STUDIES ON THE ANTI-TUMOR RESISTANCE OF B-LYMPHOCYTE-DEPRIVED MICE

A Thesis

by

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ABSTRACT

The aim of the present study was to examine the role of B-lymphocytes and their products in host resistance to chemical carcinogenesis and to a chemically-induced syngeneic tumor. To this end, host-tumor relationships were studied in mice depleted of B-lymphocytes by the continuous administration of rabbit anti-mouse IgM serum. In the first phase of the study, it was found that these mice have a heightened resistance to carcinogenesis and to the tumor. In the second phase, an *in vitro* analysis of the nature and level of cell-mediated anti-tumor reactivity was undertaken. It was observed that spleen cells from suppressed mice had an increased cytotoxicity to tumor cells. This activity was independent of local tumor size, and was not specific to the tumor injected. Fractionation procedures aimed at the selective removal of either T-lymphocytes or phagocytic monocytes indicated that these cells did not play a major role in the reaction. Additionally, it was found that spleen cells from suppressed mice were considerably more cytotoxic to an NK sensitive target and that the killer cell displayed several of the functional and morphological characteristics of the NK cell. Furthermore, using a cold target inhibition assay, a correlation could be demonstrated, between the ability of various tumor lines to specifically block NK target lysis, *in vitro* and an increased resistance to their growth in the immunosuppressed mice. It seems, therefore, that the *in vivo* resistance to tumor growth of B-lymphocyte-depleted mice, may be mediated by heightened natural killer mechanisms.

RESUME

La présente étude a pour but d'étudier le rôle des lymphocytes B et de leurs produits dans la résistance de l'hôte à une carcinogénèse chimique et à une tumeur isogénique provoquée chimiquement. Pour cela nous avons observé les relations hôte-tumeur chez des souris démunies de lymphocites B par administration continue d'un sérum anti IgM de souris provenant d'un lapin. Lors de la première phase de cette étude, nous avons découvert que ces souris présentaient une résistance accrue à la carcinogénèse et à la tumeur. Dans la seconde phase, nous avons entrepris l'analyse in vitro de la nature et du degré de la réactivité anti-tumorale à médiation cellulaire. Nous avons observé une cytotoxicité accrue envers les cellules tumorales dans des cellules de la rate de souris soumises à des immunosuppresseurs. Cette activité était indépendante de la taille de la tumeur locale et n'était pas spécifique de la tumeur provoquée. Nous servant de méthodes de fractionnement, nous avons tenté le retrait sélectif soit des lymphocytes T, soit des monocytes phagocytaires, et nous en avons conclu que ces cellules ne jouaient pas un rôle important dans la réaction.

De plus, nous avons découvert que les cellules de la rate de souris soumises à des immunosuppresseurs étaient considérablement plus cytotoxiques envers une cible sensible aux NK et que le lymphocyte K présentait plusieurs des caractéristiques fonctionnelles et morphologiques de la cellule NK. En outre, à l'aide d'une épreuve d'inhibition de cible froide, on a pu démontrer une corrélation entre la capacité, in vitro, de diverses lignes de tumeurs de bloquer spécifiquement la lyse des cibles NK, et une résistance accrue à leur développement chez des souris soumises à des immunosuppresseurs. Il semble donc qu'in vivo la résistance aux tumeurs de souris soumises à des immunosuppresseurs soit due à un accroissement des mécanismes naturels de destruction.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Our studies on the tumor-host relationship in B-lymphocyte deprived mice produced the following findings:

1. Mice, depleted of their B-lymphocytes had a heightened resistance to tumor induction by 3-MCA.

2. The depletion of B-lymphocytes could significantly enhance the resitance of the immunosuppressed mice to syngeneic, transplanted MCA-induced tumors.

3. The depletion of B-lymphocytes could not under any of the conditions tested, increase the susceptibility of the mice to the growth of MCA-induced tumors.

4. The metastatic spread of a local MCA-induced tumor was reduced in the immunosuppressed mice.

5. B-lymphocyte deprived mice displayed a heightened resistance to grafts of parental BM and a leukemia of the parental strain.

6. The increased resistance displayed by the immunosuppressed mice to malignancy was paralleled by a 2-3 fold increase (compared to normal mice) in the spontaneous cytotoxicity of their spleen cells to tumor targets in vitro. This spontaneous killing was mediated by a cell population which displayed many of the characteristics of the mouse NK cells.

7. A positive correlation was found between the enhanced resistance of the immunosuppressed mice to the growth of a tumor in vivo, and the susceptibility of this tumor to the killer cell in vitro.

8. In addition to these findings made in the course of the study of B-lymphocyte deprived mice, a method for the production of large volumes of antibody, in ascites fluid, against particulate antigens was modified and adapted to the production of large volumes of anti-tumor antibodies in normal mice. The procedure was successful in raising large volumes of anti-tumor antibodies in individual mice of the syngeneic strain.

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LIST OF ABBREVIATIONS

ACK - 0.155 M NH_4Cl + 0.1mM Na_2EDTA + 0.01 $NKHCO_3$

ADCC - Antibody Dependent Cellular Cytotoxicity

Anti-IgM - Rabbit anti-mouse Immunoglobulin M serum.

BM	Bone Marrow
BUDR	5-Bromodeoxyuridine
cAMP	Cyclic Adenosine 3'5' Monophosphate
CFA	Complete Freund's Adjuvant
cGMP	Cyclic Guanosine 3'5' Monophosphate
CM	Cell Mediated
Con A	Concanavalin A
^{51}Cr	$Na_2{}^{51}CrO_4$
DMSO	Dimethylsulfoxide-$(CH_3)_2SO$
DNAse	Deoxyribonuclease
DTH	Delayed Type Hypersensitivity
FCS	Fetal Calf Serum
FUDR	5-Fluorodeoxyuridine
HBSS	Hank's Balanced Salt Solution
Ig	Immunoglobulin
i.m.	intramuscular
i.p.	intraperitoneal
$^{125}IUDR$	5[^{125}I]Iodo-2-deoxyuridine
i.v.	intravenous
NK	Natural Killer
MCA or 3-MCA	3-Methylcholanthrene
MD	Medin-Darby Medium
MEM	Minimal Essential Medium
min.	minutes
NRS	Normal Rabbit Serum
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
PLL	Poly-L-lysine
RPMI-FCS	RPMI medium + 1% Hepes Buffer + 0.001 gentamicin sulfate + 10% FCS

R.T.	Room Temperature
s.c.	subcutaneous
SDS	Sodium Dodecyl Sulfate
SRBC	Sheep Red Blood Cells
Anti SRBC	Mouse anti-Sheep Red Blood Cell Serum
^{3}H-Thymidine - methyl	^{3}H-thymidine
YAC	YAC-1

Materials

A.	Supplier
ACK - 0.155 M HN_4Cl	Fisher Scientific Co. Ltd., Montreal, Quebec
0.1 mM Na_2 EDTA Dihydrate crystal	J.T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.
0.01 M $KHCO_3$ - crystal	" " "

Antisera

Goat anti-mouse sera, class specific (anti-IgG_1, anti-IgG_2, anti-IgA)	Meloy Lab., Springfield, Virginia, U.S.A.
Rabbit anti-mouse brain (C3H) serum, absorbed	Litton Bionetics, Kensington, Maryland, U.S.A.
Rabbit anti-mouse T cell serum (rabbit anti-brain associated Thy-1 antiserum) absorbed	Cedarlane Lab., Hornby, Ont.
Rabbit anti-mouse immunoglobulin antiserum polyvalent, lyophilized	" "

B.	
Bouins Solution - picric acid, saturated solution (15 parts)	Fisher Scientific Co. Ltd., Montreal, Quebec
- formaldelhyde 36.9/solution (5 parts)	" " "
- acetic acid glacial (2 parts)	" " "

C.	
chromium-51 (^{51}Cr) $Na_2{}^{51}CrO_4$ solution in saline	New England Nuclear, Boston, Mass., U.S.A.
complement - low tox rabbit complement lyophilized	Cedarlane Lab., Hornby, Ontario
carbonyl iron powder	GAF Co., N.Y., N.Y., U.S.A.
Concanavalin A (Con A), freeze dried	Pharmacia Fine Chemicals, Uppsala, Sweden

	Supplier
Complete Freunds Adjuvant (CFA)	Difco Laboratories, Detroit, Michigan, U.S.A.
Cobalt 60 - Theraton CII Cobalt Unit	Atomic Energy of Canada, Commercial Products, Ottawa, Ontario
Centrifuges	
IEC International Centrifuge - Model PR-2 - Model PR-6000, Damon - Model UV - Table top	IEC, Needham Hts., N.J., U.S.A.
Sorval GLC-2	Sorval, Newton, Conn., U.S.A.
Clay Adams Safety Head	Clay Adams, Parsnippy, N.J., U.S.A.
D.	
Dimethyl sulfoxide (DMSO)	J.T. Baker Chemical Co., Phillipsburg, N.J.
Deoxyribonuclease I (DNase I) non-crystalline	Sigma Chemical Co., St. Louis, Mo., U.S.A.

	Supplier
F.	
5-flourodeoxyuridine - crystalline	Sigma Chemical Company, St. Louis, Mo., U.S.A.
G.	
Gamma sample counter - Wallac 8000	LKB, Uppsala, Sweden
Glass fiber filters - reeve angel	Whatman Inc., Clifton, N.Y., U.S.A.
L-glutamine - MEM (100x) - lyophilized	Gibco, Burlington, Ont.
Glutaraldehyde 50% w/w	Fisher Scientific Co., Montreal, P.Q.
Glycine (aminoacetic acid), crystalline	Sigma Chemical Co., St. Louis, Mo., U.S.A.
H.	
Hepalean (heparin sodium)	Harris Laboratories, Brantford, Ontario
I.	
5[^{125}I] - Iodo-2-deoxy uridine (^{125}IUDR) in aqueous solution, sp. act. 5 ci/mg	Amersham Co., Oakville, Ontario
L.	
Latex particles (Dow Uniform), polystyrene latex, particle diameter 1.10 μ	Dow Chemical Co., Midland, Michigan, U.S.A.
M.	
Mash II harvester	Microbiological Associates, Bethesda, Maryland, U.S.A.
Metabolic shaking incubator (Dubnoff)	Precision Scientific Co., Chicago, Illinois, U.S.A.
3-methylcholanthrene	Eastman Kodak Co., Rochester, N.Y., U.S.A.
McNeal Tetrachrome	Sigma, St Louis, M.O.

N.	Supplier
Needles 25 g x 5/8"	Terumo Co., Tokyo, Japan
Nylon wool - LP-1 leuko-pak, leukcocyte filter	Fenwal Lab., Morton Grove, Illinois, U.S.A.
P.	
Poly-L-lysine (PLL) hydrobromide type 1-B, approx. mol. wt. 85,000	Sigma Chemical Co., St. Louis, Mo., U.S.A.
Protein A - staphyloccoccus aureus	Pharmacia Fine Chemicals, Uppsala, Sweden
R.	
Rocker platform	Bellco Glass Inc., Vineland, N.J., U.S.A.
S.	
Sheep Red Blood Cells (SRBC)	Institute Armand Frappier, Laval, P.Q.
Solimix II	ICN - Chemical and Radioisotope Divisio Irvine, California, U.S.A.
Syringes - plastipak	Becton, Dickinson and Co., Rutherford, N.J., U.S.A.
T.	
Tissue culture media and supplements	
Hank's Balanced Salt Solution (HBSS or Hank's BSS)	Gibco, Burlington, Ont.
Minimal Essential Medium (MEM), Earle's base (10x) supplemented with 3% sodium bicarbonate 7% solution	" "
RPMI 1640	" "
Medin Derby Medium (MD) - 8 gm. NaCl (sodium chloride), 0.4 gm. KCl 0.58 $NaHCO_3$ (sodium bicarbonate) 1 gm. Dextrose, 0.2 gm. EDTH in 1 liter distilled H_2O	Fisher Scientific Co., Montreal, P.Q. " "

	Supplier
Fetal Calf Serum (FCS)	Gibco, Burlington, Ont.
Hepes Buffer - 1 M solution	" "
Gentamicin Sulfate, 55% potency	Schering Co., Quebec
Tissue Culture Plasticware	
Tissue culture flasks, Falcon, growth area 25 cm^2x75 cm^2	Becton, Dickinson and Co., Oxnard, California, U.S.A.
Tissue culture plate	
1) microtest II - Falcon, 96 flat bottom wells	" " "
2) tissue culture cluster, 96 flat bottom wells	Cestar, Cambridge
Tissue culture dishes	
Thymidine - ^{3}H-methyl (^{3}H-thymidine) spec. act. 20 ci/mmole	New England Nuclear, Boston, Mass., U.S.A.
Trioctanoin oil	Eastman Kodak Co., Rochester, N.Y.
Trypsin, 2.5% in saline (10x) lyophilized	Gibco, Burlington, Ont.

CHAPTER 1

INTRODUCTION - PART I.

TUMOR IMMUNOLOGY - A HISTORICAL REVIEW

Two reports are often cited as the experimental and theoretical basis for the field of tumor immunology. The first is a demonstration by Foley in 1953 (1) that mouse tumors can be immunogenic for their host, and the second is a reformulation by Thomas in 1959 (2) of the theory of immune surveillance first proposed by Ehrlich in 1909 (3). The ensuing years have produced abundant experimental evidence, the interpretation of which is still a source of much controversy and little consensus.

The immune surveillance theory, of which Burnet has been a major advocate in recent years (4), assumes an active role for thymus-dependent cellular immune mechanisms in searching out and eliminating cancerous cells in situ. It also assumes that the appearance of a tumor indicates a failure of some sort on the part of the immune response to fulfill this function. Although this theory was strongly criticized, and its validity openly questioned in recent years (5-7), it did have its benefits in producing a wide search for cellular immune mechanisms capable of specifically recognizing and destroying cancer cells. It has also brought forth numerous theories, some of them supported by experimental evidence, attempting to explain the growth of tumors in the face of an active immune response.

A comprehensive review of the developments in the field during the last two decades is beyond the scope of this work. However, major issues and findings will be discussed with emphasis on the studies with the laboratory animal model. Where appropriate, the relevance of these findings to human cancer will also be discussed.

1.A. Tumor Associated Antigens.

Since the ability of tumors to evoke an immune response in their syngeneic host, i.e. tumor antigenicity, has been the premise (although not universally accepted) for the study of anti-tumor immunity, it seems appropriate to begin this review with a summary of the evidence available to date on tumor antigens. Three types of experimental tumors, the chemically-induced, the virally-induced, and the spontaneous, are widely used and each will be discussed separately.

Tumor antigenicity has commonly been defined on the basis of three criteria:

1) an in vivo resistance to a tumor challenge induced by previous exposure to the tumor or tumor extracts (8).

2) an in vitro sensitivity of the tumor to cellular immune mechanisms (9.), and

3) the demonstration of tumor specific antibodies (10,11,12).

Based on these methods, three types of tumor antigens are now recognized:

Tumor-Specific Antigens (TSA) are those antigens which are detectable only on tumor cells and which differ qualitatively from those expressed on normal cells.

Tumor-Associated Antigens (TAA) are antigens which are found on tumor cells but which can also be detected on other types of tissue.

Tumor-Associated Transplantation Antigens (TATA) are defined as those antigen which are capable of inducing a rejection of tumor grafts in vivo. This functional definition may refer to either of the above.

1.A.1. Antigens of Chemically-Induced Tumors. Chemically-induced tumors provided the first indication for the existence of TATA. Several reports published in the 1950's (1,13,14) demonstrated that exposure of mice and rats to a methylcholanthrene-induced tumor in a non-lethal form (either by injecting a small number of cells or by excising a small tumor) prevents the growth of a second challenge of the same, but not other MCA-induced

tumors. These findings were later extended to include tumors induced by other chemicals such as the 4-dimethyl amino azobenzene (DMAB) induced hepatomas (15), and 3,4-benzpyrene-induced tumors (15-17).

It has long been demonstrated that the antigens of a chemically-induced tumor are unique and are not shared by other similarly induced tumors. This was shown to be the case even for tumors which originated in the same host (18). The evidence for these private antigens comes both from experiments showing non-cross-reactivity of resistance to tumor transplantation in vivo (19) and from neutralization, and microcytotoxicity studies in vitro (16,20-23). Other immunological parameters that were tested, including Delayed-Type Hypersensitivity (DTH) and macrophage migration inhibition (23), confirmed the individuality of these antigens.

In addition to the unique private antigens, chemically-induced tumors were also reported to have cross reacting or common antigens (14,19,24,25). Their role in mediating in vivo tumor rejection however, is still uncertain (19,26).

It should be noted in this context that fetal calf serum and other culture medium components have been shown to modulate cell surface antigenicity of cultured cell lines. It is possible, therefore, that antigenic cross-reactivity of tumor lines is due to common medium components rather than shared intrinsic receptor sites (37-30). The expression of C-type particles on mouse cells after their long term in vitro cultivation has also been well documented (31,32).

Chemically-induced tumors have also been shown to possess fetal antigens (33,35). These antigens can be detected on a wide range of experimental tumors, regardless of their etiology, and were also demonstrated on human malignancies (35). Coggin et al., after an extensive examination of many rodent tumors proposed, in fact, that "The expression of embryonic and fetal antigens occurred as a fundamental trait of neoplastic cells" (36). Since fetal antigens are present during certain stages of embryonic development (37) and can also be detected in diminished amounts on normal tissue (38), they represent a major group of TAA.

1.A 2. Antigens of Virally-Induced Tumors. Antigenic expression on virally-induced tumors can be due to either the expression of viral antigens or the viral induction of new cellular antigens.

The virally-induced tumors can be divided into two major groups. Those induced by DNA viruses and those induced by RNA viruses.

DNA Virus (Oncodna)-Induced Tumors: The best studied viruses in this group are the Papova viruses - Polyoma and SV40 (39) and the Herpes viruses, in particular the Epstein-Barr virus (EBV) - induced tumors (40).

The reproduction of DNA virus particles and the transformation of host cells are two alternative pathways in the oncodna virus cycle. Therefore, cells that are permissive and produce complete virus particles do not undergo a malignant transformation and are lysed by the virus. On the other hand, transformed cells, which are capable of producing new, tumor-associated, antigens, rarely produce intact virus (41). This phenomenon has greatly facilitated the separation and study of viral and the distincly different, virally-induced cellular antigens.

As opposed to chemically-induced tumors, cellular antigens on virally-induced tumors are characteristically shared by all tumors induced by the same virus (42,43). This indicates a common mechanism of transformation in these cells. It does not, however, exclude individual tumor antigens which have also been reported (44).

Two types of TSA have been described in this group; the intracellular and the cell surface antigens. The intracellular, i.e. the nuclear and cytoplasmic antigen, T, of the SV40 system is one of the better studied antigens. It was first detected in 1964 (45,46). It is synthesized early in infection (12-24 hrs), does not require viral DNA synthesis, and can be found in cells undergoing both a reproductive and a transforming infective cycle (47). A second intracellular antigen located on the nuclear membrane and designated V, was also identified in the SV40 system. These antigens which are shared by all SV40 transformed cells do not appear to play a significant role in tumor rejection (48).

The cell surface antigens of the oncodna viruses, on the other hand have been shown by different methods to be capable of evoking both cellular and humoral immune responses. Sjörgen in 1965 (8) tested various oncodna virus-induced tumors and demonstrated TATAS capable of inducing a cellular

immune response and *in vivo* tumor rejection on all of them. These antigens were also shown to be immunogenic in the solubilized form (49).

Burkitt's lymphoma is believed to be a virally-induced human tumor and the Epstein-Barr oncodna virus has been implicated in its etiology (50). Its antigens have been extensively studied and reviewed (40). Several tumor antigens have been described. Among them are the intracellular antigens VCA (Viral Capsid Antigen) (51) and EA (Early Antigen) (52), as well as cell surface antigens which react with sera from Burkitt's lymphoma patients (53).

RNA virus (Oncorna) - induced tumors. Oncorna viruses have been shown to be the causative agent in a wide spectrum of experimental tumors and were implicated in several human malignancies (54). The study of TAA s in this group of tumors, has been complicated by the fact that they actively produce virus particles which are in themselves rich in antigenic structures.

TSAs on the surface of these tumors were first demonstrated by Klein et al. (55) and Sjörgen and Jonsson (56). These antigens were reportedly responsible for tumor rejection by immunized animals. Using serological techniques, it was found that several classes of cell surface antigens exist, and that they are distinct from virus particle-associated antigens (57). Different tumors, induced by the same virus, even in different species, share these antigens as well as Viral Envelope Antigens (VEA), which can be detected on the membranes of the cells.

In addition to cellular immune mechanisms which mediate graft rejection, cytotoxic, as well as neutralizing antibodies directed against these antigens can be detected in tumor-bearing mice (58,59). Similarly to the chemically-induced tumors, fetal TAAs were also detected on the virally-induced tumors (36).

1.A.3.The Tumor and Histocompatibility Antigens. The biological significance of tumor antigens is as yet poorly understood. There have been suggestions that tumor specific antigens are closely related to histocompability antigens and some evidence exists that they may indeed be altered normal histocompatibility antigens. This evidence is based on the findings that both classes

of antigens can evoke cellular and humoral immune responses (60) and that an inverse relationship can be demonstrated between the expression of H-2 and TATA on the surface of cells (61,62). The genetic evidence linking the H-2 complex and tumor antigens is at present controversial. In a study by Klein and Klein (63), no evidence could be found that the TATA of a murine MCA tumor was coded for by the histocompatibility complex. Other studies, however, imply that such a link does exist (64,65).

Additionally, it was suggested that tumor antigens may function as receptor sites on the cell surface or they may have an enzymatic function responsible for the malignant behavior of the cell (66).

1.A.4 Antigenicity of Spontaneous Tumors. Whereas, both chemically and virally-induced experimental tumors have been convincingly shown to possess TATAs, reports originating from several laboratories indicate that spontaneous rodent tumors (i.e. those that are not induced by laboratory techniques) of recent origin cannot induce immunity in the syngeneic host. These findings have in fact been the source of some recently expressed skepticism, as to the relevance of the experimentally-induced tumors as models in cancer research (67-69).

