mice injected six hours earlier with FCA, generated cytophilic Ig for T cells when added *in vitro* to a mixture of the 7S fraction of NMS and a soluble foreign protein (Orr and Paraskevas, 1974). Both the presence of Ig and foreign Ag were necessary in the mixture for generation of cytophilic Ig. The activity of the 4S fraction was called the immunoglobulin antigen complexing factor (IACF). Particulate antigens and LPS were both found to induce in *in vitro* thymocyte cultures a similar activity to that which was found in the 4S fraction of FCA sera (Paraskevas *et al.*, 1976).

The production of IACF is a result of a macrophage-T cell interaction, occurring when macrophages are present in an optimal proportion (4%) in relation to T cells (Paraskevas *et al.*, 1979). The macrophage-T cell

interaction takes place through a mediator released from macrophages. Only the supernates from BCG stimulated macrophages induced production of IACF from nylon wool nonadherent T cells. The Fc receptor negative T cell subpopulation produced IACF. Characterization of IACF indicates that it is a small molecular (10,000 to 50,000 daltons) weight substance. IACF can be released from T cells by other substances with free sulfhydryl groups such as 2-mercaptoethanol. Production of IACF is dependent on active protein synthesis but not carbohydrate metabolism. IACF is sensitive to trypsin but resistant to heating at 56^{o}C.

(iv) Cellular Uptake of Ig-Ag Complexes

The cytophilic Ig-Ag complexes have been shown to be taken up by 20% to 25% of splenic T cells (Orr and Paraskevas, 1973). More recently, Paraskevas and Lee (1979) have shown that the Ig-Ag complexes are taken up by Fc^{+} T cells. It is interesting to note that Fc^{+} T cells in the spleen make up 24% of T cells or about 9% of all spleen cells (Stout *et al.*, 1975). It was shown, however, that the Fc receptor of the T cells is not necessary for the uptake of Ig-Ag complexes since blocking of the Fc receptor still allows the Ig-Ag complexes to be taken up by the Fc^{+} T cells. The receptor for the Ig-Ag complexes is labile and is lost during culture of these cells *in vitro*. The T cells recover the receptor for the Ig-Ag complexes upon exposure *in vitro* to a macrophage supernate. Characterization of the receptor suggests that it bears determinants coded by the I region of the major histocompatibility complex.

(v) Biological Activity of Ig-Ag Complexes

T cells which have acquired surface Ig six hours after antigenic stimulation (6H T cells) exert a pronounced amplification effect on antibody synthesis in collaboration with bone marrow cells. Paraskevas and

Lee (1976) found that 6H T cells educated with SRBC, combined with normal bone marrow cells induce a six-to seven-fold increase in the number of 7S PFC and a three-fold increase of 19S PFC. In comparison, T cells collected five days after antigen stimulation amplified both the 7S response and the 19S response three-fold.

Lee and Paraskevas (1981) demonstrated that serum collected six hours after intraperitoneal injection of particulate antigens or soluble proteins in FCA contains a potent and specific enhancing factor which significantly enhances antibody formation when given with a subimmunogenic antigen challenge. The factor is antigen-specific and enhances antibody formation maximally when it is injected two hours before antigenic challenge. When SRBC is the antigen used for immunization, the enhancement of the number of 7S PFC is thirty-seven-fold and for 19S PFC is fifty-three-fold if the initial challenge dose is 5×10^8 SRBC and the subimmunogenic challenge is 10^5 SRBC. The kinetics of the enhanced antibody response and the short latent period resemble the classic secondary response (Eidinger and Pross, 1967). In the absence of T cells the enhancement of the 19S and 7S responses is revealed by different antigen doses and is smaller for both the 19S and 7S responses. This suggests that the enhancing factor can activate B cells directly or through macrophage. The factor is carrier-specific and enhances the primary anti-hapten 7S responses.

The specific antibody enhancing activity of 6HS is associated with the Ig-Ag complexes present in the serum at this time (Lee and Paraskevas, 1981). Besides Ig and Ag, the Ig-Ag complexes were found to contain Ia antigens. Passing 6HS induced by SRBC through anti-SRBC, anti-mouse Ig and anti-Ia immunosorbent columns removed the enhancing activity of the 7S response but had no effect on the 19S response. Both the anti-SRBC and anti-mouse Ig columns were also able to remove the cytophilic Ig from the

six hour sera as measured by RICA. Lee and Paraskevas (1981) suggested that one mechanism of enhancement of antibody formation by Ig-Ag complexes is through their uptake by T cells.

(vi) Effect of Syngeneic Tumour Cells on Formation of Ig-Ag Complexes

The formation of Ig-Ag complexes apparently represents a universal phenomenon since it has been observed with antigenic stimulation by particulate antigens, soluble antigens, allogenic cells, and also syngeneic tumour cells. Dular *et al.* (1978) found that complexes, presumably of Ig and a tumour antigen, are formed within six hours after intraperitoneal injection of 10^7 frozen and thawed L5178Y leukemia cells into their syngeneic host DBA/2 mice.

Chow *et al.* (1978) reported that an intraperitoneal injection of 100 live L5178Y leukemia cells into DBA/2 mice, an inocula which would eventually kill 100% of the animals, is able to abolish the ability of the animals to form cytophilic Ig-Ag complexes to horse erythrocytes or 10^7 L5178Y cells for as long as 14 days following the injection. Single intraperitoneal injections of either a tumour cell antigen preparation or 50 µg deaggregated human gamma globulin was also able to abolish the ability of the animals to form cytophilic Ig-Ag complexes to horse erythrocytes or 10^7 L5178Y leukemia cells for three days following inoculation. It was further demonstrated that both an intraperitoneal injection of 100 live L5178Y leukemia cells or a small dose of tumour cell antigen preparation abolished the enhancing activity which six hour educated T cells exert on antibody formation. The observed unresponsiveness is non-specific in the sense that Ig-Ag complexes cannot be induced against antigenic stimulation unrelated to tumour cells. In summary, the data suggested that the effect of small tumour cell inocula on the induction of cytophilic Ig-Ag complexes

is probably mediated by a product released from the tumour cells and that the product may represent a tumour cell antigen.

MATERIALS AND METHODS

I. MICE

Inbred DBA/2 male mice, six to eight weeks old, were obtained from Jackson Laboratories, Bar Harbor, ME. The DBA/2 mice were matched for sex and age in each experiment.

II. RABBITS

Outbred white rabbits, weighing approximately 5 lbs., obtained from Canadian Breeding Laboratories, St. Constant, Quebec, were used for the preparation of rabbit anti-BSA and anti-mouse immunoglobulin sera.

III. TUMOUR CELLS

The tumour used in this study was a clone, P815-16, of the methylcholanthrene-induced murine mastocytoma P815X2 syngeneic for DBA/2 mice. The P815-16 clone was obtained from Dr. A. H. Greenberg, Department of Immunology, University of Manitoba, and maintained in culture. The tumour line was cultured in Fischer's medium (Gibco Canada Ltd., Calgary, Alberta) containing 50 mg/ml streptomycin and 50 IU/ml penicillin (Gibco) and 10% foetal calf serum (Gibco). The doubling time of the P815-16 cells in culture was approximately 12 hours. The cells were washed three times with Hank's Balanced Salt Solution (HBSS; Gibco) at 4°C before use.

IV. ANTIGENS

1) Bovine Serum Albumin and Egg Albumin

Bovine serum albumin (BSA) and egg albumin (EA) were obtained from Pentex, Kanakee, IL.

2) Sheep Red Blood Cells

Sheep red blood cells (SRBC) were purchased from National Biological Laboratory, Winnipeg, Manitoba. The blood was collected in citrate buffer. The cells were washed three times in 0.15 M saline before use.

3) Human Gamma Globulin

Human gamma globulin (Hgg) fraction II was obtained from Pentex, Kanakee, IL. The method of Chiller and Weigle (1971) was used to prepare deaggregated Human gamma globulin (dHgg). A 0.60 g sample of Hgg was dissolved in 20 ml of 0.15 M saline (30 mg/ml). The solution was ultracentrifuged at 40,000 rpm (approximately 100,000 g) for three hours at 4°C using a Ti50 rotor in an L_2 preparative centrifuge (Beckman Corp., Toronto, Ontario). The upper one-third of the solution was carefully removed, the protein content determined and used immediately.

V. ADJUVANTS

Freund's complete adjuvant (FCA) was obtained from Difco Laboratories, Detroit, MI.

BCG vaccine was obtained from Connaught Medical Research Laboratories, Toronto, Ontario.

VI. PREPARATION OF SIX HOUR IMMUNE SERA AND NORMAL SERA

Mice were immunized with the appropriate antigen, with or without FCA, in a 0.2 ml volume by intraperitoneal injection. For soluble protein antigens, 250 µg of the protein in 0.1 ml of 0.15 M saline was emulsified with

0.1 ml of FCA. Controls used were 0.1 ml of 0.15 M saline emulsified with 0.1 ml FCA and 250 µg soluble protein in 0.15 M saline. For particulate antigens, the desired number of cells were injected intraperitoneally in 0.2 ml of 0.15 M saline.

Six hours after injection, the mice were anesthesized with chloroform and bled by cardiac puncture. The blood was allowed to clot at room temperature for 30 minutes. The clot was centrifuged at 3,000 rpm for five minutes to recover the supernate. The serum was stored at 4°C and was used within one day.

Normal DBA/2 serum (NMS) was obtained from non-immunized mice in the above manner.

VII. OUCHTERLONY GEL DIFFUSION

Glass slides (25 x 76 cm) (Becton, Dickinson and Company, Parsippany, NJ) were coated with 2 ml of 0.5% melted noble agar (Difco Laboratories, Detroit, MI) in deionized distilled water and dried at 80°C for four hours. The coated slides were layered with 3 ml of 1.5% melted noble agar in 0.15 M saline. The gels were allowed to solidify and wells were cut using a template. The wells were filled with antigen or antibody solution in a prearranged pattern which would allow the formation of the desired antigen-antibody precipitin lines. The slides were placed in a moist chamber at room temperature overnight. They were then washed for 24 hours in 0.15 M saline and then washed for another 24 hours in deionized distilled water. The slide was dried overnight by placing filter paper on the slides. The slides were then stained with amido black (1 gm/1,000 ml sodium acetate buffer) for ten minutes, decolorized twice in an acetic

acid solution (150 ml acetic acid:750 ml methanol:750 ml deionized distilled water) for ten minutes. The slides were then air dried at room temperature.

VIII. SEPHADEX G-200 AND G-100 GEL FILTRATION

Sephadex G-200 and G-100 columns were packed and run according to the method of Pharmacia Ltd. (Montreal, Canada). The gel was allowed to swell in excess 0.15 M borate saline buffer for three days at room temperature. During this time, twice a day, the buffer was decanted, additional buffer added and the gel resuspended by gentle mixing. To pack a column a gel to buffer suspension of one to one was used. Trapped air bubbles were removed by a vacuum pump before packing. The column was mounted vertically and the empty space under the disc and in the tubing was filled with buffer. The homogeneous slurry mixture was poured very gently down the side of the column until the column was filled, making sure to keep the outlet at the same height as the top of the gel slurry. When approximately ten centimeters of the bed was settled, the outlet was slowly lowered one centimeter below the top of the gel slurry. Approximately, each half hour as the length of the packed bed increased, the outlet was lowered to maintain an optimum pressure equal to one-tenth of the packed bed length. Excess buffer was removed and replaced immediately with fresh gel slurry. When the column was packed, a sample applicator was placed on the surface of the packed bed. The column was allowed to equilibriate for 24 hours at 4°C at a rate of 5 to 10 ml per hour. In order to check the uniformity of the gel bed and to determine the void volume, 2 ml of a blue dextran solution was passed through the column at a rate of 5 to 10 ml per hour at 3 to 4 cm pressure at a temperature of 4°C.

The G-100 column was packed at atmospheric pressure. A piece of

filter paper was applied to the packed bed to protect its upper surface. The column was run at a flow rate of 30 to 40 ml per hour.

IX. PREPARATION OF BCG STIMULATED MACROPHAGE SUPERNATE

The method used was followed as described by Paraskevas *et al.* (1978). The peritoneal cavity of DBA/2 mice was rinsed with 8 ml Minimum Essential Media (MEM; Gibco Canada Ltd., Calgary, Alberta). The collected cells were washed twice with MEM and a viable cell count using the trypan blue exclusion technique was performed. For every 20×10^6 collected cells, 0.2 ml of anti-thy-1 and 0.2 ml guinea pig complement (Miles Laboratories Inc., Elkhart, IN) were added. The total volume of the suspension was kept as low as possible. The suspension was incubated for 45 minutes at 37°C. The incubated cells were then washed twice and another viable count was performed. There was usually 40% cell death following the anti-thy-1 treatment. To an aliquot of 2×10^6 anti-thy-1 treated peritoneal cells 1 mg BCG was added and the total volume was adjusted to 1.0 ml. The cell suspension was incubated for 30 minutes at 37°C. The incubated cells were then washed twice with MEM, resuspended with MEM in a final volume of 1.5 ml and incubated for three hours at 37°C. Subsequently, the supernate was collected by centrifugation of the cell suspension at 3,000 rpm for ten minutes, and used in the preparation of immunoglobulin-antigen complexing factor (IACF).

X. PRODUCTION OF IMMUNOGLOBULIN-ANTIGEN COMPLEXING FACTOR (IACF)

Two separate methods as described by Paraskevas *et al.* (1978) were used for the production of IACF.

1) T Cells Stimulated by Macrophage Supernate

Nylon wool nonadherent spleen cells were prepared from DBA/2 mice (Materials and Methods, Section XIV) and 1×10^7 cells were suspended in

1.5 ml of supernate obtained from a culture of 2 x 10^6 macrophages stimulated by BCG (Materials and Methods, Section IX). After three hours of culture at 37°C, the supernate was collected by centrifugation and tested for the presence of IACF (Materials and Methods, Section XIII).

2) T Cells Stimulated by 2-Mercaptoethanol

Nylon wool nonadherent spleens prepared from DBA/2 mice (Materials and Methods, Section XIV), at a concentration of 1 x 10^7 cells in 1.5 ml Hank's Buffered Saline Solution (HBSS; Gibco Canada Ltd., Calgary, Alberta), were exposed for 30 minutes at 37°C to 5 x 10^{-5} M 2-mercaptoethanol (Matheson, Coleman & Bell, Norwood, OH). The incubated cells were washed twice with HBSS, resuspended in 1.5 ml HBSS, and incubated for three hours at 37°C. The supernate was collected by centrifugation of the suspension at 3,000 rpm for ten minutes and tested for the presence of IACF (Materials and Methods, Section XIII).

XI. COATING OF SHEEP RED BLOOD CELLS

Sheep red blood cells (SRBC), formalinized according to the method of Wede (1962), were used to prepare BSA-coated sheep red blood cells. The sheep erythrocytes suspended in citrate buffer were washed three times with 0.15 M saline, pH 7.0 - 7.5. Equal volumes of 8% SRBC and a 3% formaldehyde solution, adjusted to pH 7.5 with 0.1 M NaOH, were incubated at 37°C for 24 hours, shaking occasionally. After incubation, the cells were washed four times in distilled deionized water (DDW), pH 7.0 - 7.5. The cells were stored as a 10% suspension in DDW with 0.1% sodium azide at 4°C for up to six months.

The tanning and coating method of Herbert (1967) was used. Two ml of a 2% suspension of the above formalinized cells were washed three times in 0.15 M phosphate buffered saline (PBS), pH 6.4. The formalinized cells

were resuspended in 2 ml of a 0.0025% solution of tannic acid (Baker Chem. Co., Phillipsburg, NJ) in 0.15 M PBS, pH 6.4. The cells were then washed twice with 0.15 M PBS, pH 6.4 and left overnight at 4°C. The cells were centrifuged and resuspended in 2 ml of 0.15 M PBS, pH 6.4, containing 0.12 mg BSA and incubated at 37°C for one hour. The coated cells were washed three times with 0.15 M PBS, pH 6.4, and then resuspended in 1.5 ml PBS, pH 6.4, containing a drop of 2.5% human serum albumin to stabilize the cells. The resulting suspension, approximately 2.5% cells by volume, were stored at 4°C and used within three weeks.

XII. PREPARATION OF HYBRID ANTIBODY

The methods described by Paraskevas *et al.* (1970, 1971a, 1971b) were used for the preparation of a hybrid antibody. The hybrid prepared was a 5S hybrid $F(ab')_2$ rabbit antibody, one site specific for BSA, the other directed against myeloma proteins of the mouse 7Sγ1 (γF) or 7Sγ2a (γG) subclasses.

Rabbit anti-mouse γF and γG (anti-F,G) was prepared as outlined by Paraskevas *et al.* (1971a). Rabbit anti-BSA was prepared by the same method using 1.0 mg BSA in 1.0 ml of an FCA-saline emulsion as the immunogen.

The rabbit anti-F,G antibodies were purified using a BDB aggregated γF-γG aggregate. The stock solution of BDB was prepared according to the method described by Herbert (1967). The method for BDB aggregation of proteins used was that of Bernier and Cebra (1965). The rabbit anti-BSA antibodies were purified using a BSA aggregate. The ethyl chloroformate method of aggregation of proteins (Avrameas and Ternyck, 1967) was used for the preparation of the BSA aggregate.

To purify the rabbit antibodies, 10 ml of rabbit antiserum was mixed with 50 to 100 mg of the appropriate aggregated antigen and stirred continuously overnight at 4°C. The aggregate was then separated from the

supernatant by centrifugation at 10,000 rpm for 15 minutes, and washed three times with PBS, pH 6.4, at 4°C. Subsequently, the aggregate was suspended in 0.1 M glycine-HCl buffer, pH 2.5, for one hour at 4°C followed by centrifugation at 10,000 rpm for 15 minutes at 4°C. The supernatant containing the antibodies was recovered and neutralized immediately with NaOH. The aggregate was also neutralized and the process of elution repeated to recover as much antibody as possible from the serum. The antibody activity was examined by the Ouchterlony technique. The antibody preparations were stored at -20°C.

The purified anti-mouse immunoglobulin and anti-BSA antibodies were individually pepsin digested according to the method of Nisonoff *et al.* (1960) and Uteumi and Karush (1965). Digestion was carried out using a pepsin (Millipore Corporation, Freehold, NJ) to a protein ratio of 2 to 100. After incubation for five hours at 37°C in 0.1 M acetate buffer, pH 4.0, the digestion was stopped by neutralization to pH 8.0 with 1 M NaOH. The $F(ab')_2$ fragments were isolated by Sephadex G-100 gel chromatography.

Equal amounts of anti-BSA and anti-F,G $F(ab')_2$ fragments, according to their optical density at 280 nm, were mixed. Following the method of Nisonoff and Rivers (1961), the $F(ab')_2$ fragments were reduced with 0.015 M 2-mercaptoethylamine hydrochloride (Matheson, Coleman & Bell, Norwood, OH) to yield univalent $F(ab')_2$ fragments. The reducing agent was immediately removed by passage of the sample through an AL-50W-X4 (Bio-Rad Laboratories, Mississauga, Ontario) cation exchange column. The eluted protein was neutralized with 1 M NaOH and reoxidized by stirring under O_2 for two hours at room temperature. The divalent $F(ab')_2$ fragments were separated from any unoxidized fragments by G-200 chromatography.

To isolate only anti-BSA and anti-F,G recombinants, the preparation was absorbed with the γF-γG aggregate and then with the BSA aggregate. Ouchterlony gel diffusion was used to confirm the presence of only anti-BSA-anti-F,G hybrid molecules.

XIII. REVERSE IMMUNE CYTOADHERENCE (RICA)

The protocol described by Paraskevas *et al.* (1970, 1971a, 1971b) was followed for the performance of the reverse immune cytoadherence (RICA) technique. The test uses a 5S hybrid $F(ab')_2$ rabbit antibody, one site specific for BSA, and the other site directed against myeloma proteins of the mouse 7Sγ1 (γF) or 7Sγ2a (γG) subclasses. Through the former site the hybrid antibody is able to react with BSA coated SRBC and through the latter site with surface associated Ig on lymphocytes. A rosette of small SRBC around the large lymphocyte is formed. The number of Ig^+ cells were determined by counting the number of rosettes per 1,000 splenic lymphocytes.

1) Spleen Cell Suspensions

In order to prepare a suspension of normal DBA/2 spleen cells, normal DBA/2 mice were killed with chloroform and their spleens quickly removed. The spleens were teased apart with forceps in HBSS. The spleen cells were washed twice with HBSS. A total cell count was performed in 2% acetic acid in a hemocytometer. The spleen cells were then diluted with HBSS to a concentration of 50×10^6 cells per ml.

2) Detection of Cytophilic Ig-Ag Complexes in Serum

To detect cytophilic Ig-Ag complexes in DBA/2 sera the protocol as described by Paraskevas and Lee (1981) was followed. At 37°C for 30 minutes, 5×10^6 normal DBA/2 spleen cells were incubated with 0.2 ml sera. The cells were then washed twice with HBSS and resuspended with HBSS to

a total volume of 0.5 ml. The concentration of the incubated cells is 10×10^6 spleen cells per ml. An aliquot of 1×10^6 spleen cells is mixed with 0.030 ml anti-BSA-anti-F,G hybrid antibody and 0.030 ml of a 2.5% suspension of BSA coated SRBC (Materials and Methods, Section XI). The suspension is incubated overnight at 4°C. T cells do not have surface immunoglobulin detectable by RICA. Therefore, the uptake by the T cells of the cytophilic Ig-Ag complexes produces an increase of Ig^+ cells in the normal spleen population which is determined by RICA.

The following sera were tested by RICA for the presence of cytophilic Ig-Ag complexes:

(a) normal DBA/2 mouse sera;

(b) sera collected six hours after i.p. injection of 10^2, 10^3 or 10^4 live PX2-16 cells;

(c) sera collected six hours after i.p. injection of 5×10^7 or 5×10^8 SRBC;

(d) sera collected six hours after i.p. injection of 5×10^7 or 5×10^8 SRBC into mice which were injected i.p. 24 hours earlier with 10^2 live PX2-16 cells;

(e) sera collected 30 hours after i.p. injection of 10^2 live PX2-16 cells.

3) Detection of IACF in Culture Supernates

The presence of IACF in culture supernates was detected by the method described by Paraskevas *et al.* (1978). At 37°C for 30 minutes, 0.20 ml of culture supernate (Materials and Methods, Section X) was incubated with 0.10 ml DBA/2 NMS and 250 µg of egg albumin in 0.10 ml saline. Subsequently, 10×10^6 normal DBA/2 spleen cells (Materials and Methods, Section XIII) were added to the incubated culture supernate and the result-

ing cell suspension was incubated at 37°C for 30 minutes. The incubated cells were then washed twice with HBSS and resuspended with HBSS in a total volume of 0.5 ml. The concentration of the incubated cells was 10×10^6 spleen cells per ml. An aliquot of 1×10^6 spleen cells was mixed with 0.030 ml anti-BSA-anti-F,G hybrid antibody and 0.030 ml BSA-coated SRBC (Materials and Methods, Section XI) and incubated overnight at 4°C. An increase of Ig^+ cells in the normal spleen cell population is indicative of the presence of IACF.