As early as 1966 Baldwin reported that whereas immunization with MCA-induced tumors was accomplished in rats, attempts to immunize them with their spontaneous tumors were unsuccessful (70). Prehn obtained similar results with spontaneous fibrosarcomas in old mice (71,72). Furthermore, Hewitt recently reported that after tests with 27 spontaneously arising tumors in mice, they could demonstrate no evidence of immunogenicity, although a wide variety of quantitative experiments were carried out (73).

One explanation given for this phenomenon was that spontaneous tumors arise in face of an immunoselective pressure which favors the growth of non-antigenic tumors (68). This hypothesis was supported by evidence in some systems of an inverse relationship between immunogenicity of tumors and the latency period after chemical induction (15,74) and between tumor antigenicity and metastasis (75).

The existence of this relationship has been rejected, however, by several researchers on the basis of several lines of evidence. Among them were reports of considerable variability in antigenicity of tumors with the same latent period, and the demonstration of a marked resistance by antigenic tumors to negative selection in immunocompetent hosts (76,77).

Other lines of evidence indicate that tumor antigenicity is not influenced by an immune selection process (78) and may be affected by the cell cycle phase of the transformed cells. Thus Carbone and Parmiani have shown (79) that treatment of replicating cells with methylcholanthrene induces non-immunogenic sarcomas, while the same treatment given to cells in a resting phase produces immunogenic tumors.

The above findings suggest that the expression of new antigens on a tumor is not a necessary characteristic of the neoplastic transformation and may in fact be the exception rather than the rule.

These conclusions have recently brought forth several calls for a change in direction and emphasis in the field of tumor immunology (67,69).

1.B. Mechanisms of Tumor Cell Destruction.

An intense search for immune mechanisms capable of specifically eliminating maligant cells was undertaken by many laboratories in the last two decades. It was the natural product of accumulating evidence on the antigenicity of experimental tumors, the immune surveillance theory expressed by prominent researchers (4,80), and the rapid development of *in vitro* technology for the study of immune responses. The search has produced a vast amount of information on cellular and humoral effector mechanisms involved in the recognition of, and reaction to, tumor cells.

As experimental data were accumulating, it was realized that the immune reaction to tumors is by no means simple and that its net effect is influenced by mechanisms which both inhibit and favor tumor growth.

Before reviewing these data, it should be stressed that many of the findings are based on *in vitro* studies and that their relevance to the growth

of malignancies in vivo cannot be taken for granted. Furthermore, many of the cited experiments were carried out with tumors selected for their antigenicity. As already mentioned above, tumor antigenicity was claimed to represent a laboratory artifact rather than a universal biological phenomenon. The relevance, therefore, of many of the results to be reviewed to the in situ development of tumors is yet to be confirmed.

As with other immune responses, both cell-mediated and humoral reactions can be demonstrated in response to tumors. These reactions as well as the influence they exert on each other will be discussed separately.

1.B.1. Cell-Mediated Immune Responses

1.B.1.1. Methodology

In Vivo Studies. The ability to transfer immunity to a tumor with lymphocytes of an immune animal was demonstrated in the early 60's by Klein and associates and by Old et al. They showed that lymphocytes of mice immune to either chemically or virally-induced tumors can slow tumor growth when injected into x-irradiated recipients together with tumor cells (16,58,81). Similarly, it was shown that injection of animals intravenously or intraperitoneally with immune lymphocytes prior to the injection of tumors, or immediately thereafter, can protect against a tumor challenge. This was true for tumors of both viral and chemical origin (82-85).

Another in vivo method for the detection of a cellular immune response has been the elicitation of a DTH (Delayed-Type Hypersensitivity) response to tumor cells or their extracts. Several laboratories reported positive skin tests in animals preinjected with tumor cells. These results, however, could only be obtained after the growing tumor was excised (23,86,87).

In Vitro Techniques. Numerous techniques have been developed to study the reactions between immune cells and antigenic tumor cells. Among them is the colony inhibition assay originally developed by Hellström for tumor lines growing in monolayers (88) and then improved and extended to study other types of tumors by the use of agar (89). Using this method with a variety of experimental tumors, it was observed by many investigators that peritoneal and lymph node cells from animals which were injected with

tumor cells, could block the colony formation of cultured tumor cells. In some of the experiments, an inverse relationship was observed between tumor size and the ability of the lymphocytes to limit the growth of the colonies (90,91).

The microcytotoxicity assay was designed to provide a measure of the number of viable cells remaining after an interaction with immune cells. It was first described by Takasugi and Klein in 1970 (92) and subsequently used by several laboratories. In this assay, the number of viable tumor cells remaining in microtitre wells after a 48 hour interaction with lymphocytes is ascertained by visual means.

Using this method it was again demonstrated that maximal tumoricidal and tumor-inhibitory activity by lymphocytes can be detected before the tumor reaches its maximal size and after its regression.(93).

Assays involving radiolabelling of target cells. Two types of assays which employ isotope labelling of target cells for the determination of their growth or death are now commonly used. In one assay which is designed to measure target cell lysis, tumor cells are labelled by the metabolic incorporation of an isotope prior to their interaction with lymphocytes. The cytotoxicity of the lymphocytes is then ascertained by measuring the amount of isotope released by the lysed cells. Isotopes which are taken up by either cell membrane (^{51}Cr in the form of $Na_2{}^{51}CrO_4$) or cellular DNA (^{3}H Thymidine, ^{125}IUDR) have been used as markers (94-99). The assays can normally provide an objective, quick, and rather simple method for the assay of cell death. They do not, however, provide a measure of the inhibition of cell growth, and are not effective with target cells which are relatively resistant to lysis. These methods are also likely to miss cellular mechanisms which require either long periods of tumor and effector cell interaction, or preactivation of lymphocytes, presensitized in vivo. These limitations may explain the differences in the results obtained with the microcytotoxicity assay and the ^{51}Cr release assay, using the same target and effector cell combinations (97).

Some of these disadvantages can be overcome by the end point labelling assay in which target cells are labelled at the end of the incubation with effector cells. Labelling with either ^{3}H-Thymidine (100) or ^{3}H-Leucine (101) has been employed. The incorporation of labelled metabolites, after the immune interaction, provides a measure of both inhibition of target cell growth and its lysis.

Two other *in vitro* methods which were originally used in the study of cell-mediated immune responses to a wide variety of non-tumor-related antigens were applied in limited cases to the study of tumor immunity. The ability of tumor cells to stimulate lymphocytes into rapid proliferation in a manner similar to alloantigens (102) was tested. Tumor cells, as well as tumor cell extracts, were shown to have a limited capacity to stimulate either presensitized or normal lymphocytes. Different laboratories however differ considerably in the experimental results reported (103,104).

The other assay system used measures the inhibition of macrophage migration induced by tumor cells or tumor extracts. Both virally and chemically-induced tumors were tested by this method. Results reported by several laboratories demonstrate again an inverse relationship between lymphocyte reactivity and tumor growth (105,106).

I.B.1.2. The Correlation Between In Vitro Activity and In Vivo Protection

The fast proliferation of data on anti-tumor reactivity *in vitro* and the possibility that it reflects *in vivo* functions has made it necessary to assess the correlation between the two phenomena in the various tumor systems.

Early studies using chemically-induced tumors could demonstrate a correspondance between colony inhibition *in vitro* and tumor inhibition *in vivo* (16,107). Many attempts were since made to correlate specific cytotoxic responses detected *in vitro* with effective tumor resistance *in vivo*. Glaser in 1976 demonstrated such a relationship for strongly immunogenic virus-induced tumors (108). When less antigenic chemically-

induced tumors were used, however, the correlation could be demonstrated only when spleen cells from tumor-bearing mice were fractionated prior to use, or when large numbers of lymphocytic cells were mixed with the target cells (109,110).

A more recent study attempted to compare *in vivo* protection detected by three different assay systems with *in vitro* cytotoxicity in a ^{3}H-Proline release assay. Using either the *in vivo* neutralization (Winn) assay, concomitant immunity, or resistance to tumor challenge after excision, as the *in vivo* parameters, the study showed that while the *in vivo* protection was always tumor specific, *in vitro* cytotoxicity of spleen cells could be demonstrated against a range of tumor targets (111). It seems therefore that cytotoxic reactions detected *in vitro* do not always reflect and measure the immune mechanisms taking place *in vivo*.

The interpretation of *in vitro* findings therefore should not be based on an *a priori* assumption of relevance to *in vivo* immune mechanisms, and where possible, this relevance should be experimentally supported.

1.B.2 Cell Populations Mediating Anti-Tumor Reactions

1.B.2.1. The Thymus-Derived Lymphocytes

The similarities between the immune responses evoked by alloantigens and those demonstrated for tumor antigens, as well as the parallel development of these two fields of research led to the belief that they are mediated by the same cell population. The wide use of allogeneic tumors in the study of allograft rejection has further strengthened this concept. It was, in fact, this parallelism between the two immunologically-mediated rejection mechanisms, which prompted Burnet to suggest that the allograft rejection evolved as a mechanism to prevent the emergence of cancer cells, which continuously arise through somatic cell mutations (2,4).

It is now becoming clear that the role assigned to T cells in tumor immunity is not as general as originally thought and that their function is restricted to specialized types of tumors and tumor-host relationships (112). Furthermore, it has become clear in recent years that different subsets of T cells play different and opposing roles in the response to a growing

tumor (113,114) and that humoral as well as cellular mechanisms operate in regulating this response (115).

T cell-mediated anti-tumor immunity has been assayed in two systems. The first, tumor induction by viruses or chemical carcinogens, is thought to be an experimental model for the study of spontaneous malignancy. The other, tumor immunity, measures the resistance to tumor transplantation induced in the laboratory by the pre-exposure of animals to non-lethal forms of the same tumors.

Thymus-depleted mice have been the major source of information on the role of T cells in the growth of a primary tumor. The role of T cells in the process of tumor transplantation was elucidated mainly from in vitro studies and in vivo lymphocyte transfer experiments (see above).

1.B.2.1.1. The Role of T-Cells in Host Protection Against Virally Induced Tumors. The strongest support for T cell-mediated protection against primary tumors comes from the virally-induced tumors. The role of T-cells in the rejection of these tumors in vivo has been demonstrated by the effectiveness of anti-lymphocyte serum (116) anti-thymocyte serum (117) X-irradiation (118) and thymectomy (119) in abolishing this resistance. Corroborative evidence came from several studies with the nude mouse. They have shown that whereas MSV-induced tumors regressed in 85-100% of normal and nu/+animals, no such regression could be observed in nu/nu mice (120-122). The importance of T cells in the regression of MSV-induced tumors was further confirmed by the demonstration of cytotoxic T lymphocytes infiltrating the tumor (123). In addition to MSV induced tumors, increased susceptibility to polyoma virus induced tumors were also demonstrated in nu/nu mice (124,125).

Thymus-dependent mechanisms were also shown to play a role in the transplantation immunity induced by viral tumors. It was shown that the resistance to tumor induction by SV40 which could be generated in hamsters by the injection of SV40 transformed cells, could be abrogated by thymectomy (126-128). Similar findings were also reported for polyoma virus-induced tumors. It was shown that thymectomy could block the specific resistance induced by the injection of polyoma virus or by immunization with polyoma-induced tumor cells (129).

In vitro assays supported in vivo evidence of a T cell-mediated protection against virally-induced tumors. The study of Glaser et al., cited above (108), demonstrated a strong correlation between in vivo protection and in vitro cell-mediated destruction of Gross virus-induced lymphomas. In other in vitro and adoptive transfer studies, anti-θ-serum and complement were used to specifically remove T lymphocytes from the effector cell population. This treatment resulted in the elimination of the cytotoxic cell in vitro and a decrease in protection in vivo (130-133).

1.B.2.1.2The Role of T Cells in Host Protection Against Chemically-Induced Tumors. The evidence for a T cell-mediated protection against chemically-induced tumors however, is not as clear cut and in many instances contradictory. Stutman, in a review of the relationship between immunosuppression and tumor growth, and Naor, in a recent review (5,114), have shown that thymectomy, as well as other less specific immunosuppressive treatments, can be either inhibitory, stimulatory, or without effect on the development of primary chemically-induced tumors. The outcome depended on the assay system, the age and strain of animals, and the carcinogen. In reports from several laboratories, it was shown that neonatally thymectomized mice have a higher incidence of sarcomas or lung adenomas with shorter latent periods, after the application of the carcinogens methylcholanthrene, dimethylbenz(a) anthracene, and urethan (134-136). Other reports, however, demonstrated no such thymus dependence. The latter showed that after the application of methylcholanthrene, thymectomized and sham operated mice did not differ in either tumor incidence or latent period (137-139). In yet another series of reports, thymectomy reportedly resulted in a decrease in tumor incidence. In particular, this effect could be shown with chemically-induced leukemias and radiation-induced lymphomas (114,140). It is possible that the decreased incidence in this cases might have been due simply to the removal of the cells which are targets for the carcinogens.

The evidence originating from experiments with the nu/nu mouse however, is convincing in its unanimity. When compared with normal or nude/+ mice, no difference could be observed in either tumor incidence or latent period after application of the carcinogen (141-143). A recent report in fac

claims that the incidence of MCA-induced tumors is lower in nude/nude mice than in nu/+ mice (144).

T cells probably play a more important role in transplantation immunity. T cell killing has been demonstrated with the chemically-induced leukemia El-4 (145) and with a mineral oil-induced murine plasmacytoma. θ-bearing cells were essential in the transfer of immunity to the plasmacytoma and furthermore, this immunity could not be induced in nude mice (146-148). Growing MCA-induced tumors were shown to induce a specific concomitant immunity to the same but not other tumors (149) and lymphoid cells from tumor-bearing mice were shown to be cytotoxic or growth inhibiting for cultured tumor cells (150,151). This activity could be demonstrated in the early phases of tumor growth and disappeared as tumor mass increased. The specificity of these mechanisms suggests a role for T-cells, but concrete evidence to that effect is lacking in most of the reports available.

1.B.2.1.3.The Role of Host cells in Protection against Spontaneous Tumors.

The apparent lack of antigenicity of spontaneous tumors has been alluded to before. Based on evidence originating from different laboratories, it seems reasonable to conclude that spontaneous tumors of recent origin cannot induce transplantation resistance in the manner demonstrated for viral or chemically-induced tumors (70,73). Several tumor lines of spontaneously arising tumors have been shown to possess tumor-associated antigens which induce specific cytotoxic responses *in vitro* (152,153). However, the length of time these tumor lines were maintained *in vivo* or *in vitro* excludes any definite conclusion regarding the origin of the antigens. In studies with nu/nu mice and mice injected with anti-thymocyte serum, no increase in the incidence of spontaneous tumors could be demonstrated (154,155).

These findings, which suggest that T-cells do not play an important role in the prevention of primary tumors, provide one of the strongest arguments against the concept of a T cell-mediated immune surveillance in malignancy. Furthermore, the lack of evidence for a T-cell mediated protection against chemical carcinogenesis has brought forth the conclusion that the major role

played by T cells in anti-tumor immunity is that of protection against viral infection and consequently, against the virally-induced tumors which express virally-determined antigens (156).

G. Klein in a recent review rejects this suggestion (68). He supports his argument for a specific T cell-mediated anti-tumor (as opposed to anti-viral) protection by evidence from several viral tumor systems,where a clear distinction can be drawn between anti-viral and anti-tumor transplantation immunity. In these systems, an immunity to the viruses did not provide protection against the growth of the corresponding tumor.

1.B.2.1.4 The Mechanisms of T Cell-Mediated Tumor Cell Lysis. Most of the information currently available on the lytic process as mediated by T killer cells is derived from *in vitro* studies with allogeneic target cells. It is believed that from a mechanistic point of view, T cell-mediated destruction of syngeneic tumor cells is identical to the lysis of allogeneic targets.

Lysis by T-cells is characteristically specific to the sensitizing antigen and is independent of the complement system (157). Kinetic studies of the process indicate that it results from a collision between a single lymphocyte and a single target cell (the "one hit" model) and that the lymphocyte after lysis of one target cell can collide with and lyse more targets (158,159). The viability of T cells but not target cells is essential for lysis. However, *de novo* protein synthesis by the killer cells is probably not required (159,160).

The lytic process can be divided into three phases: Effector cell-target interaction, establishment of the lesion and complete lysis.

1. The requirement for an effector-target intereaction has been elucidated from experiments which showed that the reaction can be completely inhibited by either the separation of target and effector cells with a semipermeable membrane, or by the suspension of interacting cells in a viscous medium (e.g. dextran, agarose)(161,162). It was also shown that the

interaction has an energy, as well as, Mg^{++} requirement and might require membrane modulation since it is inhibited by cytochalasin b (159,163).

2. Within minutes of the cellular contact, changes in the membrane permeability of the target cells can be demonstrated (164). Once the membranous lesion has been inserted, complete lysis can be accomplished without the presence of the killer cells (162). Drugs which increase levels of cAMP in the effector population were found to decrease their lytic ability. This inverse relationship is, as yet, poorly understood (165,166).

Several lines of evidence suggest that the lesion is caused by soluble mediators released by the killer cells after their triggering. Major support for this suggestion was provided by the finding that supernatants of cultured, stimulated lymphocytes contain a soluble factor capable of lysing target cells (167). The suggestion however, is being questioned on the basis of several findings. Among them are the demonstrated specificity of the killing when lymphocytes are mixed with several target cells, which suggests a mechanism of specific recognition, and the reported finding that treatments that inhibit the lytic activity of lymphocytes fail to affect their ability to release the soluble mediators (159,168).

Although the question of cell contact vs. soluble mediators has not been resolved, attempts have been made to accomodate both models by postulating a model that requires cell membrane contact in order to induce the release by the killer cells of soluble mediators (169).

3. Once the cellular collision results in the lesion, the target cell undergoes a series of permeability changes which result shortly thereafter (10 minutes) in the exchange of inorganic ions and small molecules. Macromolecules can pass the cell membrane only after a lag period, during which cell destruction is completed by disordered osmotic regulation and water influx (170,171).

1.B.2.2 The Macrophages

1.B.2.2.1 Characterization of the Cell.

The importance of phagocytic cells in the inflammatory process and in host protection against bacterial infections was first demonstrated by Metchnikoff in 1905 (172). The role of the phagocytic mononuclear cells in these processes was later confirmed in many reports and extensively reviewed (173). These cells which originate in the BM are found in their mature, functional state either in the peripheral blood where they are known as monocytes, or in the various tissues where they were classified as macrophages. The mature monocyte is characteristically adherent to glass surfaces, highly phagocytic, actively synthesizing a variety of substances and highly motile in vivo or in vitro (174,175).

The central role played by the mononuclear phagocyte in every aspect of the immune response is now becoming clear. An abundance of literature based mainly on in vitro work demonstrates a macrophage or macrophage-product requirement in practically every stage of an immune response to antigenic challenge. Thus macrophages are required in antigen processing and presentation (176), cell-cell interaction (T cell-B cell or T cell-T cell) and effector mechanisms (175,177,178).

Whereas the macrophage can exert stimulating effects on lymphocytes, it can also respond to, and be activated by, lymphocyte signals. Among the signals shown to activate macrophages were both soluble mediators released by T-cells and B-cell products (i.e. immunoglobulins) which bind macrophages via their Fc portions (179-181).

1.B.2.2.2. The Role of Macrophages in the Defence Against Tumors.

The disillusion in recent years with the concept of T-cell-mediated defence against malignancy (see p.14) resulted in a search for alternative cell populations with tumor-inhibitory activities. Attention was then drawn to the multifunctional macrophage. The concept of the macrophage playing a role in anti tumor immunity was not a new one. It was suggested by Gorer as early as 1956 (182). Other early reports also indicated that a link existed between in vivo defence mechanisms.

operating in bacterial infection and tumor growth and the mononuclear phagocyt was later shown to provide this link. (183). But it was the demonstrated ability of macrophages, responding to lymphocytic mediators, to selectively lyse tumor cells in vitro (184) which focused the attention of many investigators on the potential of these cells in providing a natural line of defence against tumors.

The activated macrophage. Resting macrophages undergo a series of characteristic morphological, and biochemical changes both in vivo and in vitro in response to various stimuli. These changes have collectively been designated-"state of activation" and thus the stimulated macrophage is usually referred to as "activated". The activated macrophages are adherent to culture vessels on which they characteristically spread out, and exhibit a large amount of ruffled membrane activity. Their phagocytic and pinocytic activities are increased and they develop a capacity to kill viruses, bacteria, and tumor cells (185).

These morphological and functional characteristics follow, and are probably the consequence of an outburst of enzymatic activity. The activated macrophage has been shown to secrete an array of products which mediate their diverse functions, both as accessory and effector cells, in the immune response. Among the products secreted are lysosomal enzymes, metabolites, T and B cell stimulating factors, interferon, factors toxic to tumor cells, and factors lytic to intracellular parasites. The type of product released depends on the state of the macrophage prior to stimulation and seems also to be influenced by the nature of the stimuli (186). Macrophage activation can be either non-specific or initiated by the specific antigen to which the macrophage becomes reactive.(187).

Non-specific macrophage activation. It is by now a well-established fact that macrophages can be stimulated non-specifically in vivo, by either chronic intracellular infections or an array of non-specific stimulants (eg. thyoglycolate , endotoxin) to kill syngeneic, allogeneic, and xenogeneic tumor lines in vitro (175,185). This cytotoxic activity was thought initially to be directed only against cells expressing a neoplasm related characteristic

since normal cells were not sensitive (188). More recent results suggest, however, that the effect is directed at rapidly dividing cells including some non-neoplastic cell lines (175,185,189).

The killing effect is exerted via two well-characterized mechanisms, the cytocidal and cytostatic effects. In vitro, cytostasis is normally measured as the ability of the macrophage to inhibit the incorporation of DNA-seeking isotopes (^{3}H-Thymidine, ^{125}IUDR) by target cells. Lysis can be measured either by assaying for isotope released by the target cells or by counting surviving viable cells.