The supernates from the following cultures were tested for the presence of IACF:

(a) normal nylon wool nonadherent (NNA) (Materials and Methods, Section XIV) T cells stimulated by 2-mercaptoethanol;

(b) PX2-16 pretreated NNA T cells stimulated by 2-mercaptoethanol. PX2-16 pretreated cells were obtained from animals which were 24 hours earlier injected i.p. with 10^2 live PX2-16 cells;

(c) normal NNA T cells stimulated by culture supernates from BCG stimulated normal or PX2-16 pretreated macrophages (Materials and Methods, Section IX);

(d) PX2-16 pretreated NNA T cells stimulated by culture supernates from BCG stimulated normal or PX2-16 pretreated macrophage.

4) Enumeration of the Number of Ig^+ Cells

The RICA tests were read as described by Paraskevas *et al.* (1970, 1971). After the overnight incubation the treated spleen cell suspensions were gently dispersed, taken up in a Pasteur pipette and placed in a slide chamber under a cover slip (Bellco Glass Inc., Vineland, NJ). The slide was examined using a phase contrast microscope (Carl Zeiss) and a 40X objective. Approximately 700 to 1,000 nucleated cells were counted per slide. The number of rosettes were recorded and expressed as the number of rosettes

per 1,000 nucleated cells. A rosette was defined as consisting of a nucleated lymphoid cell surrounded by a minimum of four SRBC.

XIV. T LYMPHOCYTE ENRICHMENT OF SPLEEN CELLS BY NYLON WOOL COLUMNS

A modification of the methods of Schwartz *et al.* (1975, 1976) and Julius *et al.* (1973) were used to enrich suspensions of normal spleen cells for T lymphocytes by passing the normal spleen cells over nylon wool columns.

Nylon wool (FT-242, Fenwal Laboratories, Morton Grove, IL) was boiled for 30 minutes in 0.2 N HCl. The nylon wool was subsequently rinsed four times with distilled, deionized water (DDW). It was then soaked in DDW for five days at 37°C. The DDW was changed daily. The nylon wool was then dried for five days at 100°C. Approximately 1 g portions of the wool were immersed in DDW and teased with forceps to remove air bubbles. The nylon wool was carefully packed into a 10 cc Plastipak disposable plastic syringe (Beckton, Dickinson and Co. Ltd., Mississauga, Ontario) making sure there are no trapped air bubbles. The column was then flushed with 25 ml DDW.

Before use the columns were flushed with approximately 25 ml RPMI 1640 (Gibco Canada Ltd., Calgary, Alberta) with 10% heat inactivated (30 minutes at 56°C) foetal calf serum (FCS; Gibco). The column was then incubated for one hour at 37°C. Subsequently, the column was flushed with 25 ml Dulbecco's Balanced Salt Solution (DBS; Gibco) with 10% heat inactivated FCS (DBS-10FCS).

Spleens from DBA/2 mice were teased with forceps into Hank's balanced salt solution (HBSS; Gibco). The spleen cell suspensions were washed twice in HBSS and the cell pellet after the final washing resuspended in DBS-FCS. Approximately 200 x 10^6 viable nucleated cells were loaded onto the column in a 1.0 ml volume. The spleen cells were allowed to enter just into the nylon wool matrix. The column was then incubated at 37°C

for 45 minutes. At 15-minute intervals the column was eluted very slowly with the addition of 2 ml DBS-10FCS. The 2 ml volume eluted after the first 15-minute interval was discarded. The 2 ml volume eluted after the second 15-minute interval was collected. After 45 minutes the remainder of the cells were eluted slowly from the column with the addition of DBS-10FCS. The first 25 ml eluted were collected.

Nylon wool nonadherent (NNA) cells were washed twice in a large volume of HBSS. Viable cells were counted using the trypan blue exclusion technique.

XV. NYLON WOOL NONADHERENT (NNA) T CELL RECONSTITUTION OF LETHALLY X-IRRADIATED MICE

DBA/2 mice were x-irradiated with 800 rads of total body irradiation administered with an Eldorado A^{60} Cobalt therapy machine (Atomic Energy of Canada Ltd.). Twenty-four hours later groups of the x-irradiated DBA/2 mice were injected intravenously with 15×10^6 NNA T cells (Materials and Methods, Section XIV). At 48 hours after x-irradiation the mice were challenged with 5×10^8 SRBC intraperitoneally. The sera from these mice were collected six hours later at the 54-hour time point and were tested by RICA for the presence of cytophilic Ig-Ag complexes.

Lethally x-irradiated DBA/2 mice were reconstituted with T lymphocyte enriched spleen cells from the following donor animals:

(a) normal DBA/2 mice;

(b) DBA/2 mice injected i.p. 24 hours before removal of their spleens with 10^2, 10^3 or 10^4 live PX2-16 cells;

(c) DBA/2 mice injected i.p. 24 hours before removal of their spleens with 50 µg dHGG or 0.5 µg dHGG (Materials and Methods, Section IV);

(d) no reconstitution as a control.

XVI. MEASUREMENT OF THE ANTI-SRBC RESPONSE USING THE PLAQUE FORMING CELL (PFC) METHOD

The PFC technique of Cunningham and Szenberg (1968) was used. DBA/2 mice were killed with chloroform and their spleens quickly removed. The spleens were teased apart with forceps in HBSS. The spleen cells were washed twice with HBSS and then resuspended with HBSS in a final volume of 3.0 ml. For direct PFC assays 0.030 ml of the spleen cell suspension was combined with 0.030 ml of a suspension of 2.5×10^9 SRBC per ml saline and 0.030 ml of heme-10 guinea pig complement (Cedarlane Laboratories Ltd., Hornby, Ontario). For indirect PFC assays 0.030 ml of the correct dilution of rabbit anti-mouse immunoglobulin (Materials and Methods, Section XII) was added to the above mixture. Cunningham slides were flooded with the suspension and the slides were totally filled with a one-third dilution of the SRBC suspension for the direct PFC assay or with a one-fourth dilution of the SRBC suspension for the indirect PFC. The slides were sealed with molten wax and incubated for 45 minutes at 37°C. The plaques were counted using a 2.5X objective on a Zeiss microscope. For indirect PFC the proper dilution of the IgG fraction from rabbit anti-mouse immunoglobulin is the one which allows the development of a maximal number of PFC. At this dilution the antiserum gave no inhibition of the direct PFC. The indirect PFC was calculated by subtracting the number of direct PFC (without anti-mouse Ig) from the total PFC (with anti-mouse Ig).

XVII. ASSAY FOR SIX HOUR SERUM (6HS) ENHANCING FACTOR(S)

The assay for the 6HS enhancing factor(s) was carried out as described by Lee and Paraskevas (1981). Two hours before an intravenous injection of a subimmunogenic dose of antigen, 0.3 ml of the test sera was injected. The subimmunogenic dose of antigen was 10^5 SRBC. The anti-SRBC response

was measured six days later by the PFC technique (Materials and Methods, Section XVI).

The following mouse sera were tested for the presence of 6HS enhancing factors:

(a) normal DBA/2 serum;

(b) sera collected six hours after an i.p. injection of 5×10^7 SRBC;

(c) sera collected six hours after an i.p. injection of 5×10^7 SRBC into DBA/2 mice which were injected i.p. 24 hours earlier with 100 live PX2-16 cells;

(d) sera collected 30 hours after an i.p. injection of 100 live PX2-16 cells.

XVIII. STATISTICAL ANALYSIS

All statistical analyses were carried out using a paired t test program on a Hewlett Packard programmable calculator.

RESULTS

I. THE RELATIONSHIP OF TUMOUR CELL DOSE WITH BIOLOGICAL VIABILITY AND INDUCTION OF CYTOPHILIC Ig-Ag COMPLEXES

The doses of 10^2 and 10^3 live PX2-16 mastocytoma cells were tested for their ability to kill DBA/2 mice (Table I). Both the doses of 10^2 and 10^3 viable PX2-16 cells killed 100% of the animals tested within four to five weeks after injection.

Inocula of 10^2, 10^3 and 10^4 viable PX2-16 cells were also tested for their ability to induce the formation of cytophilic Ig-Ag complexes. Six hours after intraperitoneal immunization of DBA/2 mice with live PX2-16 cells, sera from the mice were collected and examined for the presence of cytophilic Ig-Ag complexes with the RICA assay (Table I). The challenge of 10^2 live PX2-16 cells did not induce the formation of cytophilic Ig-Ag complexes ($p < 0.0001$). The dose of 10^4 viable PX2-16 cells induced the formation of cytophilic Ig-Ag complexes to a level equivalent to that reported by Orr and Paraskevas (1973) following an intraperitoneal injection of either particulate antigens or soluble antigens emulsified with FCA. However, the challenge of 10^3 live PX2-16 cells induced the formation of Ig-Ag complexes to a level which was 60% of that induced by the inocula of 10^4 viable PX2-16 cells.

Since inocula of 10^2 live PX2-16 cells killed 100% of mice tested but did not induce the formation of cytophilic Ig-Ag complexes, such an inoculum was chosen for our studies on the effects of early tumour growth in formation of the cytophilic Ig-Ag complexes.

II. EFFECT OF 100 LIVE PX2-16 CELLS ON THE INDUCTION OF CYTOPHILIC Ig-Ag COMPLEXES BY A PARTICULATE ANTIGEN

To determine the effect of pretreatment with 100 live PX2-16 cells on the formation of cytophilic Ig-Ag complexes, DBA/2 mice were challenged

TABLE I

THE INDUCTION OF TUMOURS AND FORMATION OF CYTOPHILIC Ig-Ag COMPLEXES FOLLOWING THE INJECTION OF VIABLE PX2-16 CELLS

Number PX2-16 Injected	% Increase in Ig^+ Spleen Cells ± S.D.	% Animals Killed
10,000	$^+$22.5 ± 2.1 (3)	-
1,000	$^+$12.3 ± 3.1 (3)	100 (15)[a]
100	$^+$ 0.5 ± 0.9 (3)	100 (15)

a. The numbers in parentheses represent the number of animals tested.

with an intraperitoneal injection of either 5×10^7 SRBC (Figure 1) or or 5×10^8 SRBC (Figure 2) 24 hours after an intraperitoneal injection of 100 live PX2-16 cells (PX2-16[6HS-SRBC]). The sera were collected six hours following the final injection. As a positive control, serum was collected six hours after intraperitoneal injection of 5×10^7 or 5×10^8 SRBC (6HS-SRBC). As negative controls, serum was collected 30 hours after intraperitoneal inoculation with 100 viable PX2-16 cells (PX2-16-S) and from normal DBA/2 mice (NMS). The sera were tested on normal spleen cells for the presence of cytophilic Ig-Ag complexes by the RICA assay.

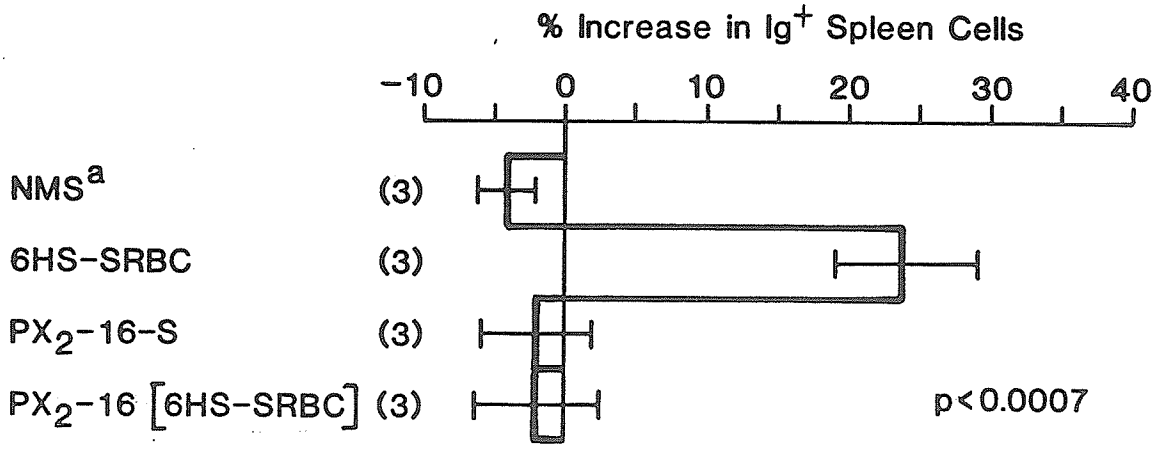
An intraperitoneal injection of either 5×10^7 or 5×10^8 SRBC induced the formation of cytophilic Ig-Ag complexes within six hours of immunization. Pretreatment of the animals with 100 live PX2-16 cells nonspecifically abrogated the formation of cytophilic Ig-Ag complexes for both the 5×10^7 SRBC ($p < 0.0007$) and the 5×10^8 SRBC ($p < 0.0002$) antigenic challenge. Neither of the control sera contained measurable amounts of cytophilic Ig-Ag complexes.

III. EFFECT OF 100 VIABLE DBA/2 SPLEEN CELLS ON INDUCTION OF CYTOPHILIC Ig-Ag COMPLEXES BY A PARTICULATE ANTIGEN

In order to determine if syngeneic normal spleen cells could nonspecifically abrogate the formation of the Ig-Ag complexes, as the 100 live PX2-16 cells did, DBA/2 mice were challenged with an intraperitoneal injection of 5×10^8 SRBC 24 hours after an intraperitoneal injection of 100 viable DBA/2 spleen cells (Spln[6HS-SRBC]). The serum was collected six hours after the 5×10^8 SRBC challenge and tested by the RICA assay for the presence of cytophilic Ig-Ag complexes (Figure 3).

Both normal and spleen cell pretreated DBA/2 mice formed Ig-Ag complexes to antigenic stimulation by 5×10^8 SRBC within six hours of immuni-

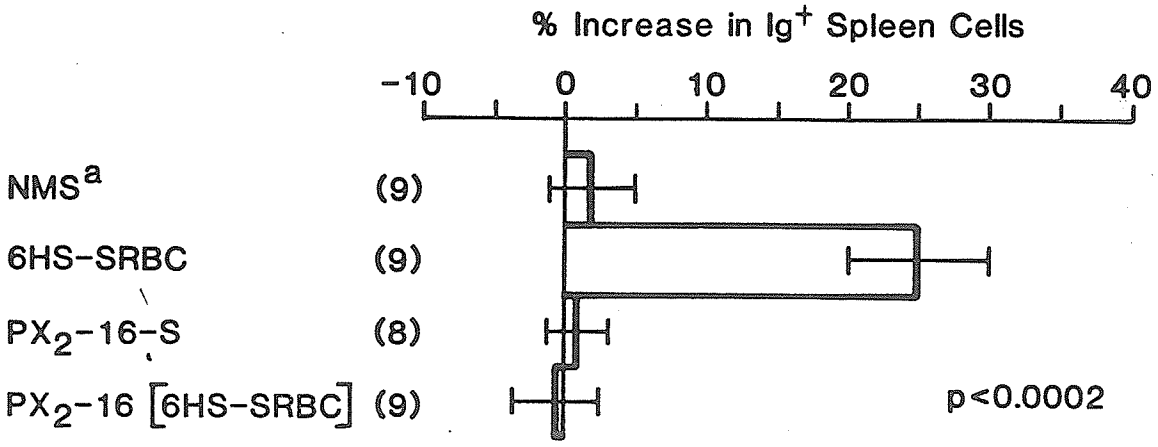
FIG 1: Effect of 100 live PX_2-16 cells on the induction of Ig-Ag complexes by $5X10^7$ SRBC



a. Serum was collected from normal mice (NMS) and tumour treated mice (PX2-16-S). Serum was also collected 6 hours following i.p. injection of 5 x 10^7 SRBC into normal mice (6HS-SRBC) and tumour pretreated mice (PX2-16[6HS-SRBC]). Each column in this Figure and following figures represents the mean ± S.D.

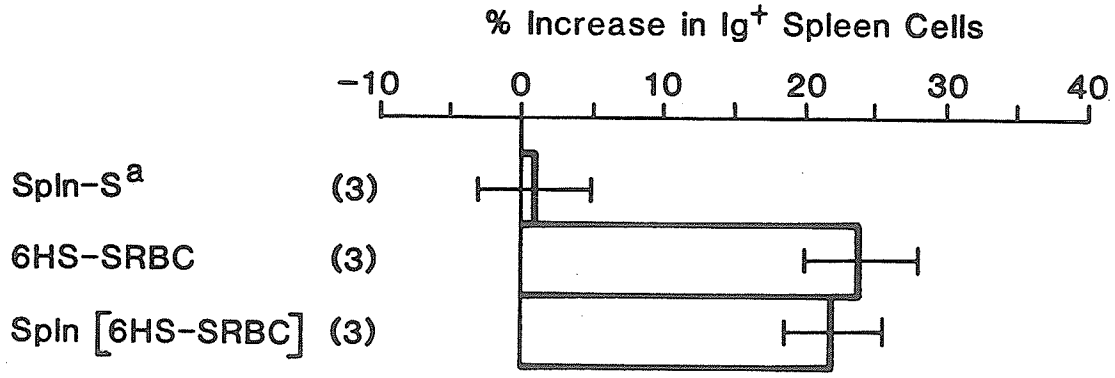
b. Numbers in parentheses represent the number of times the test was performed.

FIG 2: Effect of 100 live PX_2-16 cells on the induction of Ig-Ag complexes by $5X10^8$ SRBC.



a. Serum was collected 6 hours following i.p. injection of 5×10^8 SRBC into normal mice (6HS-SRBC) and into tumour pretreated mice (PX2-16[6HS-SRBC]). Serum was also collected from normal mice (NMS) and tumour treated mice (PX2-16-S).

FIG 3: Effect of 100 viable DBA/2 spleen cells on induction of cytophilic Ig-Ag complexes by $5X10^8$ SRBC.



a. Serum was collected 6 hours following i.p. injection of 5×10^8 SRBC into normal mice (6HS-SRBC) and spleen cell pretreated mice (Spln[6HS-SRBC]). As a control, serum was also collected from spleen cell treated mice (Spln-S).

zation. The increase in the RICA assay for the number of Ig^+ spleen cells was equivalent for the two groups. Furthermore, an intraperitoneal injection of 100 viable spleen cells (Spln-S) did not itself induce the formation of cytophilic Ig-Ag complexes ($p < 0.0007$).

IV. EFFECT OF 100 LIVE PX2-16 CELLS ON INDUCTION OF CYTOPHILIC Ig-Ag COMPLEXES BY A SOLUBLE ANTIGEN

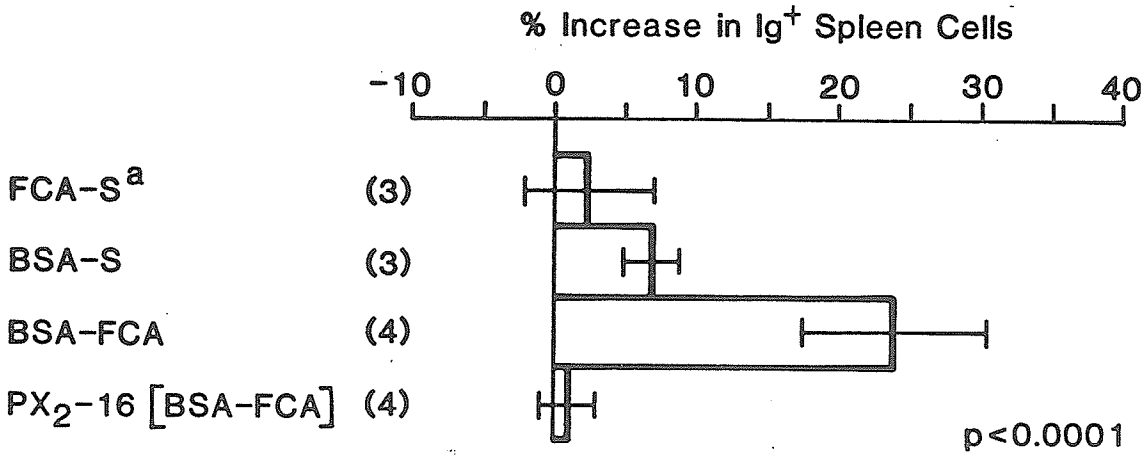
An intraperitoneal injection of 250 µg BSA emulsified with FCA (BSA-FCA) induced the formation of cytophilic Ig-Ag complexes within six hours of immunization (Figure 4). However, pretreatment of DBA/2 mice with an intraperitoneal injection of 100 live PX2-16 cells 24 hours before challenge with 250 µg BSA emulsified with FCA (PX2-16[BSA-FCA]) abrogated the formation of cytophilic Ig-Ag complexes as measured by the RICA assay ($p < 0.0001$). Neither sera collected six hours after injection of 250 µg BSA in 0.2 ml saline (BSA-S) nor six hours after intraperitoneal injection of 0.1 ml saline emulsified with 0.1 ml FCA (FCA-S) contained cytophilic Ig-Ag complexes detectable by the RICA assay.

Pretreatment of DBA/2 mice with 100 viable PX2-16 mastocytoma cells nonspecifically abrogated the formation of cytophilic Ig-Ag complexes to antigenic stimulation by a soluble antigen.