Tumor cytostasis can be detected as early as four hours after mixing target cells and activated macrophages and is normally completed at 12-24 hours. The cytotoxicity is a later event which can only be detected 24 hours after the interaction and is completed at 48-72 hours (185,187).

Studies on the mechanisms involved in the cytostatic process, have demonstrated an initial requirement for cell-cell interaction. When examined four hours after mixing, tumor cells, which normally begin to spread at that time, were still rounded and surrounded by macrophages. As the reaction proceeded they formed aggregates and their numbers began to decline. After 36-48 hours, very few tumor cells could be found although cellular debris were not evident and there was no evidence of an active phagocytosis.(ibid)

As is the case for T cell-mediated lysis, the role of soluble factors in macrophage-mediated kill is a subject of controversy. Whereas several reports demonstrate a cell-cell contact requirement (190,191), others claim that lysis can be achieved with factors released into the supernatant of cultured macrophages, and that the presence of the macrophage is not required (192,193). A reasonable explanation for this discrepancy may be the lability of the soluble mediators and their sensitivity to serum factors. The fact that they can be collected from supernatants only at restricted time intervals after macrophage culture supports this notion (175).

Several possible non-phagocytic mechanisms of lysis were suggested, all of them based on the fact that activated macrophages are characteristically rich in lysosomal enzymes. Among them is the hypothesis that a temporary cell

fusion occurs after macrophage and target cell collide, followed by the transfer of lysosomes into the target cell which results in their death. In another suggestion, peroxide is implicated as the mediator of lysis (194). Since the growth inhibitory effects exhibited by macrophages were normally found to be followed by cell lysis, it is conceivable that the two functions depend on different doses of the same inhibitory factor(s) and that whereas cytostasis is accomplished with low doses, a time-dependent accumulation of the factor(s) eventually results in cell death (185).

The role of T cells in the mediation of a non-specific macrophage activation appears to depend on the route of activation. Results obtained with the nude mouse suggest that macrophages can be activated in vivo by Freund's adjuvant in the absence of T cells (195). However, the ability of sensitized T-cells or their culture supernatants to activate macrophages in vitro, as well as, the dependance of various activation pathways on the presence of lymphocytes, imply an important mediator role for T cells in the process (185,196).

The specific (Immune) macrophage activation. Similarly to T and B lymphocytes, macrophages can participate in specific immune responses and develop a specific cytotoxicity to tumor cells if properly immunized. Evans and Alexander reported in 1970 that macrophages from the peritoneal cavity of mice either immunized to, or bearing a syngeneic s.c. tumor, were specifically cytotoxic in vitro to the immunizing tumor (198). The specific adverse effect of the macrophage is mainly a growth inhibiting effect, although depending on the route of activation, cytolytic macrophages can also be obtained (187).

The requirement for either T cells or T cell factors in the specific activation has been demonstrated in both in vivo and in vitro systems. It was shown by Evans et al. that macrophages from thymectomized and whole body irradiated mice, which were immunized to a syngeneic tumor, were not growth inhibitory to this tumor. Similarly in an in vitro system, the specific activation of macrophages which occurred upon incubation of lymphocytes sensitized by antigen and normal peritoneal macrophages, could be abbrogated by treatment of the lymphocytes with anti-θ and complement (198). Based on

these and other similar findings, Evans et al. proposed that T cells, sensitized to either allogeneic cells or syngeneic tumors, activated the macrophages by "arming" them with a factor, designated by Evans, "Specific Macrophage Arming Factor" (SMAF). This factor according to Evans has a dual recognition capacity and thus recognizes the specific target antigen and at the same time is cytophilic for macrophages (198,199).

The macrophage-binding activity of the factor was shown to have no strain-specificity when mouse factor was found to bind to rat macrophages (187). Additionally, the presence of antigen was not necessary for the arming process and could be achieved by incubating normal macrophages with supernatants of immune lymphoid cells, previously incubated in vitro with the antigen (200). The arming factor has been partially characterized and does not appear to be a conventional immunoglobulin molecule (187,201).

It is interesting to note that "armed" macrophages can turn into activated macrophages, non-specifically cytotoxic to tumors, after a second incubation with the specific antigen. This process was termed "specific activation" since it requires the specific presensitizing antigen. As a result of this activation, macrophages invariably turn cytostatic, but not cytolytic, to tumor cells (187).

A summary of the factors inducing specific or non-specific macrophage activation is presented in Tables 1 and 2. (see p.22).

The Role of Macrophages in the In Vivo Protection Against Tumors

The evidence for the participation of macrophages in host defence against tumors stems from several observations:

1. Peritoneal macrophages isolated from tumor-bearing mice are inhibitory to tumor growth in vitro (197).

2. Phagocytic mononuclear cells infiltrate the tumor site in large numbers (202)

Tables 1 and 2

Table 6.1 Some methods of obtaining macrophages which are non-specifically cytotoxic towards tumour cells

(A) *Direct – lymphocyte independent*	
1. Poly I/Poly C, endotoxin, double-stranded RNA	incubate with *'armed'* or incubate with macrophages *in vitro.*
2. Glucan, peptone, PPD	inject i.p. to animals
3. *C. parvum*	inject i.p. to animals
BCG	inject i.p. or incubate with macrophages*
4. Pyran copolymer, Complete Freund's adjuvant	inject i.p. to animals
(B) *Lymphocyte dependent*	
1. Supernatants from immune lymphocytes (syngeneic, allogeneic or xenogeneic)	incubate with macrophages *in vitro* or inject i.d. to animals
2. Aggregated IgG, antigen–antibody complexes	incubate with macrophages *in vitro* (? *in vivo*)
3. Specific antigen	incubate with *'armed'* macrophages *in vitro.* (2 stage process)
4. *Toxoplasma gondii*, *Besnoitia*, *Listeria*, *Pasteurella*, *Nippostrongylus brasiliensis*	persisting infections in animals (may follow a specifically 'armed' stage, as in 3)
5. Ascites or solid tumour	growing in animals. (Macrophages obtained from tumour site. Mechanism probably as in 3 and 4)

†Abbreviations: i.p. Intraperitoneally; i.d. Intradermally
*Ability to activate macrophages *in vivo* depends on virulence of strain

Table 6.2 Some methods of obtaining macrophages which are specifically cytotoxic towards tumour cells

(A) *In vitro*
Incubate normal macrophage monolayers with:
1. Supernatant factor(s) from immune syngeneic or allogeneic T lymphocytes from spleen or lymph nodes.
2. T lymphocytes from mice hyperimmune to tumour
3. Supernatant factor from Ig-bearing immune lymphocytes from peritoneal cavity
4. Cytophilic antibody

(B) *In vivo*
Macrophages harvested from:
5. Peritoneal cavities of mice immunized with live allogeneic tumour, or killed syngeneic tumour cells
6. Peritoneal cavities of animals bearing a progressively growing tumour at another site
7. Within tumours growing subcutaneously. (Occasionally—since these macrophages are more often non-specifically cytotoxic)

3. An inverse relationship was demonstrated between the number of macrophages in syngeneic sarcomas and their metastatic spread (204-206). Macrophages therefore, might have a function in preventing tumor dissemination. Additionally, it was shown that immunosuppressive treatments increase the incidence of metastasis (207).

4. Macrophages were shown to play a role in the concomitant immunity operating during tumor growth (208,209).

5. Treatments which are specifically inhibitory to macrophages such as silica or anti-macrophage serum injections, were shown to accelerate tumor growth in vivo (183). On the other hand, agents with a macrophage-stimulatory effect such as BCG or Corynebacterium parvum were able to slow tumor growth and reduce metastatic spread when injected systemically or into the tumor (210-212).

A correlation between the phagocytic monocyte contents of a tumor and its growth and spread was also demonstrated in several human malignancies (205).

The realization of the potential of microbial or synthetic adjuvants in non-specifically stimulating macrophages and increasing resistance to tumors, has been the major impetus in the ongoing immunotherapeutic trials in cancer therapy.

1.B.2.2.3. The Suppression of Macrophages by the Tumor

The growth of solid tumors, in spite of a macrophage-rich infiltrate capable of both in vivo inhibition of spread and in vitro lysis, raised the possibility that the tumor can exert suppressing effects on macrophages. The presence of a growing tumor has been shown to decrease the chemotactic activity of macrophages and their ability to reach inflammatory sites. This effect was specific for macrophages since other cells participating in the inflammatory process were not affected. The effect was also dependent on tumor size and increased as the tumor progressed (213,214).

The mechanism responsible for this effect is poorly understood. It was shown that the number of peripheral blood monocytes is not reduced in the presence of a growing tumor and in fact may even increase (215). It is

unlikely therefore that the deficient inflammatory response is due to the trapping of a large number of macrophages in the tumor and it may be a deficiency in the macrophages themselves rather than in their numbers.

Several possible substances can be responsible for the anti-inflammatory effect. Among them are tumor products (216,217), substances such as chemotactic inactivator, produced by the host in abnormal quantities in response to tumors (215), or a combination of products of host and tumor origin such as antibody-antigen complexes (175).

An alternative explanation for tumor growth in the presence of activated macrophages is based on the observation that in lymphocyte or antibody-mediated cytotoxicity assays, macrophages can actually function as immunosuppressors (219) and tumor growth stimulators (220).

It is possible therefore that the net effect exerted by macrophages on the host defence against tumors is the result of both growth inhibitory and growth promoting influences.

B.2.3. The Natural Killer Mechanisms

As it became clear that T cell-mediated protection against tumors is probably restricted to limited types of host-tumor systems, the search intensified for other defence mechanisms which are endogenous to the host, selectively recognize and inhibit malignant cells, and do not require presensitization in order to recognize and develop a cytotoxic capacity towards tumors.

Macrophages were shown to be inhibitory to a wide range of tumors when specifically, or non-specifically activated and they may provide one line of defence against maligancy *in vivo*. Several other cell types have been shown to possess a natural, non-induced ability to selectively kill tumor cells and they vary in their characteristics and target specificity. These cells will be discussed with emphasis on the so-called NK (natural killer) cell of the mouse. The NK cell has been the subject of intense research in many laboratories during the last five years, and is the best characterized of several reported spontaneously occurring, tumor-killer cells.

Initial reports of natural, cell-mediated cytotoxicity to tumors were based on the in vitro observation that cells from lymphoid organs of normal individuals without known prior contact with a tumor, can mediate cytotoxic reactions against a variety of tumors lines (221,222). This activity was not species-specific and could be detected with lymphoid organs of man, rats, and mice (222,223,224). The in vitro assay most commonly used for the detection of this activity was a short-term cytotoxicity assay with ^{51}Cr-labelled target cells. However, long-term, visual, microcytotoxicity assays were also employed (225).

1.B.2.3.1.Characteristics of the Response

The natural killing phenomenon, which is referred to in the literature as the NK reactivity, differs from other cell-mediated responses against tumors in several respects.

The age dependence. In rodents, NK activity was consistently shown to follow an age-dependent pattern. In mice for example, it is detectable only at 4 weeks of age, peaks at 8 weeks and then levels off and declines at 12 weeks (222,226). In the rat, the activity was also found to peak at 5-8 weeks of age and to decline at 10 weeks (227). Although results on the age of maximal activity are not unanimous, the basic finding of an age-related activity has been confirmed by several laboratories (228,229). Nude mice, however, differ from normal mice in the kinetics of the response and show a slower and more gradual decline in activity (144).

The genetic control. Early reports comparing reactivity of different mouse strains against identical target cells, suggest that the NK phenomenon is highly strain-dependent. Subsequently, the strains were accordingly classified as "low" and "high" NK strains (222). This classification was later extended to include a third group of intermediate strains (230). A genetic analysis of hybrid strains demonstrated a multiple gene control and a dominance of the "high" NK genes, and the H-2 complex was shown to be only one of the genes controlling the level of response (231,232). Reports from several laboratories have recently questioned the "high" and "low" strain division. They suggested that the response fluctuated not only as a function of the source of killer cells, but also in relation to the

target cells tested. Thus, strains exhibiting a low reactivity against a particular target cell had a good reactivity when tested with other targets (228,233,234). It was concluded therefore that the range of specificities, as well as the ability or inability to lyse target cells are controlled by genetic factors. Kiessling and coworkers in a recent report (230) presented data disputing this argument and claimed that "high" and "low" NK strains display a consistent lysis profile against a range of targets tested.

Nude mice, as well as mice with a combined T and B cell deficiency, (Lasat strain) exhibit a wider range of specificities than conventional mice, suggesting a regulatory role for T cells in the determination of the spectrum of sensitive targets (235,236).

Manipulations of NK levels. The levels and spectrum of activity of the NK are influenced by environmental, as well as genetic factors and can therefore be manipulated and boosted. Several of the treatments discussed above (p. 22) as effective in macrophage boosting were also shown to influence NK activity. Included among them are a variety of alloantigens, tumor cells, murine viruses, and the commonly used adjuvants BCG and Corynebacterium parvulum (233). The boosting was shown to be T cell-independent, when it was demonstrated in nude mice. The route of administration was found to be of importance in the outcome of the treatment. Thus, Ojo et al. reported a stimulatory effect with corynebacterium parvulum when it was injected i.p. but an inhibitory effect when it was injected i.v. (237). Although the mechanism is unknown at present, it is reasonable to assume, on the basis of this and other lines of evidence, that macrophages play a regulatory role in the response (see p. 32). The injection of tumor cells was found to augment NK reactivity whereas the growth of a tumor was reported to suppress it (226,238). Since NK activity was reportedly found in tumors, it is possible that the depressed level of activity indicated an influx of NK cells into the tumor (227).

More recent experiments made it clear that the common denominator to the various boosting agents may be their ability to induce interferon production in vivo. Thus, it was shown that Corynebacterium parvulum induces interferon synthesis in vitro and that the injection of known interferon inducers such as poly I:C and endotoxin can significantly enhance NK activity

(239,240). Similar results were also obtained with the injection of interferon itself in vivo (230,241). In vitro findings confirmed the in vivo observations when it was shown that incubation of spleen cells with interferon inducers or interferon preparations augmented their NK activity. Furthermore, both in vivo and in vitro boosting could be abrogated by anti-interferon serum (241,242). A similar effect of interferon on the NK levels of human peripheral blood leukocytes (PBL) was reported by several laboratories (243,244).

Macrophages seem to play an important role in the activation of NK cells via interferon. Thus it was shown that the in vivo boosting by interferon inducers can be abrogated by the pretreatment of mice with macrophage inhibitors, such as carrageenan and silica. In vitro studies also indicated a link between macrophages and the stimulation of NK activity by interferon inducers. Since the specific inhibition of macrophages does not effect the boosting effect of interferon itself, it seems likely that the macrophage plays a role in the production of interferon which in turn activates potential NK cells (227).

1.B.2.3.2.Characteristics of the Killer Cells

The early studies with the murine NK cells have indicated the presence of H-2 K and D but not Ia antigens on their surface (230). They also suggested that the killer cell did not belong to either the T or B lymphocyte lineage and lacked the characteristics of either granulocytes or phagocytic monocytes. Thus it was found that nude mice have high NK levels and that treatment of lymphocyte preparations with anti-θ serum and complement did not decrease NK activity (221,222,226,245). It should be noted however, that later studies were not in full agreement with the early reports and suggested that a low avidity Thy 1 antigen is present on the killer cell surface, and that in fact NK cells may be pre-T cells (227,246). Fractionation procedures, aimed at the specific removal of Ig-bearing cells, have demonstrated that the killing is independent of B cells (222). Furthermore, several reports demonstrated the lack of complement receptors on the killer cell surface (221,222,247,248). The presence of receptors for the Fc portion of immunoglobulins is however.

still a matter of controversy. Although it was originally thought that these markers are lacking as well (222), recent experiments indicated a loss of activity after the selective removal of Fc receptor-bearing cells on sheep red blood cell (SRBC) anti-SRBC monolayers (249). One explanation for this discrepancy was that the killer cell has a low avidity Fc receptor which is not easily detectable (230).

The non-phagocytic nature of the killer cells was suggested from their inability to take up iron particles (221) and their resistance to anti-macrophage serum and complement (250). The lack of adherence properties displayed by the killer population, as well as their binding to the lectin Helix pomatia A agglutinin, which does not bind monocytes, confirmed their non-monocytic nature (221,251). These data, as well as other lines of evidence such as the size and density of the cell (230) and microscopic analysis of killer and target cell rosettes (252), suggest that the killer cell is a small "null" lymphocyte.

The human NK cells differ from those of the mouse in several of their surface markers. Thus, they were reported to be sensitive to anti-T cell serum and complement and were shown to have a low affinity receptor for sheep erythrocytes. Furthermore, Fc and complement receptors could more readily be demonstrated on most human NK cells. Similarly to murine NK cells however, peripheral blood NK cells of man are non-adherent and do not bear Ig on their surface (227).

The organ distribution displayed by the NK cells is also characteristic. High efficiency killing could be demonstrated using murine spleen or peripheral blood cells, whereas lymph node, peritoneal, and bone marrow (BM) cells had a low to intermediate activity. No activity could be demonstrated with thymocytes (221).

Though BM cells show a low reactivity against NK targets <u>in vitro</u>, a BM requirement for the production of the cell <u>in vivo</u> has been amply demonstrated. Using two different approaches to selectively block hemopoiesis by the BM, namely the administration of either ^{89}Sr or high doses of estrogen, it could be shown that agents which cause a destruction of the BM <u>in vivo</u>

markedly reduce NK-activity of spleen cells in vitro (253,254). Adoptive transfer experiments in which NK cells could be transferred to non-reactive, lethally irradiated mice by the injection of BM cells from "high" NK donors, also confirmed the BM origin of these cells (255). The spleen and thymus do not seem to be essential for the maturation of these cells as the removal of both organs does not affect the NK levels in the circulation (256).

B.2.3.3. The Spectrum and Specificity of the Killing

The specificity of NK lysis, which was alluded to earlier in this section, is still very loosely defined and poorly understood. The original reports suggested that the NK cells specifically recognize and lyse lymphoid tumor cell lines carrying virally determined antigens (234,235,257). However, the increase in the number of laboratories involved in the NK study and consequently the increase in the number of cells tested, has widened the spectrum of NK specificity. Thus it was shown that viral as well as non-viral tumors of lymphoid or non-lymphoid origin were sensitive to NK lysis (258,259). Moreover, it was reported that a sensitivity to lysis was displayed by non-tumor targets such as thymocytes, BM cells, and macrophage cultures (258,260). In a recent report, evidence was presented in fact, demonstrating that thymus cells of one to two week old mice were highly sensitive to NK lysis. This sensitivity disappeared as the animal matured and was inversely related to the frequency of NK cells in the spleen (261). The suggestion of specificity to tumor lines bearing murine C-type particles, supported by several early reports (226,260), has also been questioned in view of the demonstrable lack of correlation between the expression of C-type virus proteins on various mouse lymphomas and their sensitivity to NK lysis (262). Another study has shown that infection of human cell lines with mouse C-type particles does not increase their susceptibility to lysis (263).

Contrary to the original belief, it was also shown that tumors growing in vivo as well as in vitro lines can be lysed by NK cells (230). However, tumors maintained in vitro were more sensitive and their lysis required smaller numbers of killer cells (ibid). The mechanism responsible for the increased sensitivity of cultured cells is not clear. It is possible that the relevant

surface receptors are masked in vivo or that their expression is amplified by their growth in vitro (264). Fetal calf serum (FCS) does not seem to play a role in target susceptibility since it was shown that cultures supplemented with mouse serum were as sensitive to lysis as those cultivated in FCS-containing medium (265).

An added difficulty in the attempts to define NK specificity is presented by the fluctuation of target susceptibility as a function of NK activation. Thus, non-sensitive targets could be lysed when killer cells were preactivated with interferon or interferon inducers (230). It is possible therefore, that given the right conditions, NK reactivity may broaden to include many tumors which are presently regarded as non-sensitive. This would suggest that the insensitivity of tumors to lysis may be due to small quantities of the relevant receptors on their surface, rather than to their complete absence (230).

Heterogeneity in the NK population was also suggested as a possible reason for the broad range of their specificities. Evidence in support of this hypothesis is derived from cold target inhibition assays in which non-labelled tumor cells could be shown to block the lysis of radiolabelled targets, if the two shared determinants required for recognition and lysis. Utilizing this tool, Herberman and coworkers found that NK lysis is directed against several different antigenic specificities (226). These findings however, were not supported by other laboratories. Wigzell et al. could find no heterogeneity in NK specificity using either the cold target inhibition assay or NK depletion assays with sensitive targets (230).

In view of the unsuccessful attempts to date to define the antigenic requirement for NK susceptibility, non-immune mechanisms of binding, such as enzyme-substrate-type interactions, have been suggested. Evidence in support of such an interaction has also come from human NK cells (230,266). Recently, the isolation of up to three target structures for the NK cell from NK sensitive targets has been reported (267). Although a full characterization of these molecules is not available at present, their isolation does provide a tool for the study of NK specificity(ies).

The molecular mechanisms of binding and lysis are still poorly understood. Using a combination of cytotoxicity assays and a visual analysis of target-

effector rosettes, it was recently shown that the NK-target interaction consists of two independently controlled steps (230,268). In the first step, which was essential but not sufficient for lysis, cell-cell contact took place. This step could be blocked by treatment of the killer cell with trypsin, but it was not affected by metabolic inhibitors. These observations suggested that binding may require a protein "receptor" structure on the killer cell, but no energy. (In contrast, the binding of cytotoxic T lymphocytes to their target cells has been shown to require energy). On the other hand, the second phase of the interaction, i.e. the lytic event, was energy-dependent. In addition, it could be blocked by proteinase inhibitors suggesting the involvement of an enzymatic reaction. Cells which were non-adherent to nylon wool were capable of both binding and lysing target cells, whereas the adherent cell bound to, but did not necessaril lyse the target, supporting the concept of different control mechanisms in the two events (252). It was postulated that two different "entities" on the NK cell participate in the reaction. First a recognition structure brings the two cells into close proximity. This in turn allows a second enzymatic entity to be exposed and to lyse the target.

Experiments with the interferon inducer Tilorone have shown that this NK activator exerts its influence via an increase in the individual lytic capacity of the NK cell, rather than by an increase in the number of target-binding cells. It is possible therefore that interferon augments the expression of the functional "lytic entity" (268).

The lytic mechanism of NK cells was shown to differ from that mediated by activated macrophages. Thus macrophage-mediated cytolysis was reported to be resistant to the effects of metabolic inhibitors, trypsinization and serine protease inhibitors (195,269), indicating a different mode of both binding and kill. NK cells were also shown to differ from activated macrophages in their target selectivity and genotype distribution (270). Furthermore, a mutant was recently reported which is deficient in NK but not in activated macrophage cytotoxicity (271). It seems therefore, that although both populations mediate non-specific lysis of tumor cells, their mode of action and regulatory mechanisms differ. Macrophages may play an important role, however in the regulation of NK cells as will be discussed in the following section.

1.B.2.3.4.Regulation of the NK Response

Little is known at present about the cellular mechanisms or other factors responsible for activation or suppression of NK cells. The role of macrophages in enhancing NK cell activity via interferon production was alluded to earlier. The importance of macrophages in the regulation of NK reactivity is also evident from experiments demonstrating that agents which are toxic to macrophages, such as ℓ-carrageenen and silica, cause a reduction in splenic NK activity when injected into mice (272).

Additionally, Cudkowicz and coworkers recently reported that a macrophage-like cell could suppress NK activity in vitro (273). They found that spleen cells cultured with ℓ-carrageenen could suppress the NK lysis when they were added to a mixture of NK and target cells at the onset of incubation. The cell responsible for this suppression could be detected in thymus-depleted mice and displayed several of the characteristics of macrophages such as irradiation resistance and adherence to Sephadex G-10. Similar results were also obtained by injection of ℓ-carrageenen in vivo. In this system, the suppressive activity of spleen cells could again be shown to be thymus-independent and was removed by treatment with carbonyl iron and magnet (ibid).

A second population of suppressor spleen cells with non-adherent properties was also reported by the same group. The source of these suppressors were spleens of 4-18 day old mice or irradiated animals. Suppression was again thymus-independent, and radiation-resistant, but the effector cells did not bind to nylon wool or G-10 columns (273).

Little is known about the surface markers or mode of action of these suppressor cells. However, phagocytic cells have been known to regulate cell proliferation and function in several other systems (274,275) by the release of an array of soluble factors such as prostaglandins (276) and interferon (277). In the NK system, prostaglandins were shown to be inhibitory whereas interferon was stimulatory to the killer cells (227). It seems therefore, that macrophages via their mediators may act both as activators and suppressors to regulate NK activity.

In man, a suppressor T cell has been described in the peripheral blood which is inhibitory to naturally occurring cytotoxic cells (278). The heightened NK response described in nude mice (144) suggests a regulatory role for T cells in the mouse.

1.B.2.3.5.The Relationship Between NK and Other Mechanisms Mediating Natural Resistance

In addition to the many targets which are sensitive to NK lysis, several others have been described which belong to a second class susceptible to spontaneously occurring killer cells in the mouse. Among these targets are allogeneic or semisyngeneic hemopoietic stem cells as well as cells injected with intracellular parasites (273). It is now becoming clear that the various defence mechanisms operating against these different classes of target cells share many characteristics and may, in fact, be different manifestations of a broader biological phenomenon. Reports originating from several laboratories indicate a strong parallelism between factors influencing the NK response, resistance to intracellular parasites, and the so-called hybrid resistance detected in hybrid mice against BM of parental origin. It was found that all three functions are thymus independent (226,279,280) and sensitive to th BM seeking isotope ^{89}Sr (253,281,282). Additionally, they could be passively transferred with BM cells although they could not be mediated by BM cells themselves (256,283,284). Silica and carrageenan which are macrophage-suppressing agents have been shown to reduce both the splenic NK response and hybrid resistance (272,285,286). Macrophages may therefore play a role in regulating both functions.

This evidence may suggest that the various defence mechanisms are in fact due to different maturation pathways of the same progenitor cell. This cell is likely to originate in the BM and mature in the spleen or peripheral blood (273).

Other types of cells mediating spontaneous cytotoxicity against tumors. Although the NK cell described so far is the best documented and characterized cell with a spontaneous cytotoxicity to tumors, it appears to be only one of

several cells capable of mediating this activity.

The activated macrophage has already been described in detail earlier (also see ref. 287). In addition, an adherent non-phagocytic cell with an inhibitory effect toward MCA-induced tumors (288) and a polymorphonuclear cell with a specificity for lymphoid tumor targets (257) have been reported in the literature. In a recent report, promonocytes isolated from mouse BM cultures have also been shown to spontaneously lyse tumor cells. Their specificity was similar to that displayed by NK cells (289).

It is possible, therefore, that the NK represents only one of several cell populations which provide surveillance mechanisms *in vivo* against neoplastic transformation.

1.B.2.3.6.The In Vivo Relevance of NK Cells

The evidence for an *in vivo* role played by NK cells in providing a defence mechanism against tumors is at present scarce and indirect. It consists mainly of correlative data derived from assays which were carried out in mice. In these assays the relationship between the resistance of mice to tumors *in vivo* and NK reactivity of their spleens to the same tumors *in vitro* was studied.

The following observations were made:

1) Nude mice display a relative resistance to the induction or primary tumors, to transplantation of tumor cells, and to the metastatic spread of local tumors (141-143,154,290), while *in vitro* their spleen cells can be shown to have a high NK reactivity (144).

2) Using an *in vitro* assay, many of the tumors to which nude mice displayed an increased resistance were found to be NK-sensitive, whereas several of the tumors which grew well in nude mice were NK-resistant (144).

3) Animals of "high" NK strains are more resistant to a small inoculum of NK sensitive tumors than mice from "low" NK strains. Moreover young mice are more resistant to these tumors than old mice (291-293).

4) NK cytotoxicity and the factors influencing its level parallel other natural resistance mechanisms operating in vivo against intracellular parasites or semisyngeneic BM grafts (273).

More direct evidence came from experiments reported by Herberman and coworkers (227), who used ^{125}I-labelled tumor cells to compare the rate of destruction of injected tumors cells in vivo in various mouse strains. They demonstrated that mice of "high" NK strains can eliminate injected tumor cells more rapidly than mice of "low" strains. That this elimination was NK-mediated was suggested by its rapidity (4 hours) and by the fact that the efficiency of tumor destruction correlated well with the state of NK activity in vivo. Thus, while this destruction increased in response to known NK boosters (such as the interferon inducer poly I:C), it decreased when mice were pretreated with NK inhibitors such as ι-carrageenen and silica.

Detection of NK cells in tumors also suggested that they play a role in anti-tumor responses (238).

It should be noted that in both cancer patients and tumor-bearing mice, NK activity was found to be low (238,294). More experimental data on the role of NK cells in vivo is necessary to allow a conclusion as to whether this low activity represents a cause or a consequence of tumor growth.

NK Cells in Man. The demonstration of natural killer cells with a specificity for neoplasms in the human peripheral blood suggested that they may be of clinical importance (223). A review of the human NK system is beyond the scope of this introduction. However, it should be stated that striking similarities such as cell morphology, organ distribution, and similar responses to NK activators, have been demonstrated between the human and murine NK cells (227). While the two NK cells differ in several of their surface characteristics, such as, avidity of Fc receptors and susceptibility to anti-T cell serum and complement (227), the parallelism in their behaviour increases the importance of the mouse NK cell as an experimental model in the study of anti-tumor mechanisms in man.

1.B.3. The Role Suppressor Cells in Anti-Tumor Immunity.

Numerous mechanisms have been suggested in an effort to explain the growth of tumors in the face of demonstrable cytotoxic host immune cells. The role of humoral factors in modifying cell-mediated cytotoxic responses is discussed in section B.4.2. In addition, cell-mediated modulation of anti-tumor responses has also been demonstrated and, both specific and non-specific suppressor cells have been reported in tumor-bearing hosts. It is now becoming clear that these cells play an important role in regulating the immune response to growing tumors, and may, in fact, determine the fate of the tumors.

1.B.3.1 Non-specific Suppressor Cells

Suppressor cells capable of non-specifically inhibiting various immune responses not necessarily related to tumor antigens, have been characterized in numerous tumor-host systems. Macrophages, T lymphocytes, and B lymphocytes have all been shown to function in this capacity.

Macrophage-mediated suppression. A general reduction in immune responsiveness of splenocytes from tumor-bearing mice has been demonstrated in vitro, using either mitogenic stimulation, antibody synthesis to SRBC, or the mixed lymphocyte reaction as assay systems (295,296). This impaired responsiveness was demonstrated in mice bearing either virally induced, chemically induced, or spontaneous tumors, regardless of their anatomic site or the strain in which they arose (295-298). Immune responsiveness could in some cases be restored by either removing nylon adherent and phagocytic spleen cells, or by treating the splenocytes with macrophage-inhibiting agents such as ℓ-carrageenen. Treatments aimed at the selective removal of T cells were in these cases ineffective in restoring the responses (295,296,298,299). It was therefore conclude

that the lack of response was due to suppressor macrophages. This conclusion was further strengthened when it was shown that spleen cells from tumor-bearing mice can inhibit the mitogenic responses of normal splenocytes, and that this inhibition can be abrogated by the removal of phagocytic and adherent cells (295,296,299).

It was observed furthermore, that spleen cells from tumor-bearing mice and, in the case of MSV-induced tumors, cells isolated from the tumor itself, were also inhibitory to the growth of other tumors (123,300). These cells, and those mediating the suppression of immune reactivity, were shown to share several characteristics attributed to monocytic macrophages (301). Since the suppression of cellular proliferation could only be demonstrated after the tumors reached a certain size, it was postulated that factors released by the tumor can activate the splenic macrophages to become suppressive and that a minimal level of these factors must accumulatein the spleen before activation can take place. Furthermore, it was suggested that the growth or regression of a tumor is determined by the balance achieved between the tumor inhibiting and the immunosuppressive influences of the macrophage (301).

Recently, the accuracy of these findings was questioned when it was shown that spleens of tumor-bearing mice are highly enriched by macrophages. It was suggested that the macrophage effect was a quantitative rather than a qualitative one. This argument was strengthened by the demonstration that normal macrophages added to splenocytes in similar proportions can also be suppressive (302,303).

Suppressor B Cells. A similar non-specific type of suppression by spleen cells of mice bearing virally-induced tumors was shown to be mediated by B lymphocytes. These cells, which in the presence of complement, could be removed with anti-Ig, but not anti-θ serum, could inhibit various T cell responses such as reaction to mitogens and production of MIF (Migration Inhibition Factor)(304,305).

These suppressive effects could be demonstrated with spleen cells from mice bearing a progressing tumor. Splenocytes of mice whose tumors regressed, however, could be stimulated with PHA, could inhibit macrophage migration, and were not suppressive when mixed with normal splenocytes.

Since supernatants of cultured suppressor splenocytes were equally suppressive in this system, it was suggested that B cells suppress T cell function by mediating the release of immune complexes which block F_c-receptors on T cells. The evidence in support of this interpretation, is at present inconclusive (306).

Another non-macrophage, non-T cell suppressor has been recently reported in the spleens of mice bearing a virally-induced mammary tumor. The cell was adherent to nylon wool, but not to plastic dishes, and was not sensitive to silica or anti-θ serum and complement. The suppressive activity in this system has been attributed to B lymphocytes. At the same time, the participation of null cells or non-phagocytic monocytes in the suppression could not be ruled out (307).

Suppressor T Cells. Non-specific suppression mediated by T cells was observed in several tumor systems. One which has been well characterized is the suppressor cell detected in mice bearing the Lewis lung carcinoma 3LL. Trainin and his coworkers in their study of the immune response to this tumor found that after tumor injection, but prior to tumor appearance, cytotoxic cells can be detected in the spleens of the injected mice. These cells when injected together with tumor cells to normal recipients could suppress tumor growth. However, the suppression could no longer be detected after tumors appeared. Spleen cells removed from an animal at this time and injected together with tumor cells enhanced rather than suppressed their growth (152,308,309).

Suppressor cells which were sensitive to anti-θ and complement treatment, and were not adherent to plastic or nylon wool were identified in this system. A soluble factor with suppressive effects was found in the supernatants of cultured suppressive spleen cells. It could not, however, be detected if the cultured spleen cells were depleted of T cells (152,308). Both the suppressor cell and the soluble factor(s) could enhance growth of non-related tumors, indicating a non-specific mechanism. Host cells probably played a role in this suppression as it could only be demonstrated in immunologically intact recipients (152). Among the characteristics of the cell were its affinity to histamine coated beads (310), its elimination (or the elimination of its effect) by a 24 hour culture (309), and its sensitivity to treatments directed at cells in division (e.g. light, BUDR, or hydrocortisone acetate) (311). Based on these and other lines of evidence, it was suggested that the suppressor cell was an immature, actively dividing

thymocyte which lost its activity upon maturation (311).

Similar findings were obtained in the same laboratory with other tumor systems. Thus, spleen cells from mice bearing MCA-induced sarcomas were inhibitory to tumor growth when assayed early after tumor injection but became tumor-enhancing as the tumor progressed. Whereas the cytotoxic effect was specific, tumor enhancement could be demonstrated with non-related tumors. Fractionation procedures again pointed to a thymus-dependent cell which still responded to the thymic humoral factor (THF) indicating that an immature thymocyte was involved (113,312,313). When mixed with tumor-inhibiting splenocytes, these suppressor cells could block their activity, suggesting that tumor growth in this system was influenced by a balance achieved between the two opposing immune functions, and that the suppressor function was dominant.

Suppressor T cells capable of inhibiting the cytotoxic response of spleen cells to tumors were also reported by other laboratories. They could be demonstrated in mice bearing MCA-induced tumors, as well as in a viral tumor system (314). In the latter system, it was shown that the suppressor cells were induced by the virus complex itself (315). In yet another MCA-induced tumor system both macrophages and T suppressor cells have been shown to coexist in the spleens of tumor-bearing mice (316). Non-specific suppressor cells were also reported in cancer patients and they resemble mouse suppressor cells in their ability to inhibit proliferative responses of normal human T cells (317).

It can be concluded, therefore, that the three major components of the immune response, namely the T cell, the B cell, and the macrophage can play a role in non-specific immunosuppressive mechanisms and possibly in the enhancement of tumor growth. The evidence available, however, is not sufficient to conclude whether the same mechanisms of suppression are mediating both the immunosuppression and tumor enhancement and whether the mode of suppression is common to all three cell populations.

1.B.3.2 Specific Suppressor Mechanisms

In addition to cellular mechanisms, which in the presence of a growing tumor exert a general immunosuppressive influence and thus facilitate tumor growth, other regulatory mechanisms have been demonstrated which specifically block anti-tumor reactivity toward the inducing tumor.

The cell population involved in the specific suppression was again determined on the basis of fractionation procedures and the sensitivity of suppressor cells to sera directed against known surface antigens. The specificity of the suppression was postulated on the basis of findings demonstrating either an inability of the suppressor cell to block cytotoxic reactions against non-related tumors, or their failure to block T cell responses in vitro.

Fujimoto and coworkers described one such suppressor cell (318-320). It was detected in the spleens, thymus or lymph nodes of mice bearing MCA-induced tumors, one day after tumor injection. Suppressor activity was demonstrated when it was shown that such cells can abrogate the immunity to a tumor challenge when injected into hyperimmune mice. The suppressor cell was found to be a T cell, and the suppression was reportedly mediated by a soluble factor with a specific affinity to the relevant tumor. Antibodies prepared against the suppressive factor could block cell-mediated suppression, suggesting that it was a component of the suppressor cell surface (320). Similarly to suppressor cells found in immune responses to other antigens (321,322), this suppressor cell was found to express the genes encoded in the I-J subregion of the H-2 complex (323), suggesting that the immune response to tumors is regulated by signals and mechanisms similar to these operating in other immune reactions.

Specific suppressor T cells were reported in other tumor systems such as the P815 mastocytoma and the EL-4 leukemia. Suppressive factors were demonstrated in some of these systems (324-327). It is possible that T cells can exert their suppressive influence via either a direct action on the cytotoxic effector cell or by providing help in the synthesis of suppressor factors by other cells.

In addition to the specific and non-specific suppressor cells facilitation of tumor growth, possibly via suppressor cell induction, has been attributed to a variety of other agents. Among them are u.v. irradiation (328,329) and under certain circumstances adjuvants (330) and viruses (331).

Conclusion. Suppressor cells and suppressor factors add another dimension to the complexity of the host immune response to tumors. They demonstrate that no cell acts independently in the response or mediates an isolated function. Rather, they suggest that each population can act both in an effector and regulator capacity and can thus exert either tumor supporting or tumor inhibiting influences or both. The final balance achieved between these antagonistic influences determines the fate of a developing tumor.

Having the benefit of retrospection several of the early puzzling observations made in the study of responses to growing tumors can now be attributed to suppressor mechanisms. Thus both the immune stimulation of tumors by small doses of lymphocytes described by Prehn and others (322,333) and the "sneaking through" of very small inocula of tumor cells injected into mice (334) can be explained as manifestations of a shift in the balance of the immune response from tumor-inhibiting to tumor-enhancing mechanisms.

1.B.4. The Humoral Immune Response to Tumors.

Similarly to the cell-mediated immune reactions, the humoral immune response has repeatedly been shown to play a dual role in the host-tumor relationships. Thus, depending on the assay system and the experimental conditions, antibodies could be shown to mediate both the inhibition and the promotion of tumor growth. The balance between these influences may be an important factor in the development of malignancy.

1.B.4.1. The Antibody-Mediated Inhibition of Tumor Growth.

It was shown that immunoglobulins can exert tumor-suppressing effects via two major mechanisms. They can either combine with complement to lyse

tumor cells directly, or they can "arm" or "activate" non-immune lymphocytes or monocytes to become killer cells (335,336).

1.B.4.1.1.The Cytotoxic Antibody.

The failure of several early attempts to transfer immunity to tumors by passive transfer of sera from immune mice (337,338) has led to the belief that antibodies do not play an important role in host protection against tumors. Additional experiments have demonstrated however,that experimental conditions such as dose of antibodies, timing of injections, and tumor status of the antibody donors can all influence the therapeutic effect of the sera in the recipient. Thus, protection of recipients could be achieved with sera from mice whose MSV or polyoma-induced tumors had regressed (335,339). This protection of the recipients corresponded to the appearance in the donor's serum of an anti-tumor antibody capable of a complement-mediated lysis of tumor cells *in vitro* (335). Antibody-mediated protection against tumors was also reported in a lymphosarcoma system when tumor bearers were injected with large quantities of anti-tumor antibodies (340). The mechanism of protection in this system however, was not elucidated.

Anti-tumor antibodies have also been suggested as the immune component inhibiting the dissemination of established local tumors. Thus in a study of melanoma patients, an inverse relationship was found between the ability of a patient's serum to participate in a complement-mediated lysis of tumor cells *in vitro* and metastatic spread of his tumor *in vivo* (34). It was suggested that antibodies play a protective role by inhibiting tumor spread in the circulation (342,343).

1.B.4.1.2.The Natural Antibody

Another relevant immunological phenomenon, which is rapidly gaining attention as a possible humoral immune surveillance mechanism, is that of the natural antibodies. These antibodies which can be found in the sera of normal non-immunized mice have been shown by several laboratories to mediate

an efficient complement dependent-lysis of tumor cells *in vitro* (344-346). The natural cytotoxic activity of the serum, could be demonstrated using several mouse strains, and various lymphoid and non-lymphoid tumors. It was demonstrated in normal as well as in nude mice and identified as predominantly IgM-mediated (345). Its relevance in the *in vivo* protection against tumors was ascertained indirectly by comparing tumor growth in mice whose sera exhibited high levels of natural antibodies to that in mice whose sera had only low levels of *in vitro* activity. Using a small inoculum of tumor cells (10^2 cells), it was found that randomly selected mice which were more resistant to tumors *in vivo* also showed higher levels of natural antibodies *in vitro*. Similarly, thymus-depleted and old mice which exhibited higher than normal levels of natural antibody *in vitro*, were more resistant to tumors *in vivo* (347). These and other findings have, in fact, prompted several authors to suggest that natural antibodies may be mediating an *in vivo* immune surveillance against neoplasia (ibid).

An association between natural antibodies and the natural killer cell has not been demonstrated. The age-dependence of the two natural mechanisms was shown to be remarkably different. Thus, whereas natural killer cell levels in the mouse were shown to peak at 8 weeks of age and to decrease rapidly thereafter (227), natural antibody levels were found to be low in young (8 week old) mice and considerably higher in old (8 month old) mice (346). This difference in the age dependence suggests that natural antibodies and natural killer cells are exerting their effects independently of each other.

It should be borne in mind that mouse IgM, which is the predominant class of natural antibodies, is relatively inefficient in fixing mouse complement (348) and penetrates poorly into tissues (349). These factors may in fact hinder their tumor-inhibitory capacity *in vivo*.

1.B.4.1.3. The Antibody-Mediated Cellular Cytotoxicity.

In addition to their complement-dependent lysis of tumor target cells, antibodies have been shown to participate in, and mediate, cellular cytotoxic responses against tumor cells and other targets (350-352).

Pollack et al. first reported in 1972 that sera of mice immune to various tumor cells can "arm" normal lymphocytes to become killer cells with a target specificity in vitro (351). In addition to lymphocytes, macrophages have also been shown to participate in this form of target lysis (353). The common characteristic of the potential killer cells in this system was later shown to be their receptors for the Fc portion of IgG (336). Many laboratories have since reported similar observations and the reaction is now commonly known as ADCC (Antibody-Dependent Cellular Cytotoxicity). The assay system most commonly used for the detection of this activity has been the incubation in vitro of target cells coated with specific antibody together with non-primed effector cells derived from normal animals. Similarly to the methods used in the study of cell-mediated cytotoxicity, lysis of target cells in the assay of ADCC can be measured as specific release of radioisotopes by lysed cells or in terms of the inhibition of uptake of labelled nucleotides (336,354). In addition to tumor cells, allogeneic targets, as well as non-nucleated targets, such as red blood cells, were shown to be susceptible to this form of lysis (336).