V. EFFECT OF 100 VIABLE PX2-16 CELLS ON SPECIFIC ANTIBODY ENHANCING ACTIVITY ASSOCIATED WITH CYTOPHILIC Ig-Ag COMPLEXES

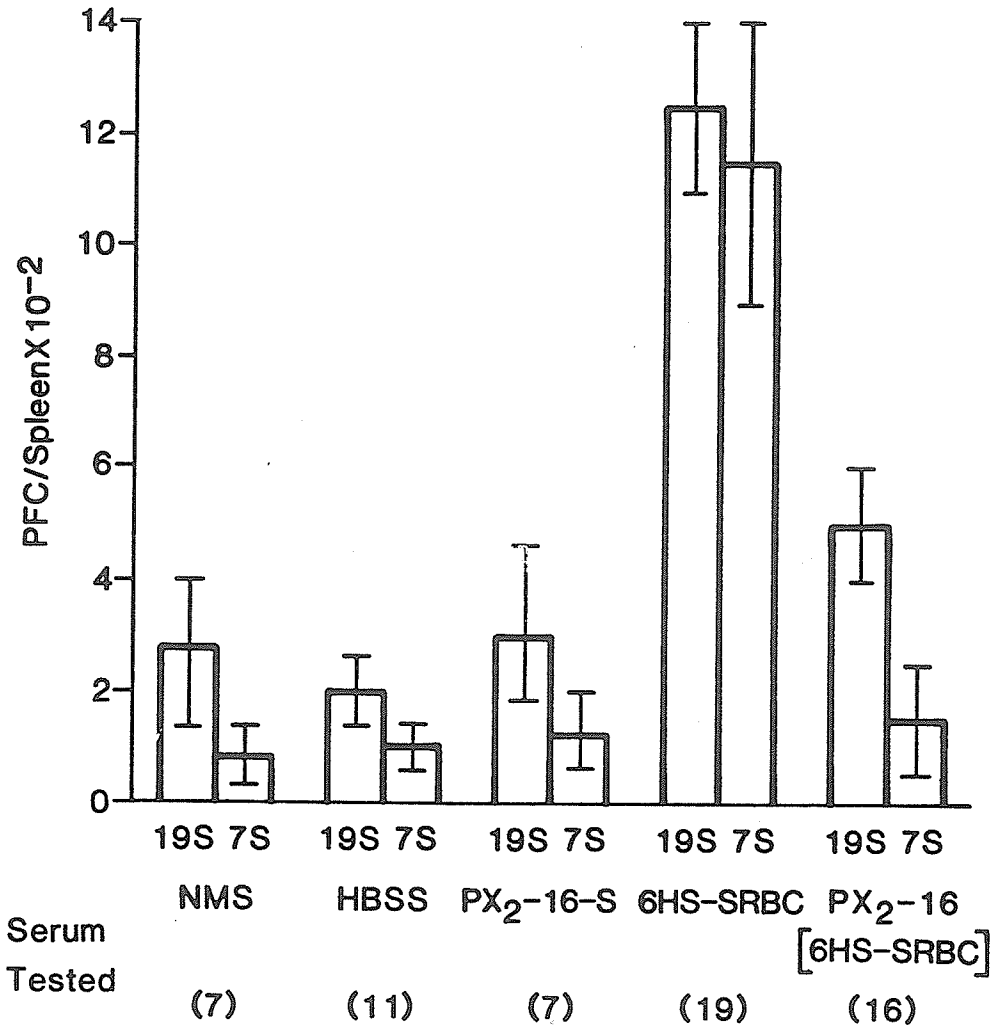
Serum which contains cytophilic Ig-Ag complexes induced by an intraperitoneal injection of 5×10^7 SRBC (6HS-SRBC) was able to specifically enhance antibody synthesis for both the 19S and 7S responses as measured by the PFC assay (Figure 5). Pretreatment of DBA/2 mice 24 hours before the 5×10^7 SRBC challenge with an intraperitoneal injection of 100 live PX2-16 cells (PX2-16[6HS-SRBC]) abrogated the enhancing activity of the

FIG 4: Effect of 100 live PX_2-16 cells on induction of cytophilic Ig-Ag complexes by 250µg BSA



a. Serum was collected 6 hours following i.p. injection of BSA in FCA (BSA-FCA), or BSA in saline (BSA-S), or FCA in saline (FCA-S) into normal mice and BSA in FCA into tumour pretreated mice (PX2-16[BSA-FCA]).

FIG 5: Effect of 100 viable PX_2-16 cells on specific antibody enhancing activity associated with cytophilic Ig-Ag complexes.



a. NMS = normal mouse serum

b. HBSS = Hank's Balanced Salt Solution

c. PX2-16-S = Serum collected after i.p. injection of 100 live PX2-16 cells

d. 6HS-SRBC = Serum collected 6 hours after i.p. injection of 5×10^7 SRBC.

e. PX2-16[6HS-SRBC] = Serum collected 6 hours after i.p. injection of 5×10^7 SRBC into tumour pretreated mice

serum for the 7S response ($p < 0.003$). Paraskevas and Lee (1981) had reported that the Ig-Ag complexes are involved in the enhancement of the 7S response, while the enhancement of the 19S response is probably due to another factor. Antibody enhancing activity was not observed in the PFC assay when sera from normal DBA/2 mice (NMS), sera collected 30 hours after injection with 100 live PX2-16 cells (PX2-16-S) or HBSS was tested.

Previously, Lee and Paraskevas (1981) reported a 33 times enhancement of the 19S response and a 37 times enhancement of the 7S response when the antigenic challenge responsible for the formation of the Ig-Ag complexes was 5×10^8 SRBC. The enhancing activity associated with the antigenic challenge of 5×10^7 SRBC in this experiment was four times for the 19S response and 15 times for the 7S response. When the challenge dose of 5×10^8 SRBC was used in this system, a higher degree of antibody enhancement was observed. However, tumour pretreatment did not have a consistent or reproducible effect on the antibody enhancing activity associated with the 5×10^8 SRBC challenge. This could be due to a dose-dependent effect.

VI. UPTAKE OF CYTOPHILIC Ig-Ag COMPLEXES BY TUMOUR PRETREATED SPLEEN CELLS

Since pretreatment of DBA/2 mice with 100 viable PX2-16 mastocytoma cells nonspecifically suppressed the formation of cytophilic Ig-Ag complexes to antigenic stimulation by a particulate antigen or a soluble antigen, the ability of tumour pretreated spleen cells to take up cytophilic Ig-Ag complexes formed in DBA/2 mice was examined with the RICA assay (Table II).

Cytophilic Ig-Ag complexes were formed in normal DBA/2 mice within six hours of intraperitoneal immunization with 5×10^8 SRBC (6HS-SRBC).

TABLE II

UPTAKE OF CYTOPHILIC Ig-Ag COMPLEXES

BY TUMOUR PRETREATED SPLEEN CELLS

Group	Challenge	% Change in Ig^+ Spleen Cells from Tumour Pretreated DBA/2 Mice ± S.D.
NMS (4)[a]	None	$^+0.4 \pm 0.9$
6HS-SRBC (4)	5×10^8 SRBC	$^+22.5 \pm 3.7$

a. The numbers in parentheses represent the number of times the test was performed.

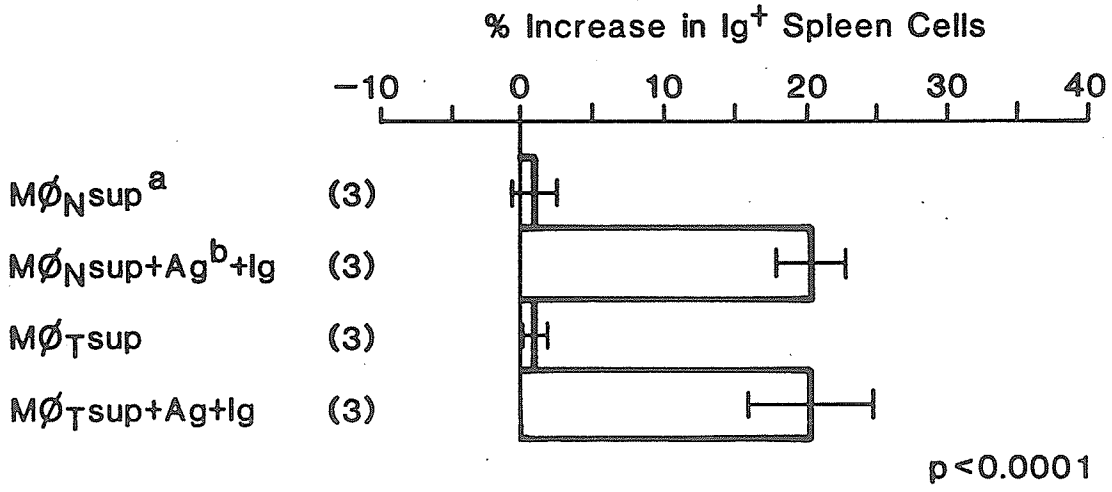
Spleen cells from DBA/2 mice which were 24 hours earlier injected intraperitoneally with 100 live PX2-16 cells were able to take up *in vitro* the cytophilic Ig-Ag complexes formed in normal DBA/2 mice ($p < 0.006$). The cytophilic Ig-Ag complexes were taken up by the spleen cells from the tumour pretreated animals to the same degree as by spleen cells from normal animals. Serum from normal DBA/2 (NMS) did not increase the number of Ig^+ spleen cells from tumour pretreated DBA/2 mice.

VII. ABILITY OF PX2-16 TUMOUR PRETREATED MACROPHAGES TO COOPERATE WITH NORMAL NNA T CELLS TO PRODUCE IACF

An intraperitoneal injection of 100 live PX2-16 cells 24 hours before a strong immunogenic challenge abrogated the production of cytophilic Ig-Ag complexes. The complexes are formed through the mediation of IACF, a T cell derived factor, produced as a result of interactions between T cells and macrophages (Paraskevas *et al.*, 1979). Therefore, the cell population in which the defect was induced by the inoculation of a small number of tumour cells was examined.

Peritoneal exudate macrophages from normal DBA/2 mice which had been injected intraperitoneally 24 hours earlier with 100 live PX2-16 cells were tested for their ability to cooperate *in vitro* with nylon wool nonadherent T cells from normal DBA/2 mice to produce IACF (Figure 6). Addition of a foreign soluble protein antigen and normal mouse immunoglobulin to a culture supernate containing IACF generates cytophilic Ig which can be detected by the RICA assay (Paraskevas *et al.*, 1979). Both macrophages from normal and PX2-16 tumour pretreated DBA/2 mice were able to successfully cooperate *in vitro* with nylon wool nonadherent T cells from normal DBA/2 mice to produce active IACF which could generate cytophilic Ig ($p < 0.0001$).

FIG 6: Production of IACF by PX_2-16 pretreated macrophages in co-operation with normal NNA T cells.



a. Production of IACF by cultures of macrophages obtained from normal ($M\emptyset_N$ sup) and tumour pretreated ($M\emptyset_T$ sup) mice. T cells from normal mice were used in all experiments.

b. Ag is egg albumin.

VIII. ABILITY OF NORMAL MACROPHAGES TO COOPERATE WITH PX2-16 TUMOUR PRETREATED NNA T CELLS TO PRODUCE IACF

Since macrophages from PX2-16 tumour pretreated DBA/2 mice were able to cooperate successfully *in vitro* with nylon wool nonadherent T cells from normal DBA/2 mice, the ability of NNA T cells from PX2-16 tumour pretreated DBA/2 mice to cooperate with macrophages from normal DBA/2 mice was tested. Nylon wool nonadherent T cells from normal DBA/2 mice and from DBA/2 mice which were 24 hours previously injected intraperitoneally with 100 live PX2-16 cells were examined for their ability to cooperate *in vitro* with peritoneal exudate macrophages from normal DBA/2 mice to produce IACF (Figure 7). The RICA assay was used to test the culture supernates for the presence of IACF.

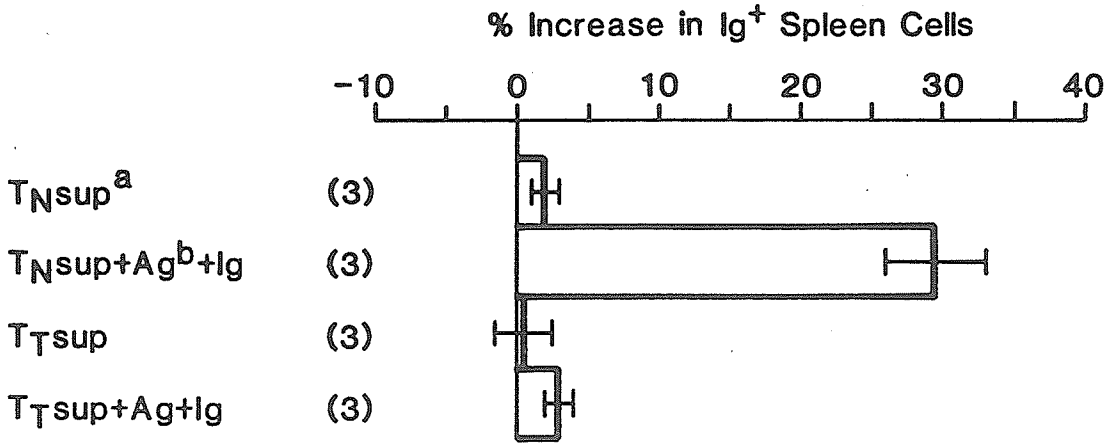
The PX2-16 tumour pretreated NNA T cells were unable to cooperate with normal macrophages to produce active IACF ($p < 0.0003$). Normal NNA T cells and normal macrophages could cooperate successfully to produce active IACF, which generated cytophilic Ig when a foreign soluble protein antigen and normal mouse immunoglobulin were added to the culture supernate.

IX. EFFECT OF PX2-16 TUMOUR PRETREATMENT ON THE PRODUCTION OF IACF BY NNA T CELLS STIMULATED WITH 2-MERCAPTOETHANOL

Paraskevas *et al.* (1979) reported that NNA T cells from normal Balb/c mice in the absence of macrophages can be stimulated *in vitro* by 2-mercaptoethanol to produce IACF. The ability of NNA T cells from DBA/2 mice which were injected 24 hours earlier with 100 viable PX2-16 cells and NNA T cells from normal DBA/2 mice stimulated *in vitro* by 2-mercaptoethanol to produce IACF was compared (Figure 8). The RICA assay was used to test the culture supernates for the presence of IACF.

Nylon wool nonadherent T cells from normal DBA/2 mice stimulated with 2-mercaptoethanol produced active IACF which could generate cytophilic

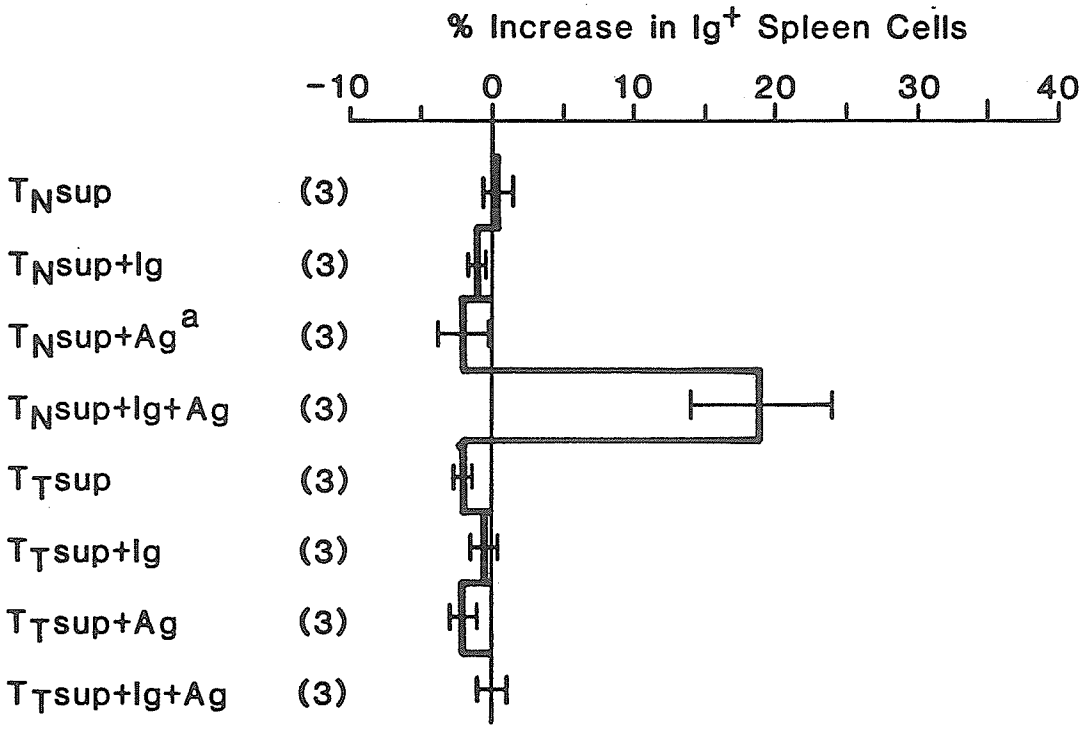
FIG 7: Production of IACF by normal macrophages in co-operation with PX_2-16 pretreated NNA T cells.



a. Production of IACF by cultures of macrophages from normal mice and NNA T cells from normal (T_N sup) and tumour pretreated (T_T sup) mice.

b. Ag is egg albumin.

FIG 8: Effect of PX_2-16 tumour pretreatment on the production of IACF by T cells stimulated with 2-mercaptoethanol.



a. Ag is egg albumin.

b. NNA T cells used for cultures were obtained from normal (T_N sup) and tumour pretreated (T_T sup) mice.

Ig. However, the PX2-16 tumour pretreated NNA T cells stimulated with 2-mercaptoethanol did not produce detectable IACF activity ($p < 0.0003$). The addition of both a foreign soluble protein antigen and of normal mouse immunoglobulin to the culture supernates was necessary for the generation of cytophilic Ig by IACF.

X. ABSENCE OF ACTIVITY INHIBITORY TO IACF IN SUPERNATES FROM CULTURES OF PX2-16 TUMOUR PRETREATED NNA T CELLS

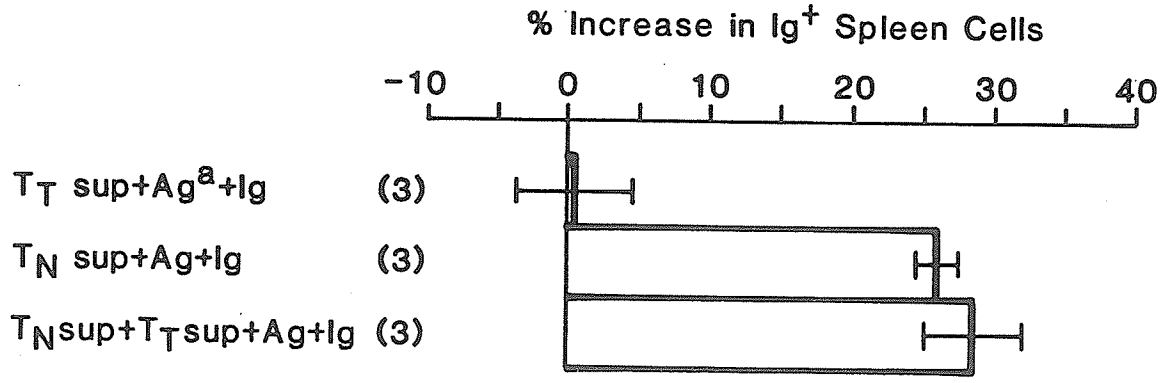
In order to determine if a substance antagonistic to the activity of IACF is present in the supernates of cultures of NNA T cells from tumour pretreated animals, equal volumes of supernates from cultures of normal NNA T cells stimulated by 2-mercaptoethanol and cultures of PX2-16 tumour pretreated NNA T cells stimulated by 2-mercaptoethanol were mixed and tested for the presence of IACF activity (Figure 9). The NNA T cells were pretreated with the PX2-16 mastocytoma by injecting intraperitoneally 100 live PX2-16 cells into DBA/2 mice 24 hours before the preparation of the NNA T cells. Normal NNA T cells were obtained from normal DBA/2 mice. The RICA assay was used to test for the presence of active IACF.

Supernates from cultures of normal NNA T cells stimulated by 2-mercaptoethanol contained IACF activity, while supernates from cultures of tumour pretreated NNA T cells stimulated by 2-mercaptoethanol did not contain measurable IACF activity. The mixture of the two culture supernates contained active IACF, capable of generating cytophilic Ig when both a soluble foreign protein antigen and normal mouse immunoglobulin were present ($p < 0.0004$).

XI. RECONSTITUTION OF LETHALLY X-IRRADIATED DBA/2 MICE WITH NORMAL AND PX2-16 TUMOUR PRETREATED NNA T CELLS

Lee and Paraskevas (1972) reported that lethally x-irradiated Balb/c

FIG: 9 Absence of inhibitory activity to IACF in culture supernate of NNA T cells from mice pretreated with tumour cells.



a. Ag is egg albumin.

b. NNA T cells used for cultures were obtained from normal (T_N sup) and tumour pretreated (T_T sup) animals.

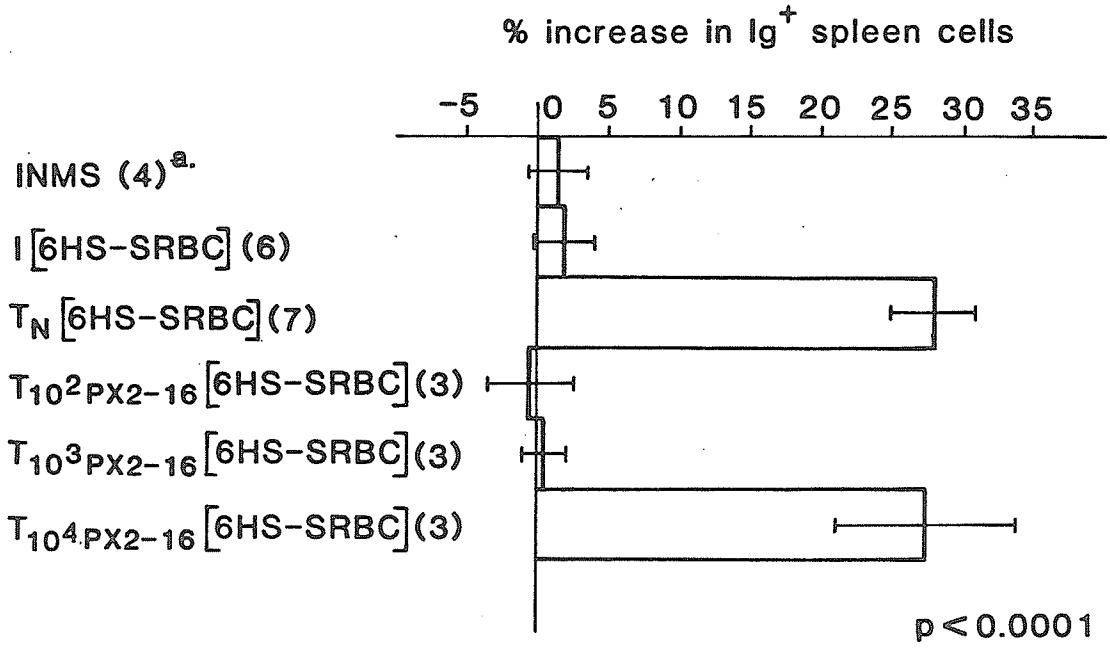
mice could be reconstituted with the thymocytes from normal Balb/c mice to restore the ability of the x-irradiated mice to produce cytophilic Ig-Ag complexes. The ability of NNA T cells obtained from DBA/2 mice which were previously immunized with an intraperitoneal injection of either 10^2, 10^3 or 10^4 viable PX2-16 mastocytoma cells to restore the production of cytophilic Ig-Ag complexes in lethally x-irradiated DBA/2 mice was examined by the RICA assay (Figure 10). The antigenic challenge of 5×10^8 SRBC was used for the induction of the cytophilic Ig-Ag complexes. As controls, lethally x-irradiated DBA/2 mice were reconstituted with NNA T cells obtained from normal DBA/2 mice or were not reconstituted.