Using the methods described and a variety of target cells, 2 major classes of effector cells have been identified and characterized. One, commonly referred to as the K (killer) cell was shown to be a non-phagocytic and non-adherent lymphocyte, while the other displayed the characteristics of a phagocytic monocyte. Other populations of mononuclear cells, such as platelets, polymorphonuclear cells, and mast cells, were also shown to possess Fc receptors. The possibility that they too can lyse Ig-coated targets is supported by recent data which demonstrate that platelets can participate in ADCC in an adoptive transfer system (336,355).

The K-cell and its cytotoxic reactions. Using the various fractionation procedures described elsewhere in this chapter (see p. 27) K cells in the mouse were originally shown to lack T or B lymphocyte characteristics (351,352,356). Recent experiments however have suggested that a subpopulation of T cells can also mediate the reaction (357,358). Additionally, K cells were found to lack phagocytic characteristics. However, subpopulations of the non-phagocytic killer cell differ in their adherence properties (356).

In order to bind to and lyse target cells, K cells were originally thought to require a specific antibody of the IgG class with an intact Fc portion (336,359). However, some recent reports indicate that IgM molecules can also participate in the cytolysis and that in this case the lysis is probably mediated by a subpopulation of T cells with receptors for IgM (360-362). Minute quantities of the antibody, when either added to the effector and target mixture or preincubated with the target cells, are sufficient to trigger the reaction (336).

Mechanism of target cell destruction. The initiation of the lytic reaction was shown to require cell-cell contact and a metabolically active effector cell. Once contact was established however, the reaction could proceed to completion even with a metabolically inactive effector cell (336).

Unlike the cytotoxic activity of T cell, K cell activity is enhanced rather than blocked by pretreatment of the effector cell with trypsin or neuraminadase, indicating the involvement of different recognition sites. Cytocholasin b, however, blocks K cell as well as T cell mediated lysis, probably by inhibiting cell-cell contact (336). Although the recognition and binding phases differ in the K and T cell-mediated lysis, the lytic event itself appears to be similar. Thus both events were shown to be energy-dependent, were blocked by high levels of cAMP, and were enhanced by high levels of cGMP. Contrary to T cell mediated lysis, however, soluble, cytotoxic mediators have not been reported in the K cell system (ibid).

K cell and the natural killer cell. The similarities between the K and NK cells, i.e. their lack of either T or B lymphocyte markers, the presence of Fc receptors on their surface, their age and strain-dependence, and their inability to mediate phagocytosis, have prompted several investigators to suggest that the 2 cells are identical and that NK cells are essentially K cells armed in vivo with natural anti-tumor antibodies which are bound to their Fc receptors (350,363,364). However, several lines of evidence dispute both the possibility of identity between the two killer systems and the presence of Fc-bound immunoglobulins on NK cells. Thus, high levels of NK activity could be found in mice depleted of B cells and

antibodies by the chronic administration of heterologous anti-IgM sera (227,365, and see Chapter 4 of the thesis). Moreover, no evidence could be found in mice or rats for natural antibodies capable of sensitizing NK target cells (366). It was also shown that pretreatment of mouse lymphocytes with either anti-Fab reagents, or the $F(ab')_2$ portion of anti-IgG, did not effect their natural cytotoxicity (227). Cold target inhibition assays with human lymphocytes have also suggested that the 2 lytic activities are mediated by different mechanisms and different cells (367).

In a recent report by Ojo and Wigzell, it is claimed that whereas K cells mediating lysis against antibody-coated chicken red blood cells (CRBC) differ markedly from the natural killer cell, the K cell mediating lysis of the antibody-coated mastocytoma P815 cells, exhibits a striking similarity and may be identical to the NK cell (350). It is possible therefore that, depending on the target cell assayed and given the right experimental conditions, NK cells are capable of mediating ADCC reactions.

Based on the available data on NK and K cells, Herberman and Holden, in a recent review, proposed a model in which both cells are placed as intermediates in the maturation pathway of T cells. The authors suggest that both cells are prethymic cells which originated in the BM, express Fc receptors and low density θ antigens and are on their way to further differentiation in the thymus. During the thymic period, the cells loose their Fc receptor, as well as their NK and K functions and gain θ and TL antigens. The authors further suggest that the mature post-thymic T cell, although incapable of mediating NK or K reactivity can revert back into cells with pre-T cell characteristics, thus explaining the presence in the secondary lymphatic organs of NK and K cells (227). This model is supported by several lines of evidence such as the finding that nude mice have low density θ-bearing cells and increased NK reactivity (144,368) and that mature T cells in culture reexpress Fc receptors while loosing θ antigens (369).

The macrophage mediated ADCC. Macrophages represent the second major cell population capable of mediating tumor inhibitory reactions in the presence of specific antibodies.

The ability of macrophages to lyse antibody coated tumor cells was demonstrated in both in vitro and in vivo systems (370,371). Using an in vitro assay to measure the uptake of radiolabelled nucleotides by tumor target cells, it was shown that macrophages can suppress DNA synthesis by tumor cells when they are incubated in the presence of specific antisera. Pretreatment of the killer cells with anti-Thy-1 serum and complement did not affect this inhibition. Neither phagocytosis nor target cell lysis were observed in this reaction. However the suppressed tumor cells, once affected by the macrophage, could not resume proliferation and eventually died. Similarly to the antibody-independent interaction of macrophage and tumor, cell-cell contact was shown to be a requirement for the suppression of DNA synthesis. However, shortly after the interaction took place suppression could proceed in the absence of cellular contact. Similarly to K cell-mediated lysis, soluble mediators were not detectable in the supernatants of the reaction mixtures (354,370).

The evidence for the relevance of macrophage mediated ADCC in host protection against tumors in vivo, is presently inconclusive. Yamamura and Coworkers in a series of reports demonstrated that passively transferred sera from tumor-bearing mice can protect the recipients from the growth of the same tumor (a mammary adenocarcinoma). They also showed that this protection was dependent on the intact immune capacity of the recipients. On the basis of the sensitivity of the adenocarcinoma to macrophage-mediated ADCC in vitro, they claimed that this mechanism is also operating in vivo (353,372). Although a positive characterization of the killer cell is lacking in these reports, the finding of macrophages capable of mediating ADCC reactions in vitro in the tumors, may support this claim (373).

1.B.4.2 Humoral Factors Enhancing Tumor Growth.

Similarly to the cell-mediated immune response to tumors, the net effect of antibodies in the immune reaction to a tumor is influenced by a duality in their function. Thus, it was demonstrated by many investigators that in addition to lysing tumor cells, antibodies, by themselves or in

combination with other serum factors, can block the cytotoxic activity of killer T cells.

Hellström and Hellström (115), using the MSV-induced tumor system, fisrt observed that sera from mice whose tumor had regressed were cytotoxic to tumor cells in vitro and that this cytotoxicity could not be detected in sera from mice bearing a progressively growing tumor. Furthermore, they observed that, when added to a mixture of tumor cells and immune, killer cells sera from tumor-bearing mice could block the cytotoxic reactions.

This observation was followed by numerous investigations undertaken in an effort to elucidate the nature of the serum component which was capable of blocking cell-mediated (CM) lysis of tumors and consequently was mediating what became known as "tumor enhancement". The blocking activity could normally be detected using in vitro assays of CM cytolysis, which are commonly used in the study of tumor immunology. These included the microcytotoxicity assays (115) and the isotope release assays (374).

It became clear that the blocking phenomenon was not restricted to the MSV tumor system but was operating in a range of virally and chemically-induced, as well as spontaneous, tumors (375-378). The blocking activity was shown in many instances to be specific for the individual unique tumor antigens (374,378). However, sera with blocking activity against cross reacting or common, tumor antigens were also reported (22,379).

Several reports have also demonstrated the presence of blocking factors in sera of patients with a variety of neoplasms including carcinomas of the lung, colon and breast and various sarcomas and melanomas (380,381).

It is important to note in this context that the nature of the blocked killer cell was not determined in most of the systems tested. In some systems the killer cell was identified as a T-cell (382). However, it cannot be assumed that the blocking was directed against this cell in all host-tumor systems, where blocking was reported.

Blocking Antibodies. Originally, several lines of evidence suggested that the blocking component in the sera was a tumor specific antibody. Among these lines of evidence were the observations that the blocking activity (1)

could be removed from the serum by an absorption with the specific tumor (115), (2) could be neutralized by goat anti-mouse immunoglobulin serum (ibid), and (3) could be removed together with the 7S fraction of the serum (383).

Several findings, however, were incompatible with the suggestion that antibodies alone mediated the blocking activity. Thus, it was found that following either the excision or the regression of a tumor blocking activity rapidly disappeared from the serum (150,384). Moreover, such serum, devoid of blocking activity could neutralize or "unblock" the blocking activity of serum from tumor-bearing mice (384,385). These findings suggested that a tumor-derived component was participating in the blocking activity. It was subsequently claimed, in fact, that antigen-antibody complexes may be mediating this activity.

Blocking by antibody-antigen (ab-ag) complexes. The evidence in suppor of the blocking capacity of ab-ag complexes was again derived from the analysis of sera from tumor-bearing mice or cancer patients and is mainly indirect. Thus, by employing ultrafiltration techniques, blocking sera, or blocking factors eluted directly from tumors, could be separated into 2 fractions of low and high molecular weight. These fractions could not block cell lysis when applied separately to mixtures of tumors and immune cells. However blocking did occur when the recombined fractions were used (386,387) Although these findings do suggest a requirement for 2 "entities" in the blocking, they do not provide sufficient information on the nature of these entities.

More direct evidence in support of the blocking capacity of immune complexes is derived from experiments which demonstrated that papain extracts of a tumor, in combination with anti-tumor antibodies, could mediate blocking of killer cells (388). Additionally, it was found that sera passed through immunoabsorbents which selectively bound either tumor antigen or antibody lost their blocking activity (389,390).

Blocking by tumor antigen. In addition to immune complexes, tumor antigens could also be shown to block cell-mediated cytotoxicity. Thus, it was originally observed that repeated washings of peripheral blood

lymphocytes of cancer patients increased their cytotoxicity in vitro to the specific tumor (391). It was suggested that the washings resulted in the exposure of masked cytotoxic activity, and that circulating antigens were masking the active sites of the cytotoxic cells (392). Subsequently it was shown in several tumor systems that pretreatment of cytotoxic effector cells with tumor antigen preparations or tumor extracts blocked their inhibitory effect (374,382,393,394).

The presence of tumor antigens in the circulation of tumor-bearing hosts has been demonstrated both in man and in experimental animals (395-397). The mechanism of antigen shedding by the tumor and the role of immune factors in the process are not clear. It has been shown that antigen release may be due to either the normal metabolic activity of a tumor or its death (398). Immune mechanisms resulting in tumor cell cytolysis are likely to participate in the latter. Additional experimental evidence suggests that in the course of tumor growth, antibodies bind to the tumor cell surface (399,400) and may mediate a release of antigens by non-lytic mechanism, such as antigen modulation and shedding (401,402). Other lines of evidence support the notion that the immune response may be actively contributing to antigen modulation and release, and consequently may be providing an escape route for tumor cells. Among these lines of evidence are a demonstrated decrease in circulating antigen in irradiated mice (403) and the findings that the ability of a tumor to metastasize is influenced by, and correlates with, its antigen shedding (404-406).

It seems, therefore, that antibodies can influence the inhibition of cell-mediated cytotoxicity not only by direct intervention, i.e. by masking of tumor antigens, but also by inducing the release of tumor antigens into the circulation.

The in vivo relevance of serum blocking factors. The role of blocking factors in vivo is at present poorly understood.

Sjörgenand coworkers demonstrated that the blocking activity of sera from tumor-bearing mice, originally observed in vitro, can also occur in vivo. Thus injection of mice with these sera could be shown to facilitate the growth of their implanted tumors (407). Moreover, the same group has

also shown that "unblocking" sera obtained from mice whose tumors had regressed can induce a regression of an established tumor when injected in vivo (408). The unblocking mechanism is not clearly understood, but it was suggested that anti-tumor antibodies in the serum, bind to the blocking factor which may be an antigen or have antigenic determinants and thus allow cell-mediated cytotoxic reactions to resume (398).

The injection of antigenic extracts of tumors into mice, which has been shown in many instances to lead to the induction of humoral and cellular immunity, (409-412), could also be shown to prevent subsequent immunization with irradiated tumor cells and could lead to enhanced tumor growth (413-415). These findings suggested that the duality of the humoral response to a tumor is operative in vivo and that it may be influenced by the mode of antigen administration.

In addition to tumor antigens and antibodies, other blocking factors have been demonstrated in the serum of tumor-bearing animals. Nepom et al. recently reported that blocking factors detected in the sera of tumor-bearing mice were glycoprotein molecules with an affinity for both anti-tumor antibodies and homologous tumor cells. Although the possibility that this factor is a tumor-specific antigen has not been excluded, the authors also suggest that it may be an immunosuppressive molecule released by suppressor T cells (416). Suppressor cells have in fact been demonstrated in tumor bearing mice and it was suggested that they are triggered by either immune complexes or other soluble factors released into the circulation by the tumor (417,418).

In conclusion it seems therefore, that a growing tumor can induce the production by the host of an array of suppressive factors of which antibodies shed antigen and antibody-antigen complexes are only a few examples. These factors in turn may enhance tumor growth by the generation of suppressor cells.

SUMMARY

The complexity of the immune response to an antigenic tumor cannot be overemphasized. As shown in this review of the literature, this response is influenced and controlled by a multitude of factors, some of which have only recently been elucidated. The understanding of other mechanisms awaits further developments in the tools available for the study of the immune phenomena.

It seems, that the net response of a host to its tumor consists of, and is determined by, many reactions and counter reactions mediated by both the cellular and humoral arms of the immune apparatus. Thus a tumor stimulus may encounter natural (spontaneous) resistance mechanisms and/or is capable of inducing the generation of cytotoxic cells and antibodies capable of its destruction. The appearance of these destructive mechanisms, however, seems to trigger a multitude of other immune reactions which curtail or inhibit their activity, thus facilitating tumor growth.

The evaluation of these responses and counter responses is complicated by the fact that: (1) they differ according to the types of tumors and hosts assayed and (2) subpopulations of the same cell can function in the capacities of both tumor inhibitors and tumor stimulators. Thus, the T cell response during tumor growth fluctuates between a tumor inhibitory effect exerted by cytotoxic T cells and a tumor stimulatory effect mediated by T- suppressor cells. The macrophage, which was shown to inhibit tumor growth *in vitro* and *in vivo*, could at the same time support tumor progression by suppressing the proliferation of cytotoxic T cells. Similarly, antibodies which can mediate tumor lysis, either directly in the presence of complement, or by "arming" non-sensitized cells in an ADCC-response, were also shown to enhance tumor growth by blocking cell mediated cytotoxicity.

This dichotomy in the role played by the immune response, during tumor growth, may explain the limited success, up to date, of the clinical trials of the immunotherapeutic approach to the treatment of cancer. It also calls for great care in the design of these treatments.

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CHAPTER 2

INTRODUCTION - PART II

THE B -LYMPHOCYTE DEPRIVED MOUSE - A MODEL FOR THE STUDY OF ANTI-TUMOR IMMUNITY

2.A. Review of the Literature

2.A.1 The Suppression of Immunoglobulin (Ig) Synthesis by Heterologous Sera

One of the most fruitful approaches to the study of the immune response and the intricate relationship between its different components has been the selective removal of one known component coupled with the study of the remaining immune capacity. Thus thymectomy, anti thymocyte serum and the nude mouse served as valuable tools in the study of T cell functions, whereas treatments such as silica, ζ-carrageenen or anti-macrophage serum which are selectively toxic to macrophages were helpful in illuminating the importance of these cells in an array of immune phenomena.

For several years now, a similar approach is available for the *in vivo* and *in vitro* study of B-lymphocyte functions. Thus, several laboratories demonstrated in the early 70's that treatment of mice *in vivo* or their lymphocytes *in vitro*, with heterologous anti-heavy chain antisera, results in the suppression of antibody synthesis. The immunoglobulin class shown to be affected by this treatment was dependent on the antiserum injected. However anti-μ antiserum was shown to have the most profound effects and when injected into neonatal mice or into chicken in ovum, caused a general suppression of all Ig classes (1 - 4). The extent of suppression attained *in vivo* was shown to be dependent on both the schedule of serum administration and the doses injected (5). If administered neonatally and in high doses, anti-μ serum not only caused a marked reduction in all Ig classes but also an elimination of all Ig bearing cells from the spleen and peripheral blood. This, in turn, led to parallel reductions in the formation of germinal centers in the spleen and in the spleen size (6, 7).

Whereas anti-μ serum was repeatedly shown to cause a pan-specific suppression and to inhibit both IgM and IgG responses, if administrated during the early stages in the development of an immune response (4), antiserum to other classes of heavy chains were shown to have more restricted effects and to reduce only the synthesis of immunoglobulins against which they were directed. Thus, anti α antibodies were shown to suppress primarily IgA responses when administrered to neonatal or several days old mice (3, 5, 8). This suppressive effect could not be demonstrated if treatment was initiated when mice were older than 3 weeks (9). Similarly, treatment of mice with anti-γ antibodies caused a reduction in IgG levels which frequently was only partial. Even when a more severe suppression could be accomplished by using athymic nude mice, it was restricted to IgG and did not affect levels of other Ig classes (5, 10, 11). It was originally though that IgE could not be suppressed by either anti-γ or anti-μ treatments (12) however recent experiments have shown that anti-μ antibodies can suppress IgE synthesis both in the mouse and in the rat (13, 14).

The suppressive effects of anti-δ antiserum were recently studied. In vitro assays have shown that anti-δ antibodies can severly suppress the growth of B-lymphocyte colonies in agar (15). When allogeneic antiserum was administered to neonatal mice it caused a selective suppression of the expression of IgD on the B-cell surface with a simultaneous increase in the number of "null" lymphocytes in the spleens. It was also shown that in addition to a modulating effect on cell surface IgD, anti-δ antiserum could exert a degree of pan-specific suppression. Thus, it was found to suppress the expression of cell surface IgM and cause a reduction, but not an elimination, of IgM bearing cells. Furthermore anti-δ treated mice were shown to have a depressed IgG response to antigenic stimuli (14, 15).

While the mechanism for this pan specific suppression is not clear, the results support the hypothesis that lymphocytes bearing μ and δ receptors participate in T dependent antibody responses leading to the synthesis of IgG antibodies. Lymphocytes which express only μ determinants, on the other hand, may participate in T cell-independent responses giving rise to IgM antibodies (16).

2.A2. The mechanism of the pan specific suppression by anti-μ

2.A.2.1 The target cell.

The presence of immunoglobulins on the surface of lymphocytes and in particular on the surface of B-cells has been well documented (17, 18). It has also been shown that surface Ig on B-cells can function as receptor sites for antigen binding, which triggers cell differentiation and results in antibody synthesis (19).

Several lines of evidence indicate that B -lymphocytes serve as the target cells for the suppressive effects of anti-μ serum. Both *in vivo* and *in vitro* studies have indicated that the presence of T cells is not essential for the suppression. Thus, it was shown that antibody synthesis by nude mice could be suppressed by anti-IgM (5, 11) and that immunosuppression of cultured splenocytes could be achieved even after they were depleted of T cells by anti-θ serum and complement (3). The importance of B-cells in the suppression was further demonstrated when it was shown that immunocompetence can be restored *in vitro* to suppressed spleen cells by the addition of normal B but not T lymphocytes (20).

Other lines of evidence also suggest that helper T cells mediating humoral immune responses, are not targets for suppression by anti-IgM. Thus, it was shown that treatment of lymphocytes *in vivo* or *in vitro* with doses sufficient to severely depress B-cell functions, did not impair the ability of T cells to provide normal B-cells with help in antibody synthesis in culture (21).

In this context it should be noted however, that a recent report by Janeway and coworkers suggests that helper T cells may be indirectly affected by the absence of B-cells and serum immunoglobulins in the suppressed mice. They found that a subpopulation of helper T cells which requires Ig for its priming was absent in mice suppressed by anti-μ (22).

The role of macrophages in the suppression is poorly understood. Macrophages have been shown to retain their ability to participate in immune reactions *in vitro* even after they were treated with anti-μ (3).

Preliminary results obtained in our laboratory also suggest that macrophage function in the suppressed mice is unimpaired (not published).

The B-lymphocytes which are sensitive to anti-μ were shown to be relatively mature cells capable of responding to an antigenic stimulus. Using both in vivo and in vitro systems, it was demonstrated by several laboratories that spleen, but not BM cells, are susceptible to the immunosuppressive effect of anti-μ (20) and that contrary to anti-μ treated BM cells, anti-μ treated splenocytes can adoptively transfer suppression to lethally irradiated mice (2, 23).

Plasma cells were also shown to be resistant to the suppressive effects of anti-μ when it was found that an in vitro immune response could no longer be abbrogated by anti-μ serum when the serum was added after the introduction of antigen and shortly prior to antibody synthesis (3, 24, 25).

These findings, coupled with the fact that anti-μ antibodies are likely to bind to IgM molecules, suggested that the target for the pan-specific suppression is an IgM bearing lymphocyte. The pan specificity of this suppression led to the hypothesis that IgM-bearing lymphocytes give rise to B-cells producing other classes of immunoglobulins including IgE-producing cells (13). Direct evidence for this notion was recently obtained when using immunofluorescent techniques, it was demonstrated that during the ontogeny of B-cells intermediate cells appear which express on their surface IgD, IgA and IgG, in addition to IgM (26).

2.A.2.2 Mode of Suppression.

The events which lead to suppression of humoral responses by anti-IgM are not fully understood. Several mechanisms have been suggested but many of them lack convincing experimental evidence.