Both normal NNA T cells and NNA T cells pretreated with 10^4 live PX2-16 cells were able to reconstitute the ability of lethally x-irradiated mice to produce cytophilic Ig-Ag complexes. The NNA T cells obtained from DBA/2 mice pretreated with either 10^2 or 10^3 live PX2-16 cells were unable to reconstitute the ability of the lethally x-irradiated animals to produce cytophilic Ig-Ag complexes ($p < 0.0001$). Lethally irradiated DBA/2 mice which were not reconstituted with T cells were unable to produce cytophilic Ig-Ag complexes in response to an antigenic challenge of 5×10^8 SRBC. An interpretation will be given in the Discussion concerning the absence of inhibition of the formation of cytophilic Ig-Ag complexes by the inoculation of 10^4 tumour cells.

XII. RECONSTITUTION OF LETHALLY X-IRRADIATED DBA/2 MICE WITH NORMAL AND dHGG PRETREATED NNA T CELLS

In order to determine if the ability to nonspecifically inhibit the formation of cytophilic Ig-Ag complexes is restricted to inocula of small numbers of tumour cells, the ability of NNA T cells pretreated with a tolerogenic dose of dHGG to reconstitute the formation of Ig-Ag complexes

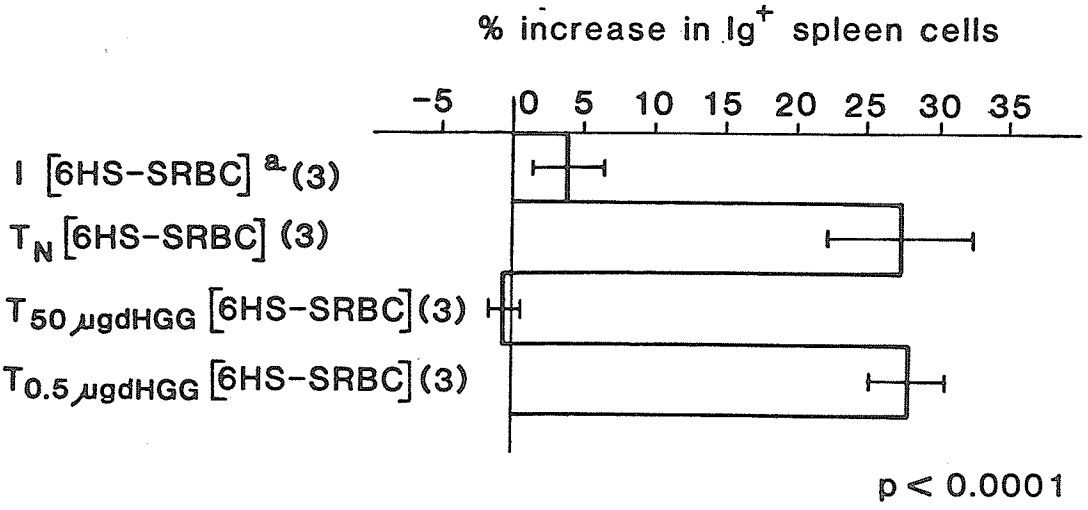
FIG. 10: Production of Ig-Ag complexes by PX 2-16 tumour pretreated T cell reconstituted DBA/2 mice.



a. Serum was collected from lethally x-irradiated mice reconstituted with NNA T cells (T_N[6HS-SRBC]) or reconstituted with NNA T cells treated with 10^2 PX2-16 cells ($T_{10^2PX2-16}$[6HS-SRBC]), 10^3 PX2-16 cells ($T_{10^3PX2-16}$[6HS-SRBC]) or 10^4 PX2-16 cells ($T_{10^4PX2-16}$[6HS-SRBC]) 6 hours after an i.p. injection of 5 x 10^8 SRBC. Serum was also collected from lethally x-irradiated mice (INMS) and from lethally x-irradiated mice 6 hours after an i.p. injection of 5 x 10^8 SRBC (I[6HS-SRBC]).

in lethally x-irradiated animals was investigated. Lethally x-irradiated DBA/2 mice were reconstituted with NNA T cells obtained from DBA/2 mice which were 24 hours earlier given an intraperitoneal injection of either 50 µg dHGG or 0.5 µg dHGG (Figure 11). Weigle *et al.* (1972) have reported that a dose of 50 µg dHGG induces immunological unresponsiveness of T cells while a dose of 0.5 µg dHGG does not. Both normal NNA T cells and 0.5 µg dHGG pretreated NNA T cells reconstituted the ability of the animals to form cytophilic Ig-Ag complexes in response to antigenic stimulation by 5×10^8 SRBC. However, NNA T cells obtained from animals pretreated with 50 µg dHGG were unable to reconstitute the formation of cytophilic Ig-Ag complexes in lethally x-irradiated animals ($p < 0.0001$).

FIG 11: Production of Ig-Ag complexes by dHGG pretreated T cell reconstituted DBA/2 mice



a. Serum was collected 6 hours after an i.p. injection of 5×10^8 SRBC into lethally x-irradiated mice reconstituted with NNA T cells treated with 50 µg dHGG ($T_{50\ \mu g\ dHGG}$[6HS-SRBC]) or 0.5 µg dHGG ($T_{0.5\ \mu g\ dHGG}$[6HS-SRBC]). See Figure 10 for other symbols.

DISCUSSION

A significant paradox found in tumour immunology is the observation that antigenic tumours can grow progressively in immunocompetent hosts. In a large number of tumour-host systems oncogenesis is accompanied by the expression of tumour-associated neoantigens. There is considerable evidence indicating that in both man and animals immunocompetent cells not only recognize tumour-associated antigens but also react to them (Herberman, 1974). The ineffectual immune response to neoplastic cells in tumour-bearing animals has been attributed to interference with immune effector mechanisms. Tumours may also escape immune elimination by the host by subverting the induction of an effective anti-tumour immune response.

The aim of this study was to examine the host's immune reactions in the early stages of tumour growth and development. An analogy to the conditions of spontaneous tumourigenesis was sought by studying the effect on the immune system of small numbers of live tumour cells. There is a large body of literature involving transplantable tumours which indicates that inocula of small numbers of tumour cells interact with the host's immune system differently than inocula of large numbers of tumour cells.

The results of this study suggest that a tumour in its initial phases of tumour growth can induce nonspecific immunosuppression.

The tumour system investigated was a clone (P815-16), of the methylcholanthrene-induced murine mastocytoma P815X2 syngeneic for DBA/2 mice. The P815X2 tumour has been reported to lack expression of "strong" tumour-specific transplantation antigens, since it is very difficult to raise a significant level of transplantation immunity in syngeneic hosts either by immunization with irradiated cells or by resection of early tumours (Al-Rammahy *et al.*, 1980). However, the P815X2 mastocytoma does express

a form of tumour-specific transplantation antigen indicated by the demonstration of T cell mediated cytoxicity against the P815X2 tumour in splenocytes of mice bearing subcutaneous early localized P815X2 tumours (Takei *et al.*, 1976, 1977).

Orr and Paraskevas (1972) have demonstrated that within six hours after immunization the serum of mice contains cytophilic complexes of immunoglobulin and antigen. The complexes are formed through the mediation of a T cell derived factor and are cytophilic for T cells. The formation of Ig-Ag complexes represents a general phenomenon since it has been observed with antigenic stimulation by particulate antigens (Paraskevas *et al.*, 1972), by soluble antigens emulsified with FCA (Paraskevas *et al.*, 1972), by syngeneic tumour cells (Dular *et al.*, 1978) and by allogeneic cells (Paraskevas, pers. comm.). As a result of the formation of the cytophilic Ig-Ag complexes which are taken up by T cells in the spleen and lymph nodes, there is an increase of Ig^+ cells six hours after immunization. Such an increase is called for convenience the "six hour response". Furthermore, the formation of cytophilic Ig-Ag complexes has been demonstrated in numerous murine strains, such as Balb/c, DBA/2, AKR, C3H/He, SJL/J, C57BL/6 (Lee and Paraskevas, 1981; Dular *et al.*, 1978).

The formation of cytophilic Ig-Ag complexes is one of a host's earliest immune responses to antigenic stimulation. The Ig-Ag complexes are involved with the regulation of the 7S antibody response. Serum collected six hours after antigenic stimulation, with either a particulate antigen or a soluble protein antigen emulsified with FCA, was demonstrated to contain an antigen-specific factor(s) capable of significantly enhancing 19S and 7S antibody formation when given with a subimmunogenic antigen challenge (Lee and Paraskevas, 1981). The factor responsible

for the antigen-specific enhancement of the 7S response was found to be the cytophilic Ig-Ag complexes. Paraskevas and Lee (1981) suggested that one of the mechanisms of enhancement of antibody synthesis by Ig-Ag complexes was through their uptake by T cells. Previously, Paraskevas and Lee (1976) had demonstrated that T cells which have acquired surface Ig within six hours after antigenic stimulation, 6-h T cells, exerted a significant amplification effect on antibody synthesis in collaboration with bone marrow cells.

An intraperitoneal inoculation of 10^4 live PX2-16 cells can induce the formation of cytophilic Ig which presumably represents complexes of Ig and tumour antigen. However, an inoculum of 10^3 live PX2-16 cells induced only a partial six hour response as measured by RICA. An inoculum of 10^2 viable PX2-16 cells did not induce a detectable six hour response (Table I). Both an inoculum of 10^2 and 10^3 live PX2-16 cells could kill 100% of the mice so injected. Previously, Chow (1975) reported that an intraperitoneal injection of 10^3 viable L5178Y lymphoma cells into their syngeneic host, DBA/2 mice, produced a partial six hour response while an intraperitoneal injection of 10^2 viable L5178Y cells did not induce a measurable six hour response. Since an inoculum of 10^2 live PX2-16 cells failed to induce the formation of Ig-Ag complexes, it is reasonable to postulate that responses of the immune system dependent on this phenomenon would be altered.

An analogy to the conditions of spontaneous tumourigenesis was sought by inoculating DBA/2 mice with an intraperitoneal injection of 10^2 live PX2-16 cells. This dose of cells does not induce formation of Ig-Ag complexes but still killed 100% of mice inoculated. Neither an intraperitoneal injection of a particulate antigen, 5×10^7 or 5×10^8

SRBC, or of a soluble antigen, 250 µg BSA emulsified with FCA, 24 hours after tumour pretreatment were any longer able to induce the formation of Ig-Ag complexes. If DBA/2 mice were pretreated with an intraperitoneal injection of 10^2 live DBA/2 spleen cells, the animals were still able to produce a full six hour response 24 hours later when challenged with 5×10^8 SRBC. Therefore, the initiation of tumour growth in DBA/2 mice from an inoculum of a small number of tumour cells nonspecifically abrogated the formation of cytophilic Ig-Ag complexes. Similarly, Chow *et al.* (1978) reported that DBA/2 mice bearing L5178Y tumours were unable to produce a six hour response either to a challenging dose of 10^7 L5178Y cells or to 5×10^8 HRBC from 24 hours to at least seven days following inoculation of 10^2 live L5178Y cells.

Since pretreatment of DBA/2 mice with 100 viable P815-16 mastocytoma cells 24 hours before antigenic stimulation nonspecifically abrogated the formation of Ig-Ag complexes which have been shown to enhance the 7S antibody response, the effect of such an inoculum on the antigen specific antibody enhancing activity of six hour serum was examined. An intraperitoneal inoculum of 100 live PX2-16 cells 24 hours preceeding an intraperitoneal inoculum of 5×10^7 SRBC inhibited the enhancing activity of the serum collected six hours after the SRBC challenge by 90% for the 7S response and by 55% for the 19S response (Figure 5). The abrogation of the enhancing activity for the 7S antibody response, normally detected in serum six hours following antigenic stimulation, further suggests that a small growing tumour nonspecifically suppresses the formation of Ig-Ag complexes. This observation is corroborated by the report of Chow *et al.* (1978) who found that the injection of 100 live L5178Y lymphoma cells into DBA/2 mice abolished the enhancing effect which 6-h primed

T cells exerted on antibody formation.