The complement mediated lysis: Complement mediated lysis of mouse B-cells treated with heterologous anti-Ig sera in vitro, has been demonstrated (27). However, several reported findings are incompatible with the notion that anti-μ antibodies mediate a complement dependent lysis of their

target cells. Among them are the demonstrated reversibility of suppression in vitro (3), the ability of anti-mouse IgM produced in chicken to suppress mouse B-cells in vitro, although chicken antibodies cannot bind mammalian complement (28), and the suppressive effects of the Fab fragments of anti-μ serum (28, 29).

Receptor sites blockade: Another possibility which was considered was that anti-μ serum blocks the antigenic receptors on the surface of B-cells, thus preventing their stimulation and differentiation (6). However, this suggestion lacks experimental support. Furthermore recent experiments in our laboratory, failed to demonstrate rabbit immunoglobulins on the surface of lymphocytes from either spleen or BM of mice suppressed by the chronic administration of rabbit anti-mouse IgM serum (30).

Opsonization: Opsonization of B-cells coated with heterologous antisera was also suggested as a possible mechanism and could be demonstrated in vivo using antisera with an intact Fc portion (31). Opsonization, however, is unlikely to be the mechanism in vitro where viable unresponsive B-cells have been demonstrated after the suppressive treatment (32).

Several other proposed mechanisms were: (1) Inhibition by anti-IgM of the cell-cell contact required for antibody synthesis, (29) ; (2) blast transformation induced by cell bound anti-IgM (33) ; and (3) the modulation of the structure of antigenic receptors on B-cell surface (28). Although attractive, none of these suggestions is experimentally supported. Another hypothesis, that of anti-μ induced capping of antigen receptors on B-cells, has in fact been contradicted by experimental evidence showing that monovalent Fab fragments (of anti-μ antiserum) which cannot induce capping, can bring about the immunosuppression (28).

Suppressor cells are unlikely to be involved in the inhibitory effects of anti-μ. This was demonstrated both in vivo and in vitro when splenocytes from immunosuppressed mice failed to induce suppression when either incubated with normal spleen cells or adoptively transfered to sublethally irradiated recipients (20, 30).

To summarize, it seems that the experimental data available on the mechanism(s) of suppression by anti-μ are mainly negative in nature. The elucidation of this mechanism may be complicated by the fact that several of the events suggested above may be taking place simultaneously. Furthermore, it is possible that different mechanisms are operating *in vivo* and *in vitro* necessiating separate investigations of the two assay systems.

2.A.3. The Immune Status of the Suppressed Mouse.

The immune responsiveness of mice neonatally suppressed by anti-IgM has recently been described by J. Gordon (30). It was shown that these mice lacked Ig-bearing lymphocytes in both their spleens and BM and that they were incapable of mounting an antibody response to a battery of test antigens. As expected, their sera lacked detectable levels of either IgM or IgA, whereas the low levels of IgG and IgG_2 initially detected declined progressively as the treatment with anti-IgM proceeded. The B-cell deficiency in the lymphatic organs of these animals was also indicated by the marginal response of their spleen and lymph node cells to the (B-cell) mitogen LPS.

Several reports, based on both *in vivo* and *in vitro* studies, indicated that suppressed mice are capable of mediating effecient thymus dependent functions. Thus they were shown to successfully reject skin and tumor allografts (30, 34) and to mediate a normal Delayed Type Hypersensitivity (DTH) response (30). *In vitro*, spleen and lymph node cells from suppressed mice could respond to T cell mitogens PHA and Con A and to an allogeneic stimulus in a Mixed Leukocyte Culture (MLC) system (30, 32). Additionally it was shown that treatment of parental mouse lymphocytes *in vitro* with anti-μ serum did not affect their ability to induce a Graft Versus Host (GVH) response in neonatal F_1 hybrid recepients (35).

It should be noted that, whereas antisera with a specificity to heavy chain μ were not suppressive to T cell responses, anti-light chain

antibodies have been reported to affect some of T cell as well as B-cell functions. Thus, they have been shown to block DTH responses in vivo, and to reduce the ability of lymphocytes to respond in an MLC or cause a GVH reaction when pretreated in vitro (32).

Reports in the literature on the effects of the immunosuppression by anti-μ on macrophage function are scarce. However, preliminary experiments from our laboratory suggest that their function in the suppressed mice is intact (not published).

In summary, it seems therefore, that with the exception of an Ig-dependent subpopulation of T helper cells, the effect of heterologous anti-μ serum is restricted to B-cells and that cell mediated immune responses are not affected.

2.A.4. The Use of B-Cell Depleted Mice as a Model in Tumor Immunology

The complexity of the immune response to an antigenic tumor, generated by the intricate relationships among its various components, has already been discussed earlier in this chapter and cannot be overstated.

B-cell deprived mice, similarly to the nude mice, can serve as a valuable tool in the study of this response since they lack one of its components capable of mediating both tumor inhibitory and tumor enhancing functions. The selective removal of this component can facilitate the analysis of its role during tumor development as well as the unhindered study of the role of other components, such as T cells and macrophages.

Several attempts to study the effects of B-cell depletion on tumor growth have been reported in the literature and they vary in their findings and conclusions.

In one such study reported in 1972, the injection of mice with an anti-plasma cell serum, which selectively inhibited B-cell functions in vivo, could be shown to reduce the incidence of a transplantable MCA-induced fibrosarcoma, and a virus-induced rhabdomyosarcoma. Additionally, this treatment could prolong the latent period of tumor induction and reduced tumor incidence after the injection of MSV (Moloney Sarcoma Virus) (36). Using a similar approach, similar results were recently reported with mice bearing an

anaplastic carcinoma. It was shown that the injection of these mice with anti-plasma cell serum caused a reduction in the rate of growth of their tumors and consequently could prolong their mean survival time (37). J.W. Jutila in another communication reported (38) that anti-μ treatment appeared to retard the growth of spontaneous "mammary" tumors of Balb/c mice and provided protection against a transplantable IgM producing myeloma. It also prevented the development of a typical leukemia of Balb/c possibly by removing one of the target cells of the virus.

These results, however, are in disagreement with findings reported by another laboratory using a similar approach (39, 40). An antiserum raised against the Balb/c myeloma MOPC 104E cells was used in this study. It was shown to react primarily with Ig-bearing and plasma cells and not to react with mature T cells. A reactivity against a subpopulation of immature thymocytes was also detected. When injected into mice, this serum was shown to cause an acceleration of the growth of an allogeneic sarcoma. The authors of this report interpreted these results as indicative of the activation of suppressor cells by the injected antiserum.

In our study, mice were depleted of B cells and their products by the continuous injection of rabbit anti-mouse IgM serum. As already indicated earlier, these mice were found to mediate thymus dependent as well as macrophage functions but were deficient in all B-cell parameters tested (30).

We used these mice to explore the role of B-cells and their products in host protection against tumors, by examining the effect of the treatment on both tumor induction and tumor transplantation. We found that the depletion of B-cells did not have an adverse effect on host resistance to tumors. Moreover, B-cell deprived mice displayed a heightened resistance to both a transplantable syngeneic MCA-induced tumor (41) and to tumor induction by 3-methylcholanthrene.

The mechanism responsible for the heightened resistance to malignancy was then investigated using <u>in vitro</u> techniques for the study of cell mediated immune responses against tumors.

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CHAPTER 3

IN VIVO STUDIES

MATERIALS AND METHODS

A. Immunosuppression by anti-IgM.

Animals: Unless otherwise specified (C57BL/C3H)F_1 designated ($B6C3F_1$) male or female mice were used in all experiments. Adult F1 mice or pregnant C57BL/6 mated with C3H were purchased from an SPF colony from BioBreeding Laboratories of Canada (Ottawa, Ontario). They were housed in sterilized cages with filter caps in rooms supplied with filtered air. Their food, water, and bedding were sterile.

DBA/2 mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

Antisera: The procedure for the production of rabbit anti-IgM antisera is described here as published elsewhere (1). Specific precipitates of mouse IgM (purified preparations purchased from Litton Bionetics Inc., Kensington, Maryland) and a rabbit anti-IgM were prepared in agar, washed, and injected with Freund's complete adjuvant twice, two weeks apart. One week later, 1 ml of Balb/c serum was injected intravenously, and the animals were bled out five days later. Pooled serum was twice precipitated with ammonium sulfate, first at 50% then at 33% saturation. The final preparation, concentrated 3-4-fold relative to the original serum, was dialyzed against phosphate-buffered saline, then clarified by centrifugation (100,000 x g), and frozen in small aliquots.

A normal rabbit serum pool, purchased from Pel-Freez Biologicals, Inc. (Rogers, Arkansas), was processed in an identical manner. Before use, antibodies against mouse red cells which were present in both the normal

and in the antiserum preparations were removed by absorption with rat and mouse erythrocytes fixed with 0.5% glutaraldehyde.

The antibody preparations were analyzed by immunodiffusion in agar. They gave precipitin bands against purified IgM in dilutions of 1:64 - 1:128, gave a faint line against purified λ but not κ chains, nor against IgG or IgA. Since the anti-μ antibodies were not specifically purified, the serum preparations used are referred to as anti-IgM throughout.

Anti-IgM-Mediated Suppression

Neonatal immunosuppression was achieved by i.p. inoculation of 24 - 48 hour old F1 mice with 5 - 10 mg antisera in 0.05 - 0.1 ml. The injections were given three times weekly until the termination of the experiment.

The immunosuppressed status of the mice was routinely confirmed at five to six weeks of age by assay of serum immunoglobulin levels. This was done using an agar immunodiffusion test with class-specific antisera purchased from Meloy Laboratories (Springfield, Virgina). Throughout this study mice treated with anti-IgM in this manner will be referred to as suppressed, immunosuppressed or anti-IgM-treated interchangebly.

Non-treated mice or mice injected with normal rabbit globulins (referred to as NRS) were used as controls. NRS was prepared from a pool of rabbit serum, purchased from Pel-Freez Biologicals, Inc. (Rogers, Arkansas). Before use, antibodies against mouse red blood cells which were present in both serum preparations, were removed by absorption with mouse and rat erythrocytes fixed with 0.5% glutaraldehyde.

B. Tumors

T-10, a metastasizing 3-methylcholanthrene-induced sarcoma, was used in many of the experiments. The tumor was induced in our laboratory in a (C57BL/6XC3H)F_1 male by the procedure described below.

Tumors MCA-1, MCA-2, MCA-3, and MÁ were induced by the same procedure. Tumors were maintained in vivo by serial subcutaneous passages in F1 male mice and were also grown as monolayers in vitro, as described below.

EL-4, the C57BL leukemia, originally induced by dimethylbenzanthracene (2) was obtained in our laboratory courtesy of L. Scarlock, (Div. of Immunology, Duke University, Durham, North Carolina). It was maintained in the ascites form in C57BL/6 females or was grown as suspension cultures in vitro.

B-16, a spontaneous C57BL/6j metastasizing melanoma (4) was obtained courtesy of Dr. Gilles Lamoureux (Institute Armand Frappier, Laval, Quebec). It was maintained as a solid tumor in C57BL/6 females and was grown as an adherent monolayer in culture.

C3H/HeJ, a spontaneous mammary adenocarcinoma was a kind gift of Dr. R. Kerbel ,(Dept. of Pathology, Queen's University, Kingston, Canada). It arose in an 18 month old female retired breeder and was maintained in tissue culture as an adherent monolayer.

P815-X2, a methylcholanthrene-induced mastocytoma of DBA/2 origin was a kind gift from Dr. Robert Philips of the Ontario Cancer Institute. The tumor was maintained in the ascites form in DBA/2 males, and in suspension cultures in vitro.

YAC-1, a Moloney leukemia virus (MLV) induced lymphoma of A/Sn origin (5) was a kind gift of Dr. G. Dorval (Royal Victoria Hospital, Montreal, Quebec). Tumor cells were maintained in vitro in suspension cultures.

Maintenance of Tumor Lines in Vivo

MCA-induced tumors, which grew as solid tumors, were excised monthly and trypsinized using the procedure described by Holden et al. (6) with slight modifications. After excision, the tumors were cut into pieces of 1 - 2 mm^3. The pieces were washed once in Hank's Balanced Salt Solution (HBSS) and resuspended in 30 ml Medin Darby (MD) medium (7) containing

0.1% trypsin and a trace amount of DNase I.

The suspension was agitated gently with a magnetic stirrer bar for 20 minutes at room temperature. The supernatant containing the dispersed cells was collected into a tube containing FCS (final concentration of FCS in the trypsinized tumor suspension was 5%). Twenty ml of fresh trypsin solution were added to remaining tumor pieces for a second digestion period of 20 minutes. Dispersed cells were pooled, washed once, and resuspended in HBSS. Viable cells were enumerated using trypan blue, and 5×10^5 viable tumor cells were injected s.c. into each of two B6C3 F1 male recipients. The procedure reproducibly yielded a high proportion of viable cells (> 70%).

B-16 melanoma was passaged every two to three weeks. Single cell suspensions were prepared by teasing tumor chunks in HBSS and passing dispersed tissue through a stainless steel mesh filter. Cells were washed once with HBSS and 5×10^5 viable cells were injected s.c. into two C57BL/6 female recipients.

EL-4 and P815-X2 were maintained in their strain of origin in the ascites form. Ascites was collected weekly. The cells collected were washed twice in HBSS and $5 \times 10^5 - 10^6$ viable cells were injected i.p. into the respective hosts.

Maintenance of Tumor Lines in Vitro

All tumors were cultured in RPMI-1640 containing 1% Hepes buffer (1 M solution), 0.001% gentamicin sulfate and 10% FCS. This medium will be referred to as RPMI-FCS throughout this work. They were incubated at 37°C in a humidified 5% CO_2 atmosphere. Tumors EL-4, P815, and YAC-1 grew as suspension cultures and were fed with fresh medium three times weekly. Feeding normally consisted of the removal of 50 - 80% of the cell suspension and its replacement with fresh RPMI-FCS medium.

MCA-induced tumors and the B-16 melanoma, which grew as monolayers, were trypsinized twice weekly using a 0.25% solution of trypsin in MD-medium (7). Trypsinization was continued for 20 minutes at 37°C. Cells were centrifuged, resuspended in RPMI-FCS and 1 x 10^5 - 5 x 10^5 viable cells reinoculated into 2-3 Falcon, 25 cm^2 tissue culture flasks, containing 8 ml fresh medium.

For freezing, all tumors were suspended in ice cold RPMI-1640 containing 15% FCS and 10% DMSO and stored at -80°C, in an Ultra Low-Revco freezer.

Induction of tumors with 3-methylcholanthrene

Tumor induction was as described by Klein et al. (8). Seven week old B6C3 F1 male mice weighing 15 - 20 gm were injected i.m. in the right hind leg with 0.1 ml of Trioctanoin oil containing 0.5 mg of 3-methylcholanthrene. Tumors were palpable 50 or more days after injection, grew progressively, and resulted in 100% mortality 4 - 8 weeks after their appearance.

C. Comparative Studies of Tumor Growth in Immunosuppressed and Normal Mice

Tumor T-10: Primary tumor T-10 was adapted to tissue culture as previously described. Unless otherwise specified, cultured cells from the 29th *in vitro* passage and on, were used in this study.

Additionally, a T-10 line maintained *in vivo* (designated T-10-V) was used in some experiments. The tumor was excised and trypsinized as described above and maintained in culture for two weeks (3-4 passages) prior to injection into animals, in order to remove infiltrating host cells.

Primary tumors MCA-1, MCA-2, and MCA-3 were passaged *in vivo* for at least six generations before use in experiments. Cells from these passages were frozen. Tumors from subsequent passages were trypsinized and maintained in culture for two weeks prior to injection into animals. This was also the case when B-16 and EL-4 were used in experiments *in vivo*. Before injection, cultured cells were collected, washed twice, and resuspended in HBSS.

Unless otherwise stated, the desired dose was injected s.c. into the right hind leg.

Tumors were measured three times weekly with calipers. Mean tumor diameters for individual mice were calculated from measurements in two planes at right angles. Tumors were registered as positive only if their mean diameter was at least 0.3 cm. The mean tumor diameter for a group was computed by dividing the sum of tumor diameters by the number of tumor-bearing mice in the group.

Tumor metastasis: Pulmonary metastasis was evaluated using the method of Wexler (9). Immediately after removal from the animals, lungs were placed in Bouin's solution for 24 hours. They were then transferred to and kept in a 70% ethanol solution. The number and size of nodules were evaluated by two independent observers.

Elimination of ^{125}I-labelled Tumor Cells in Vivo

The procedure used was that described by Herberman et al. (10). Tumor cells in the exponential growth phase were incubated at 37°C for 3-4 hours in RPMI-FCS containing 2 µci/ml ^{125}IUDR and 10% 10^{-4}M FUDR (11).

Cells growing in suspension were collected, centrifuged for 10 minutes at 1,000 rpm in an International Clinical centrifuge, Model CL, and washed three times with fresh medium. Cells growing in monolayers were trypsinized with 0.25% trypsin in MD medium prior to washing. A suspension of tumor cells was prepared in HBSS and the desired tumor dose injected into normal recipients.

Mice were sacrificed 30 minutes or 6 hours after injection of the tumor. Spleens, lungs, and livers were collected, placed into a 10% Buffered Formalin solution, and the level of ^{125}I in these organs determined using a gamma counter, Model LKB.

The Hybrid Resistance Assay

The ability of lethally irradiated B6C3 F1 hybrids to accept or reject a parental bone marrow graft was tested using the assay system described by Bennet and Cudkowicz (13,14).

Six to eight week old F1 female mice which were either non-treated or inoculated with anti-IgM or NRS from birth, were lethally irradiated using a ^{60}cobalt unit and a dose of 925 rads. Four to six hours after the irradiation, mice were injected i.v. with bone marrow cells from either C57BL/6 or B6C3. The number of nucleated cells injected ranged from 1×10^6 to 1×10^7 viable cells in a volume of 0.5 ml HBSS. One control group was injected with 0.5 ml of HBSS only.

Five days after irradiation, mice were injected i.p. with 0.1 ml of a 10^{-6}M solution of FUDR in saline followed by a second i.p. injection, one hour later of 0.5 µci ^{125}IUDR (specific activity 5 mCi/mg) in 0.1 ml saline. Mice were sacrificed 8 hours later and their spleens removed and placed in a 70% ethanol solution. ^{125}IUDR uptake by the spleens was determined using a gamma counter model LKB.

Statistical Analysis

Unless otherwise stated the student t-test was used for analysis of the data.

RESULTS

3.A. Tumor Induction in Immunosuppressed and Normal Mice.

Tumor induction by 3-methylcholanthrene was studied in anti-IgM-treated and normal mice. Two experiments were performed. In the first-a preliminary experiment - tumor incidence in a group of 12 anti-IgM treated males was compared to that in a group of 8 NRS-treated, age, weight and sex matched mice. The resuls are shown in Figure 3.1. They suggest that tumors appeared in the immunosuppressed group later than in the control group.

In the second experiment, 22, 20 and 30, anti IgM-treated, NRS-treated and normal mice respectively, were injected with the carcinogen. Tumor measurements were initiated 35 days later and continued 3 times weekly until all animals in the study developed tumors.

The results, shown in Figure 3.2 are expressed as the probability of mice remaining tumor free at various intervals following the injection of 3-MCA. They confirm the preliminary observation and indicate that up to 84 days following the injection of the carcinogen, immunosuppressed mice had a significantly lower probability than their immunocompetent counterparts of developing tumors ($P < 0.01$). No difference in tumor incidence in the NRS-treated and non-treated groups was observed.

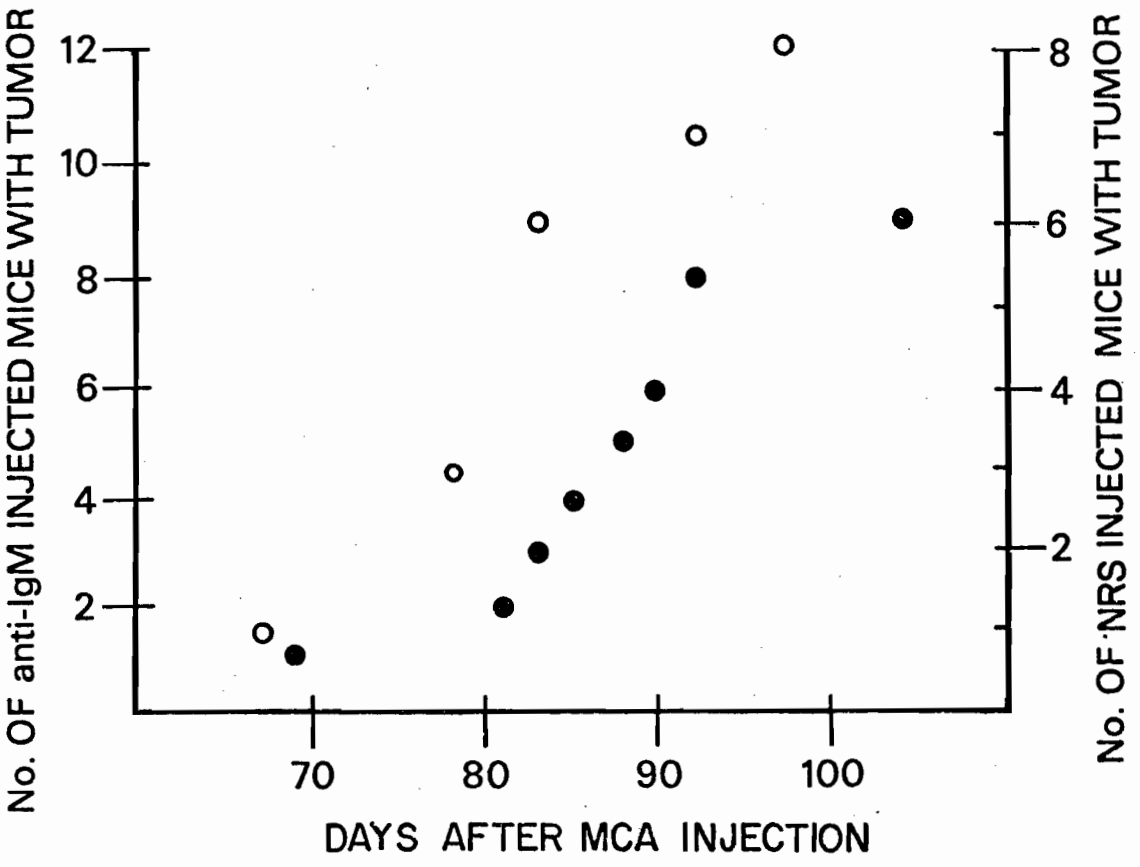
SUMMARY AND CONCLUSIONS

The results described demonstrate that immunosuppressed mice had a heightened resistance to primary, MCA-induced tumors. The statistical analysis of the results obtained in the first experiment was inconclusive ($0.10>P>0.05$) due probably to the small number of animals in the study. In the second experiment, therefore, larger groups were studied. The difference in tumor incidence in immunosuppressed and normal mice was highly significant in this experiment ($P<0.01$).