FcR^+ Ia^+ T cells is the normal T cell subset responsible for the uptake of Ig-Ag complexes as shown *in vitro* (Paraskevas and Lee, 1979). Furthermore, it was demonstrated that the Ia antigen rather than the Fc receptor acts as the cellular receptor for the Ig-Ag complexes. The RICA technique was used to determine if spleen cells from DBA/2 mice which 24 hours earlier had received an intraperitoneal injection of 100 viable PX2-16 cells were capable of taking up cytophilic Ig-Ag complexes induced in normal DBA/2 mice by SRBC. Spleen cells from tumour pretreated DBA/2 mice were able to take up cytophilic Ig-Ag complexes to the same extent as spleen cells from normal DBA/2 mice (Table II). The early stages of tumour growth did not appear to affect the ability of the FcR^+ Ia^+ T cell subset of the normal spleen cell population to take up Ig-Ag complexes.

Chow *et al.* (1978) have suggested that the T cells involved in the uptake of Ig-Ag complexes are affected by an inoculation of a small number of tumour cells. They reported that while cytophilic Ig-Ag complexes were detected in serum of mice after immunogenic challenge 14 days following pretreatment of the mice with 100 live lymphoma cells, there was no increase in Ig^+ cells in the spleens of the animals. However, the authors did not test for the presence of a defect in the T cell subpopulation responsible for uptake of Ig-Ag complexes at earlier phases of tumour growth.

The complexes of Ig and antigen found within six hours after immunization in the serum of mice are formed through the mediation of a T cell derived factor termed immunoglobulin-antigen complexing factor (IACF) (Paraskevas *et al.*, 1976, 1979). IACF is a product of an interaction

between T cells and macrophages. Paraskevas *et al.* (1979) have demonstrated that the interaction between T cells and macrophages occurs through a mediator released from the macrophages as a result of stimulation by particulate or aggregated substances. The mediator subsequently stimulates Fc receptor-negative nylon wool nonadherent T cells to produce IACF.

The effect of a small growing tumour on the ability of macrophages and T cells to successfully cooperate in the production of IACF was investigated. Macrophages from DBA/2 mice which 24 hours previously were injected with 100 live PX2-16 cells could successfully cooperate *in vitro* with nylon wool nonadherent T cells from normal DBA/2 mice to produce detectable IACF activity (Figure 6). However, tumour pretreated nylon wool nonadherent T cells could not successfully cooperate with normal macrophages to yield measurable IACF activity (Figure 7). The data suggests that a tumour in its earliest stages of growth nonspecifically induces a defect in T cells which disallows the successful interaction between macrophages and T cells necessary for the production of IACF. Macrophages from animals with a small growing tumour can still produce an active mediator capable of stimulating T cells to produce IACF.

Paraskevas *et al.* (1979) have found that Fc-receptor negative T cells in the absence of macrophages can be stimulated *in vitro* by sulfhydryl containing substances resulting in IACF production. Nylon wool nonadherent T cells from DBA/2 mice which were injected 24 hours earlier with 100 viable P815-16 cells could not be stimulated by 2-mercaptoethanol to yield measurable IACF activity (Figure 8). When supernates from normal nylon wool nonadherent T cells stimulated by 2-mercaptoethanol were added to supernates from tumour pretreated nylon wool nonadherent T cells sti-

mulated by 2-mercaptoethanol, the IACF activity in the supernates from the normal T cells was not inhibited (Figure 9). The data indicates that molecules antagonistic to the activity of IACF are not present in the supernates of T cells from tumour pretreated animals.

The *in vitro* studies of IACF production suggest that a tumour in its early stages of growth nonspecifically suppresses the generation of active IACF, the mediator necessary for the formation of Ig-Ag complexes.

Thymocytes from normal animals are able to reconstitute the ability of lethally irradiated animals to produce cytophilic Ig-Ag complexes (Lee and Paraskevas, 1972). The ability of nylon wool nonadherent T cells, from normal DBA/2 mice and from DBA/2 mice pretreated with live P815-16 mastocytoma cells, to reconstitute the formation of Ig-Ag complexes in lethally irradiated DBA/2 mice was examined. Both nylon wool nonadherent T cells from normal mice and from DBA/2 mice pretreated with 10^4 live P815-16 cells reconstituted the ability of lethally irradiated DBA/2 mice to produce Ig-Ag complexes in response to antigenic stimulation by SRBC. However, nylon wool nonadherent T cells from DBA/2 pretreated with either 10^2 or 10^3 viable PX2-16 cells could not reconstitute the formation of Ig-Ag complexes (Figure 10). The data indicates that tumours developing from inocula of 10^2 or 10^3 tumour cells can induce a defect in T cells which precludes successful *in vivo* cooperation between T cells and macrophages necessary for the production of Ig-Ag complexes. The dose of 10^4 tumour cells does not inhibit the formation of cytophilic Ig-Ag complexes because it may present sufficient antigenic stimulation to the immune system to induce an immune response rather than inhibit an immune response.

An important consideration is whether the ability to nonspecifically suppress the formation of Ig-Ag complexes is restricted to tumours in

their early phases of growth and development. As previously discussed, injections of normal syngeneic cells had no effect on the generation of Ig-Ag complexes. The ability of nylon wool nonadherent T cells pretreated with a tolerogenic dose of dHGG to reconstitute the formation of Ig-Ag complexes in lethally irradiated animals was examined. Chiller and Weigle (1971) demonstrated that an intraperitoneal injection of 50 µg dHGG renders mice specifically unresponsive to challenge with aggregated HGG to a degree of 75% within six hours. Two days after the tolerogenic stimulus the T cells were found to be almost totally unresponsive to aggregated HGG, while B cells were 100% responsive and remained so until three days following tolerization (Weigle *et al.*, 1972). A single injection of 50 µg dHGG, which itself does not induce Ig-Ag complexes, abrogated the ability of T cells to reconstitute the production of Ig-Ag complexes in lethally irradiated mice (Figure 11). The data from these experiments indicates that nonspecific suppression of the generation of Ig-Ag complexes is not a property unique to inocula of small numbers of tumour cells. The phenomenon may be a consequence of stimulation of the immune system by tolerogenic doses of soluble antigen.

The nonspecific suppression of the formation of Ig-Ag complexes is associated with a defect in a T cell subpopulation. The generation of cytophilic Ig-Ag complexes and their uptake by FcR^+ Ia^+ T cells suggests that antigenic stimulation affects a population of cells much larger than the antigen sensitive cells. It is, therefore, reasonable to postulate that interference with this early immune response would have far reaching effects on subsequent immune responses. An area for further study, presently being examined by other investigators in this laboratory, is the effect of the described T cell defect on tumour-specific immunity.

Previously, another investigator from this laboratory correlated inhibition of the production of cytophilic Ig-Ag complexes with a decrease in immune reactivity. Chou (1978) reported that a daily injection for five days of soluble HRBC hemolysate nonspecifically inhibited the six hour response to antigen from day 7 to day 14. The injection of HRBC lysate itself did not induce a six hour response. The treatment resulted in specific inhibition of both 19S and 7S PFC responses to antigenic challenge and nonspecific depression of delayed type hypersensitivity reactions, cell mediated cytoxicity, and PHA reactivity of spleen cells.

The hypothesis of immunostimulation suggests that although a specific immune reactivity may be adequate to control a neoplasm, lesser degrees of immune reactivity may promote the growth of nascent tumours (Prehn and Lappé, 1971; Prehn, 1976). The theory proposes that an immune reaction may result in better tumour growth than would have occurred in the total absence of an immune response. It is possible that one mechanism by which a nascent tumour may depress immune reactivity to a level which would be beneficial to its growth is by inhibiting the formation of Ig-Ag complexes.

Numerous reports in the literature have indicated that immune responses following the injection of a large number of tumour cells is not the same as that observed following injection of a small number of tumour cells. In several different tumour-host systems inocula of very small numbers of either syngeneic or allogeneic neoplastic cells have been observed to grow progressively in host animals, while inocula of a larger number of cells of the same tumours were rejected or grew very slowly (Old *et al.*, 1962; Humphreys *et al.*, 1963; Bonmassar *et al.*, 1971, 1973, 1974; Kölsch *et al.*, 1973; Mengersen *et al.*, 1975; Cihak *et al.*, 1981).

Kolsch *et al.* (1973) reported than an inocula of 10^2 to 5 x 10^2 BM3 mastocytoma cells into their syngeneic host, Balb/c mice, produced a higher incidence of tumours than inocula of 10^3 to 10^4 BM3 cells. In addition, Mangersen *et al.* (1975) demonstrated that pretreatment of Balb/c mice with 10 to 5 x 10^2 irradiated BM3 cells facilitated tumour take after injection of 10^5 living BM3 cells. Since determinant-specific suppression was observed following the injection of 10^2 viable BM3 cells, the authors suggested that the inocula of a small number of tumour cells induced low zone tolerance mediated by suppressor cells. These observations were corroborated by Cihak *et al.* (1981) who reported specific suppression mediated by suppressor cells was induced by injection of ADJ-PC-5 plasmacytoma cells into their syngeneic host, Balb/c mice, at a dose 10^3 times lower than that which induced protective immunity.

Bonmassar *et al.* (1971, 1973, 1974) reported that pretreatment of mice with a single injection of a small number of tumour cells facilitated the growth for up to 90 days of an inoculum of tumour cells which would normally have been rejected. The H-2 antigens of challenging or conditioning tumours did not have to be the same. The authors suggested that a diffusable factor that they found to be associated with lymphomas and other tumours nonspecifically depressed the anti-lymphoma reactivity of the mice. Consequently, the initial inadequate antigenic stimulus from the small number of tumour cells in concert with nonspecific immunosuppression allowed the outgrowth of the second tumour inoculum. This data agrees with our observations of nonspecific suppression following inoculation of a small number of tumour cells.

Argyris (1978) reported that a soluble factor released from P815 tumour cells inside cell-impermeable diffusion chambers implanted intra-

peritoneally in DBA/2 mice could activate nonspecific suppressor T cells which could inhibit mixed lymphocyte cultures. The suppressor cells were detected five days after tumour transplantation, but were not tested for their presence at earlier stages. Fujimoto *et al.* (1977a, 1977b) found specific T suppressor cells which developed 24 hours after tumour transplantation.

Furthermore, a study by Kamo *et al.* (1975) also indicated that a T cell population is directly affected by immunosuppressive factor(s) from mastocytoma cells. Ascites from mastocytoma-bearing mice or mastocytoma cell homogenates suppressed the *in vitro* immune responses of DBA/2 spleen cells to SRBC. The suppressed immune response of the pretreated splenocytes could be returned to normal levels by addition of T cells activated to SRBC but not by B cells or peritoneal exudate cells. The authors suggest that the target of the immunosuppressive factor from the mastocytoma cells may be non-activated T cells involved in helper functions.

In conclusion, there is a substantial amount of experimental evidence in the literature which details the induction of immunosuppression by small numbers of tumour cells and by factors associated with tumour cells. The data presented in this thesis indicates that a tumour in its early stages of growth and development can nonspecifically suppress the formation of cytophilic Ig-Ag complexes, one of the immune system's earliest responses to antigenic stimulation. This suppression of one of the immune system's mechanisms for specifically enhancing immune responses may prematurely limit the immune response to neoplastic cells, thereby allowing nascent tumours to escape from immunological surveillance.

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