In the presentation of the data (Figure 3.2) as well as in their statistical analysis (both experiments), the life table approach normally applied to the study of survival rates was used (15, 16). Thus, the appearance of a tumor was marked as a death in the population, whereas the death of a tumor-free mouse was considered a withdrawl. Results were then tabulated accordingly and the cumulative probabilities of the mice to remain tumor free at different intervals was calculated.

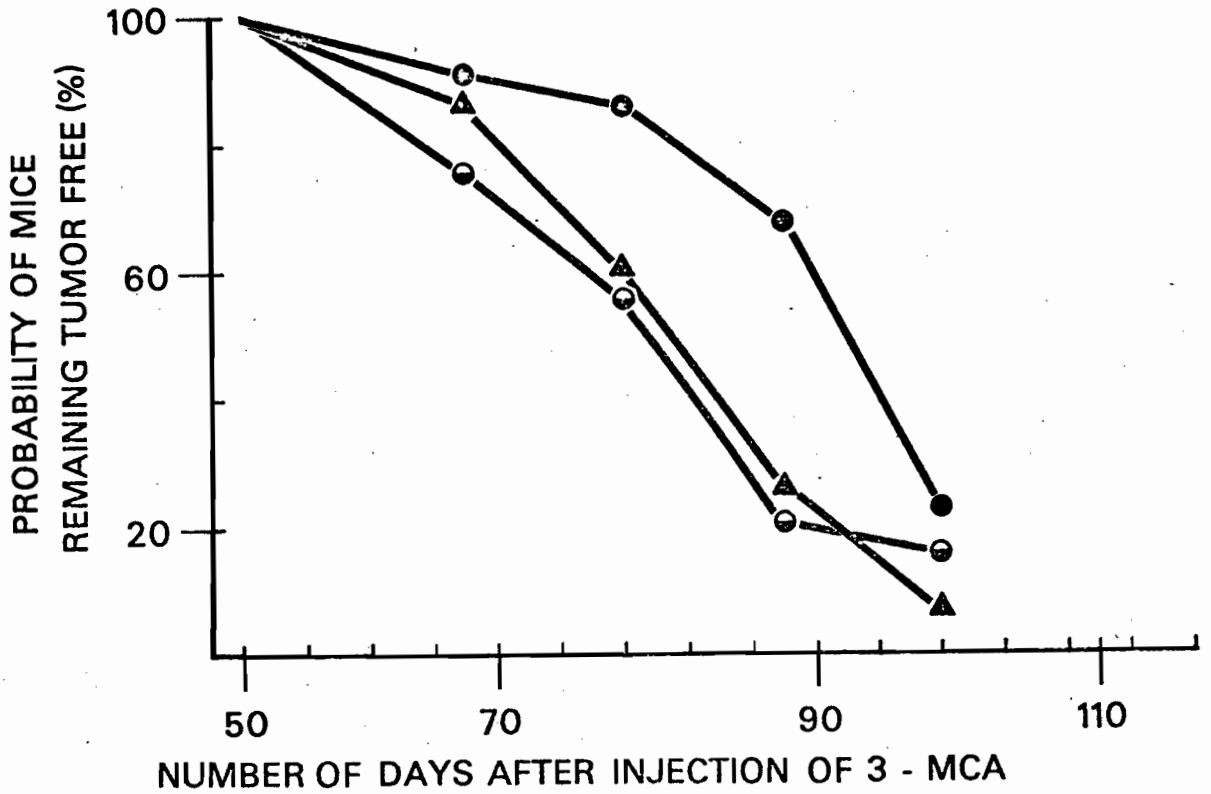
This approach facilitated the analysis of the data otherwise complicated by a higher death rate of both tumor-bearing and tumor-free immunosuppressed mice. The difference found in the survival rates was not surprising since it was comparable to that normally observed between immunosuppressed and normal mice of comparable ages. (mice were approximately 4 months old when palpable tumors were first detected). It is unlikely therefore that the higher death rate in the anti-IgM-treated group was due to differential effects of the carcinogen on the different study group.

FIGURE 3.1: THE EFFECT OF SUPPRESSION BY ANTI-IgM ON TUMOR INDUCTION BY 3-MCA. No.1



Twelve-7 week old, anti-IgM treated males (•) and 8 NRS-treated controls (o) were injected i.m. in the hind leg with 0.1 ml Trioctanoin oil containing 0.5 mg of 3-methylcholanthrene. The results are expressed as the number of mice in each group which bore a measurable tumor (0.3 cm or more) at the time intervals indicated. The two ordinates, representing the total number of mice in each group, have been scaled so as to indicate not only the number but also the relative proportion of tumor-bearing mice at each of the indicated intervals.

FIGURE 3.2: THE EFFECT OF SUPPRESSION BY ANTI-IgM ON TUMOR INDUCTION BY 3-METHYLCHOLANTHRENE No.2



Seven week old males of which 22 were treated with anti-IgM, (●——●) 20 were treated with NRS (○——○) and 30 were non-treated (▲——▲), were injected with 3-MCA as described in the legend to Figure 3.1.Their body weight at time of injection ranged from 15-20 gram. The cumulative probability of mice remaining tumor free was computed from % tumor incidence at each time point illustrated.

3.B. Tumor Transplantation in Immunosuppressed and Normal Mice.

3.B.1. Studies with the Tumor T-10.

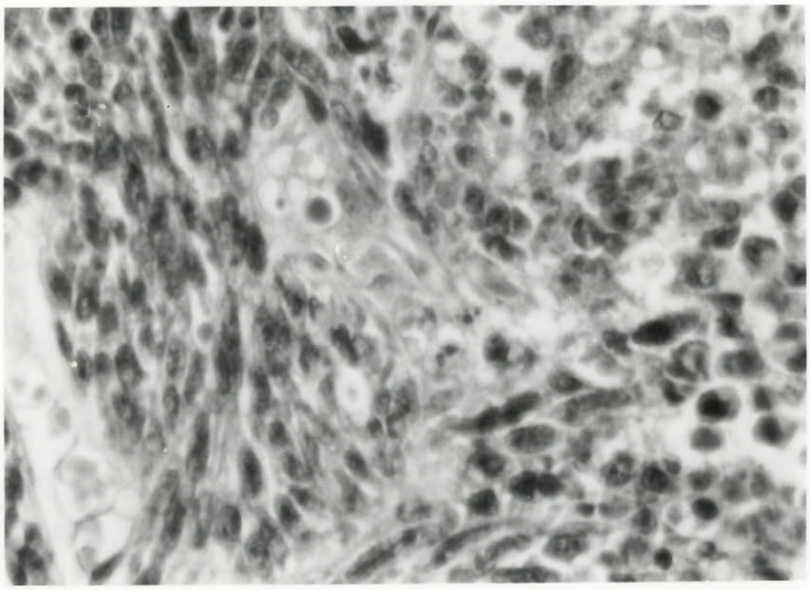
General patterns of tumor growth in vivo. Many of the experiments to be described in this chapter were performed with the MCA-induced tumor T-10. A cross section of the subcutaneous tumor and of a lung, removed from a tumor bearing mouse are shown in Figure 3.3 and 3.4 respectively. It can be seen that the tumor is a fibrosarcoma which can metastasize into the lung.

In all the experiments described, T-10 was injected s.c. in the hind leg. In normal animals a tumor inoculum of 1×10^5 cells was sufficient to yield a 100% tumor incidence. Tumors were palpable within 10 days following the injection of this dose and grew progressively, killing their host 30-50 days after the injection. Unless otherwise specified the T-10 line which was maintained in vitro (passages 29-129) was used in all experiments.

Comparative study of tumor growth in immunosuppressed and normal mice. Mice treated from birth with either anti-IgM or NRS were inoculated subcutaneously in the right hind leg with 1×10^6 tumor cells. Tumor diameters were measured until they reached 2.0 cm. As can be seen in Figure 3.5., the rate of tumor growth was significantly reduced in the suppressed mice ($P = 0.001 - 0.01$). Furthermore, a comparison of tumor incidence 17 and 26 days after tumor inoculation suggests that the tumors regressed in three of the anti-IgM-treated mice. No regression was observed in the control group. The tumor growth curve of a second control group that consisted of untreated mice was essentially identical to that of NRS-treated mice and is not shown.

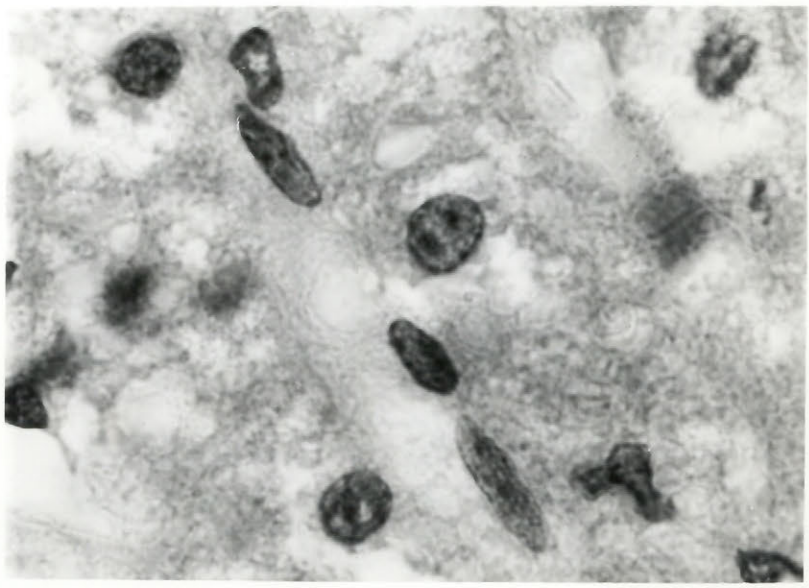
In the second experiment, suppressed and untreated mice were injected with 2.5×10^5 tumor cells. Results shown in Figure 3.6 confirm the initial observation and indicate that tumor growth was significantly ($P<0.001$) slower in the group treated with anti-IgM.

FIGURE 3.3: A CROSS-SECTION OF THE TUMOR T-10.



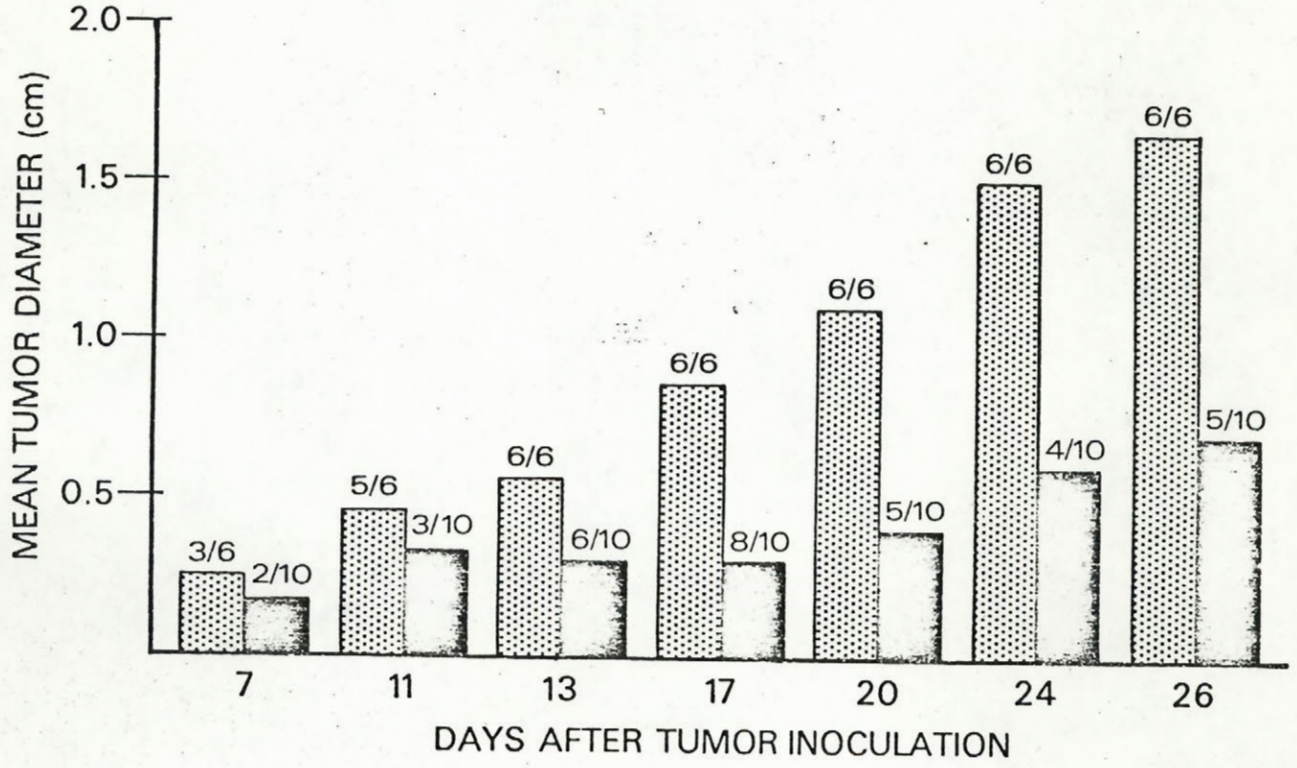
Cross-section was prepared of a tumor T-10 growing subcutaneously in the hind leg. Magnification was X1740.

FIGURE 3.4. A CROSS-SECTION OF A LUNG FROM A T-10 BEARING MOUSE.



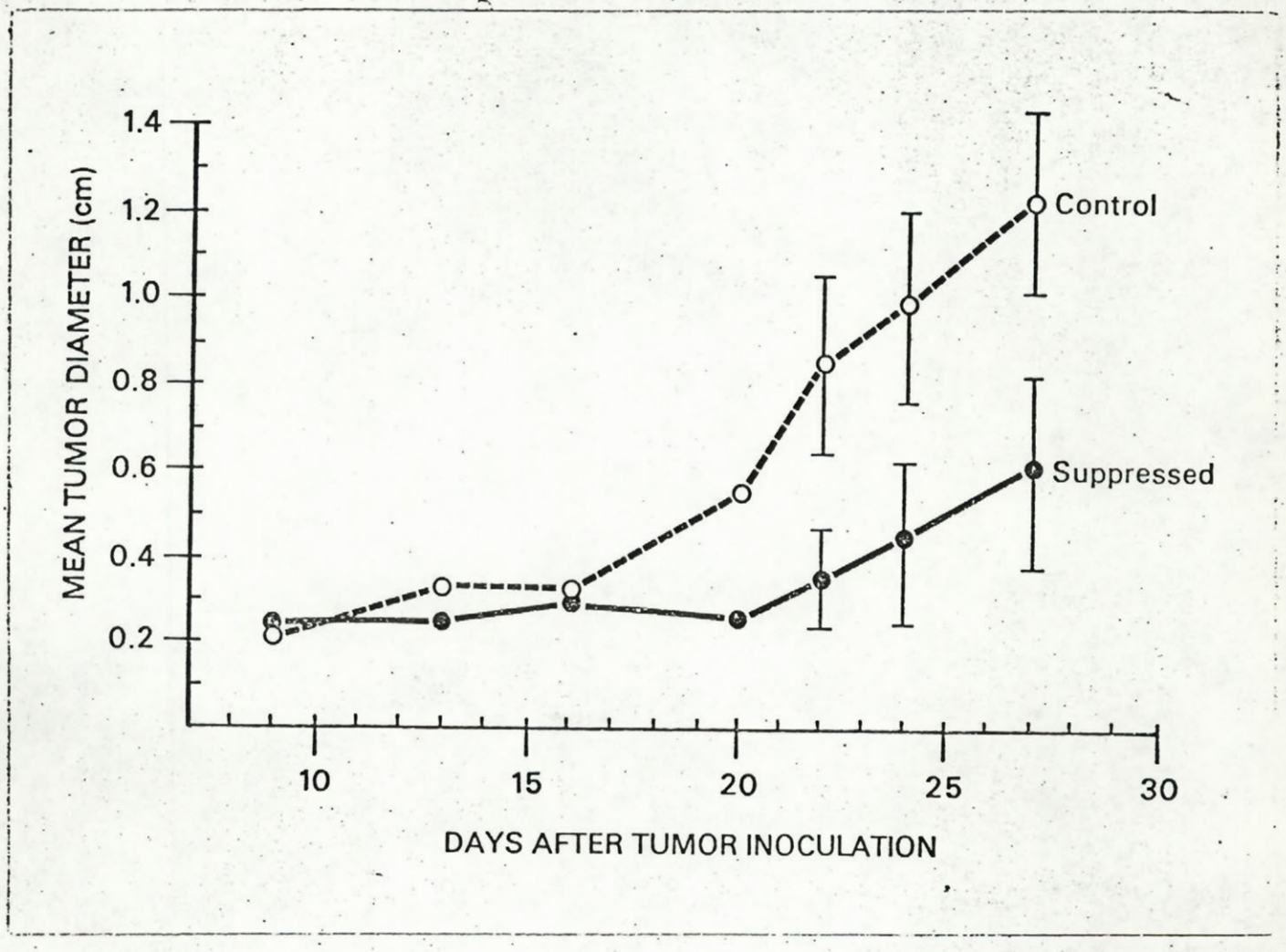
Section was prepared of a lung from a mouse bearing a s.c. T-10 tumor in the hind leg. Mean tumor size was 2 cm. Magnification was X1740.

FIGURE 3.5: THE RATE OF GROWTH OF T-10 IN IMMUNOSUPPRESSED AND IN NRS-TREATED MICE No.1



Seven week old male mice treated with either anti-IgM (solid bars) or NRS (dotted bars) were injected s.c. in the right hind leg with 1×10^6 T-10 cells. The numbers above each column indicate the number of mice with tumor over the total number of mice per group.

FIGURE 3.6: THE RATE OF GROWTH OF T-10 IN IMMUNOSUPPRESSED AND NORMAL MICE No.2



Nine-11 week old anti-IgM-treated male mice and ten 9-week old untreated controls were inoculated with 2.5×10^5 T-10 cells s.c. in the right hind leg. Bars indicate the S.D. of the mean.

The effect of anti-IgM injections on tumor growth: The following experiment was undertaken in an attempt to test the possibility of a direct cytotoxic effect on T-10 by rabbit anti-mouse IgM serum. Suppressed mice were divided into 2 groups of 13 mice each and serum injections were discontinued in one group. Seven days later, when anti-IgM could no longer be detected in the serum of mice from this group, all suppressed mice, as well as a third group of normal recipients, were injected s.c. with 5×10^5 T-10 cells.

The results, described in Figure 3.7, demonstrate that the reduction in the level of anti-IgM in the circulation did not affect the resistance of the mice to T-10. Thus, both tumor incidence and the rate of tumor growth were significantly lower ($P = 0.001 - 0.01$) in this group than in normal animals and were comparable to the results obtained with suppressed mice which were injected with anti-IgM throughout the experiment (not shown).

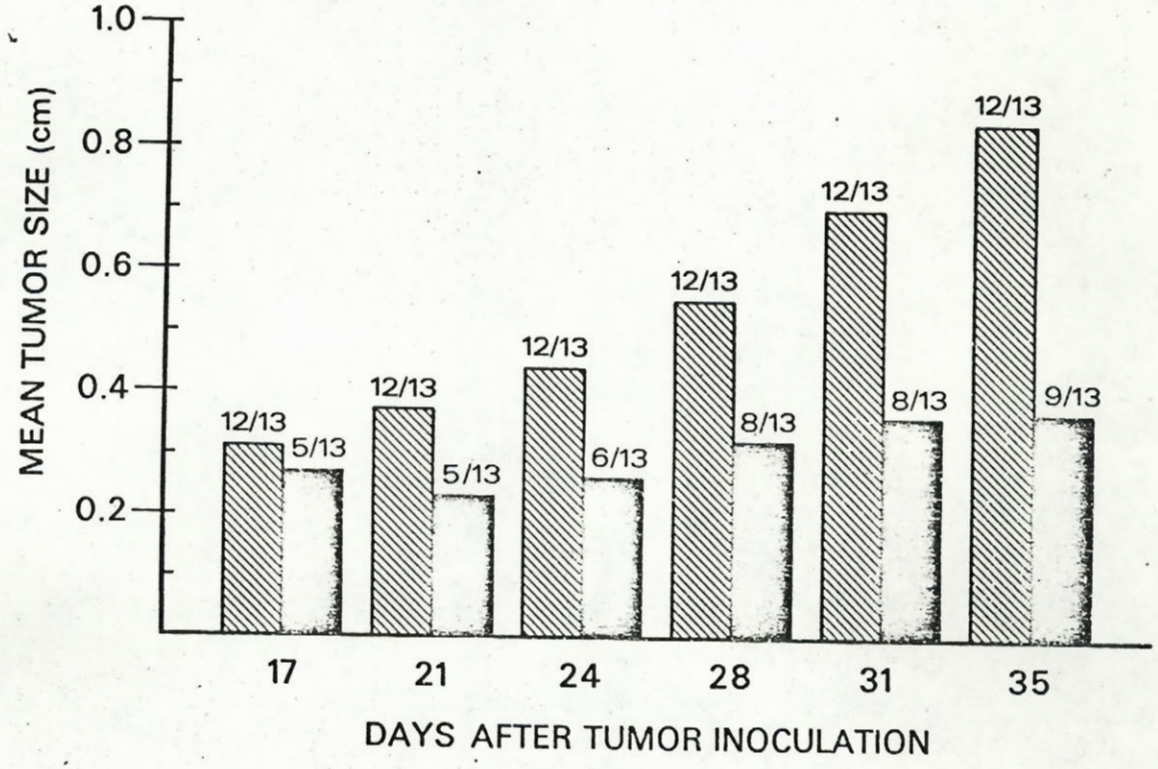
It should be noted that, despite the discontinuation of anti-IgM injections, the levels of circulating immunoglobulins remained suppressed in all the animals throughout the experiment.

Pulmonary metastasis: The effect of suppression by anti-IgM on the metastatic spread of T-10 was examined. Anti-IgM treated and normal male mice were inoculated with 1×10^6 T-10 cells. 34-36 days later the mice were sacrificed and their lungs examined for the presence of tumor nodules.

A typical tumor-infiltrated lung 24 hours after it was placed in Bouin's solution is shown in Figure 3.8. The results described in Table 3.1 demonstrate that the number of nodules detected in the lungs of immunosuppressed mice was significantly lower than that found in normal mice. ($P<0.05$). There was no significant difference in the mean size of individual nodules observed in the lungs of either of the study groups.

In a second experiment, carried out in female mice, 2.5×10^5 or 5×10^5 T-10 cells were injected s.c. into immunosuppressed and normal mice. Lungs were removed 21-24 days later and metastatic nodules counted.

FIGURE 3.7: THE EFFECT OF DISCONTINUATION OF ANTI-IgM INJECTIONS ON THE GROWTH OF T-10 IN SUPPRESSED MICE.



Twelve week old males treated with anti-IgM (solid bars) or non-treated (hatched bars) were injected with 5×10^5 T-10 cells. The injections of anti-IgM were discontinued 7 days before inoculation of the tumor. The numbers above each column indicate the number of mice with tumor over the total number of mice per group.

FIGURE 3.8. PULMONARY METASTASIS OF T-10

Four lobes of a tumor infiltrated lung derived from a mouse bearing a large s.c. T-10 tumor are shown in the right side of the figure. The lobes on the left were from uninjected controls.

Table 3.1. Effect of Suppression by Anti-IgM on Pulmonary Metastasis of T10[a]

Mice[b]	No. of Lungs Counted	Average No. of Nodules/Lung S.E.
Anti-IgM-treated	16	5.7 ± 1.65
Control	8	16.1 ± 5.28

[a]Lungs were removed 34-36 days after the s.c. inoculation of 1×10^6 tumor cells.

[b]Eight week old male mice were used.

The results, shown in Table 3.2, indicate that anti-IgM-treated mice had a significantly lower incidence of pulmonary metastasis ($P<0.05$). This suggests that the heightened resistance to metastatic spread, was not restricted to immunosuppressed males.

The subcutaneous growth of the tumor line T-10-V. The resistance of immunosuppressed mice to the tumor T-10 which was passaged in vivo (T-10-V) was studied in a subsequent experiment. Male mice were inoculated s.c. with 5×10^5 T-10-V cells. The results, shown in Figure 3.9, demonstrate that the rate of growth of the local tumor was significantly lower in the immunosuppressed mice ($P = 0.0005 - 0.005$), indicating that the heightened resistance exhibited by anti-IgM-treated mice was not restricted to the line of T-10 which was passaged in vitro.

The effect of the injection of serum from tumor-bearing mice on the growth of T-10: The effect of serum from tumor-bearing, immunosuppressed or normal mice on the growth rate of T-10 was studied in normal recipients. This was done in an effort to determine whether the serum of normal mice, contained (blocking) factors absent from suppressed mice, which could facilitate the growth of the tumor.

The sera collected from individual tumor-bearing mice were pooled and inoculated i.p. into normal recipients either prior to, or together with and following the s.c. injection of T-10. The different schedules are specificied in the legend to Figure 3.10.

The results shown in Figure 3.10. suggest that the sera pooled from normal tumor-bearing mice did not affect tumor growth differently than sera pooled from suppressed, tumor-bearing mice and that both caused a slight increase in the rate of tumor growth.

3.B.2. Studies with other Chemically Induced Tumors.

The following experiments were undertaken in an attempt to determine whether the heightened resistance to tumor T-10 observed in the suppressed mice was restricted to this tumor or whether it represented a broader phenomena of resistance to chemically induced tumors.

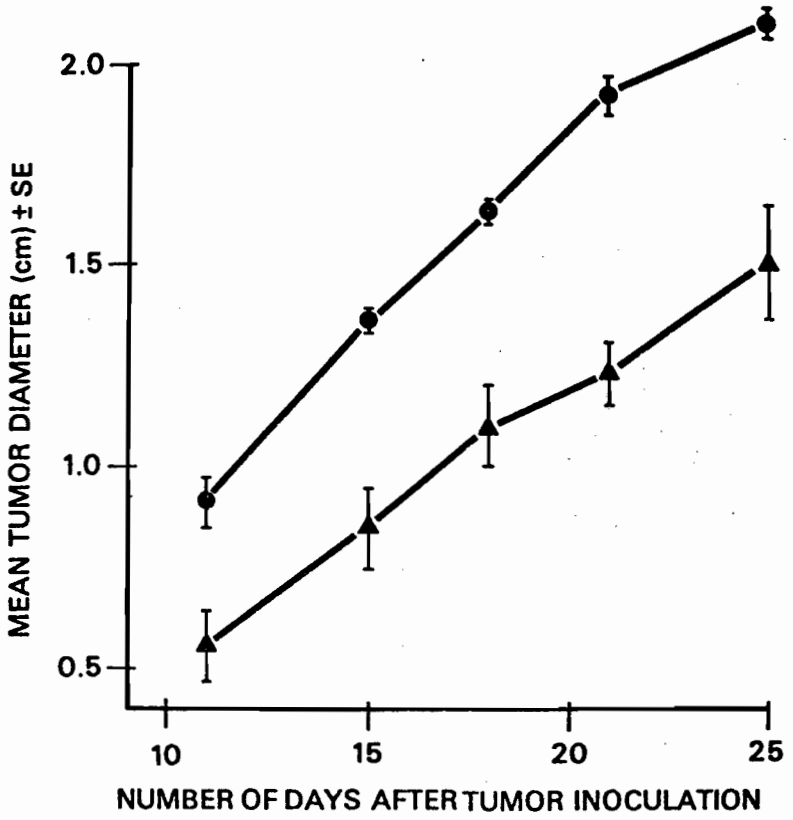
Table 3.2. Pulmonary Metastasis in Female Mice Injected with T-10-V[a]

Experiment No.	Animals	Lungs[b]	Number of Nodules/Lung ±S.E.	Mean Nodule Diameter (μ)	Mean s.c. Tumor Diameter
1	Anti-IgM-treated	7	5±2	70	1.8±0.8
	Normal	8	17.5±5	65	1.9±0.7
2	Anti-IgM-treated	7	6±1.0	52	1.94±.23
	Normal	10	17±4	73	1.95±.13

[a]15 weeks old anti-IgM-treated and normal female mice were injected s.c. with 2.5×10^5 T-10-V cell in experiment 1 and 5×10^5 T-10-V cells in experiment 2.

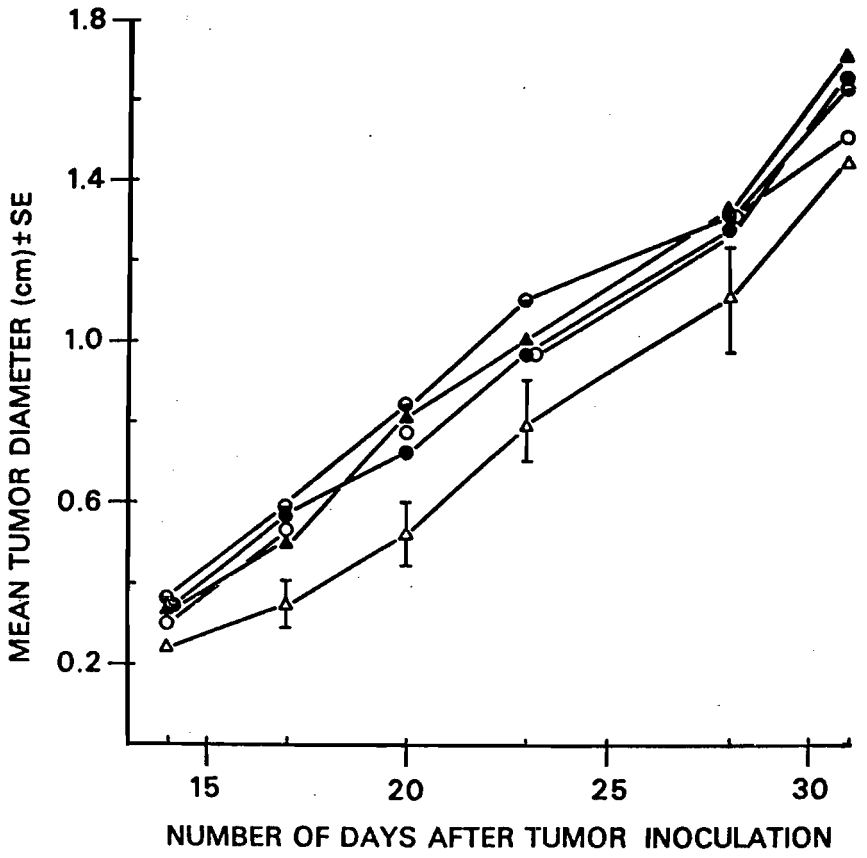
[b]Lungs were removed 31 days following tumor inoculation in experiment 1 and 34 days following tumor inoculation in experiment 2.

FIGURE 3.9: THE RATE OF GROWTH OF T-10-V IN ANTI-IgM AND NRS-TREATED MICE.



Seven week old males of which 10 were treated with anti-IgM (▲——▲) and 10 with NRS (●——●) were injected s.c. with 5×10^5 T-10-V (T-10 passaged *in vivo*) cells.

FIGURE 3.10: THE EFFECT OF THE INJECTION OF SERUM FROM TUMOR-BEARING MICE ON THE GROWTH OF T-10.



Eight week old males were divided into 5 groups of 10 mice each. One group (Δ——Δ) was injected s.c. with 2.5×10^5 T-10 cells. The other groups received the same dose of tumor cells in addition to 3 weekly i.p. injections of 0.1 ml of the following:

(▲——▲) serum from immunosuppressed tumor-bearing mice {Injections started one week prior to Injection of T-10 and continued for another week.

(o——o) serum from normal tumor-bearing mice

(●——●) serum from immunosuppressed tumor bearing mice Injections started 2 weeks prior to injection of T-10 and then discontinue

(●——●) serum from normal tumor bearing mice

General patterns of tumor growth in vivo. Tumors MCA-1, MCA-2 and MCA-3 which were induced in our laboratory were used in the study within 7-10 months of induction. Preliminary dose response studies, performed with the tumors, indicated that a dose of 1×10^5 cells, injected s.c. into the hind leg, was sufficient to give rise to local tumors in all the injected mice. Pulmonary metastasis was occasionally observed in mice injected with tumors MCA-1 and MCA-2. Macroscopic nodules could not be detected, however, when lungs of 25 animals bearing large MCA-3 tumors were examined. The chemically induced leukemia EL-4 was maintained in the ascites form. When injected s.c. a dose of 5×10^4 tumor cells resulted in 100% incidence. No metastatic growth could be observed in the lungs of mice bearing either a s.c. or an ascitic tumor.

Tumor Growth in Immunosuppressed and Normal Mice.

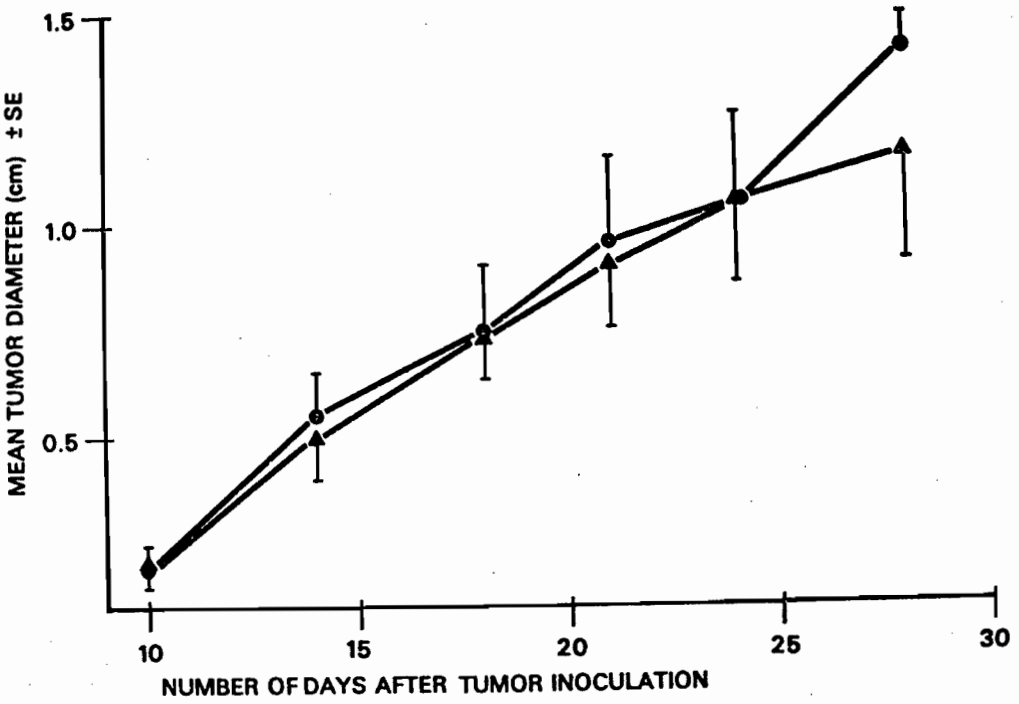
MCA-1: 5×10^5 MCA-1 cells were injected s.c. into 6 anti-IgM-treated and 6 NRS-treated mice. 5/6 suppressed mice and 6/6 control mice developed tumors.

The results shown in Figure 3.11 indicate that tumors in both groups progressed at a comparable rate. This rate did not significantly differ in a 3rd control group of 6 mice which were not injected with rabbit serum. (results not shown)

MCA-2: 6 suppressed and 6 NRS-treated mice were injected s.c. with 5×10^5 MCA-2 cells. 5/6 suppressed and 6/6 NRS-treated mice developed tumors.

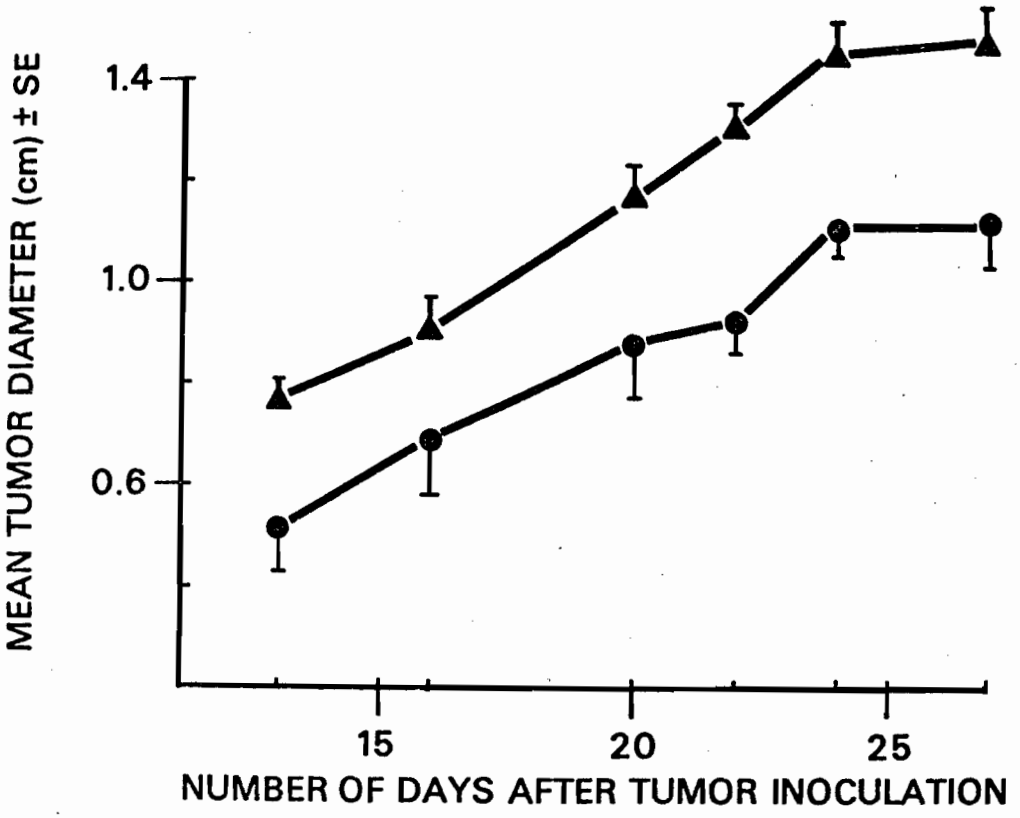
The results shown in Figure 3.12 demonstrate that tumors progressed at a significantly slower rate in the immunosuppressed group. (P was 0.002-0.05 from day 20 until the termination of the experiment). All mice were sacrificed on day 28 and lungs were examined. No metastatic growth could be detected in either of the study groups.

FIGURE 3.11: GROWTH RATE OF THE TUMOR MCA-1 IN IMMUNOSUPPRESSED AND NRS-TREATED MICE.



Eleven week old male mice, 6 treated with anti-IgM (●——●) and 6 with NRS (▲——▲) were injected s.c. with 5×10^5 MCA-1 cells. 5/6 suppressed mice and 6/6 controls developed tumors.

FIGURE 3.12: GROWTH RATE OF THE TUMOR MCA-2 IN IMMUNOSUPPRESSED AND NRS-TREATED MICE.



Thirteen week old males, 6 treated with anti-IgM (●——●) and 6 with NRS (▲——▲) were injected s.c. with 5×10^5 MCA-2 cells. 5/6 anti-IgM and 6/6 NRS-treated mice developed tumors.

MCA-3: 6 suppressed and 7 NRS-treated mice were injected with 5×10^5 MCA-3 cells. All mice developed tumors within 13 days of the injection.

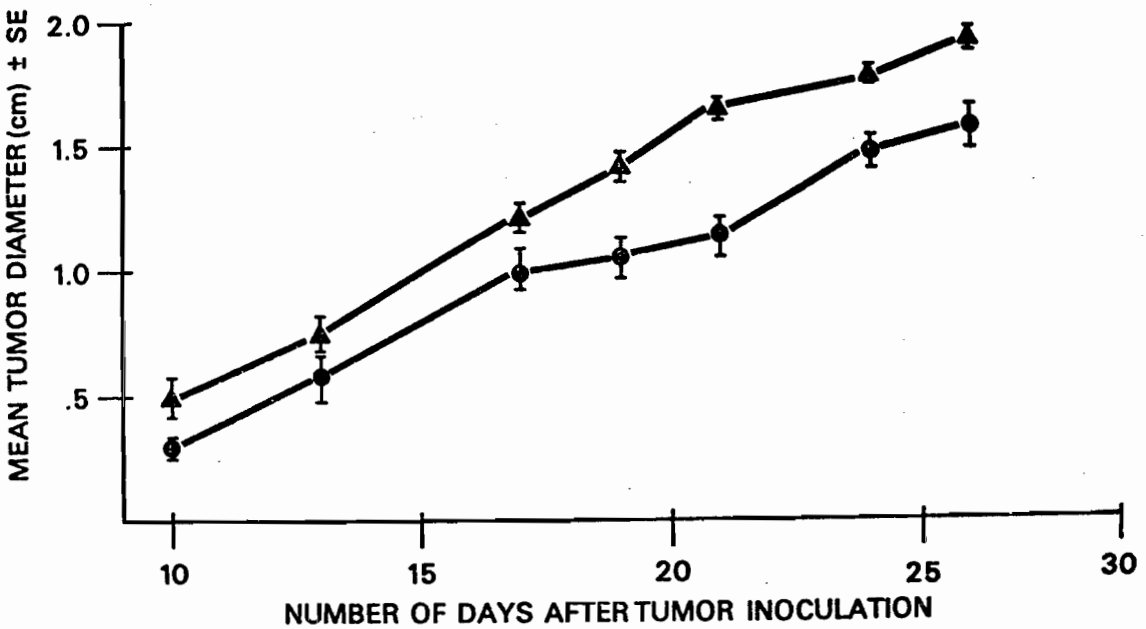
The rate of growth of the tumors in the different groups is illustrated in Figure 3.13. It can be seen that tumors of the immunosuppressed mice grew at a significantly slower rate than these of normal mice. (P was 0.0005 - 0.005 from day 19 until the end of the experiment). Mice were sacrificed on day 27 and their lungs removed and examined. No metastatic nodules could be detected.

In a second experiment 1×10^5 MCA-3 cells were injected into anti-IgM and NRS-treated mice. The results shown in Figure 3.14 support the initial observation of a heightened resistance to this tumor in the suppressed mice. It should be noted however that, using this dose, a significant difference in mean tumor diameter of the two groups could only be detected up to day 19 following tumor injection.

Leukemia EL-4: 5×10^5 EL-4 cells were injected s.c. into 6 anti-IgM and 6 NRS-treated female mice. Tumors were all measurable one week after the injection and grew rapidly, killing their hosts within 20 days. Results shown in Figure 3.15 indicate that there was no difference between suppressed and NRS-treated mice with respect to the rate of growth of their tumors. Similar results were obtained in a second experiment (not shown) in which suppressed and NRS-treated mice were injected with 5×10^4 EL-4 cells.

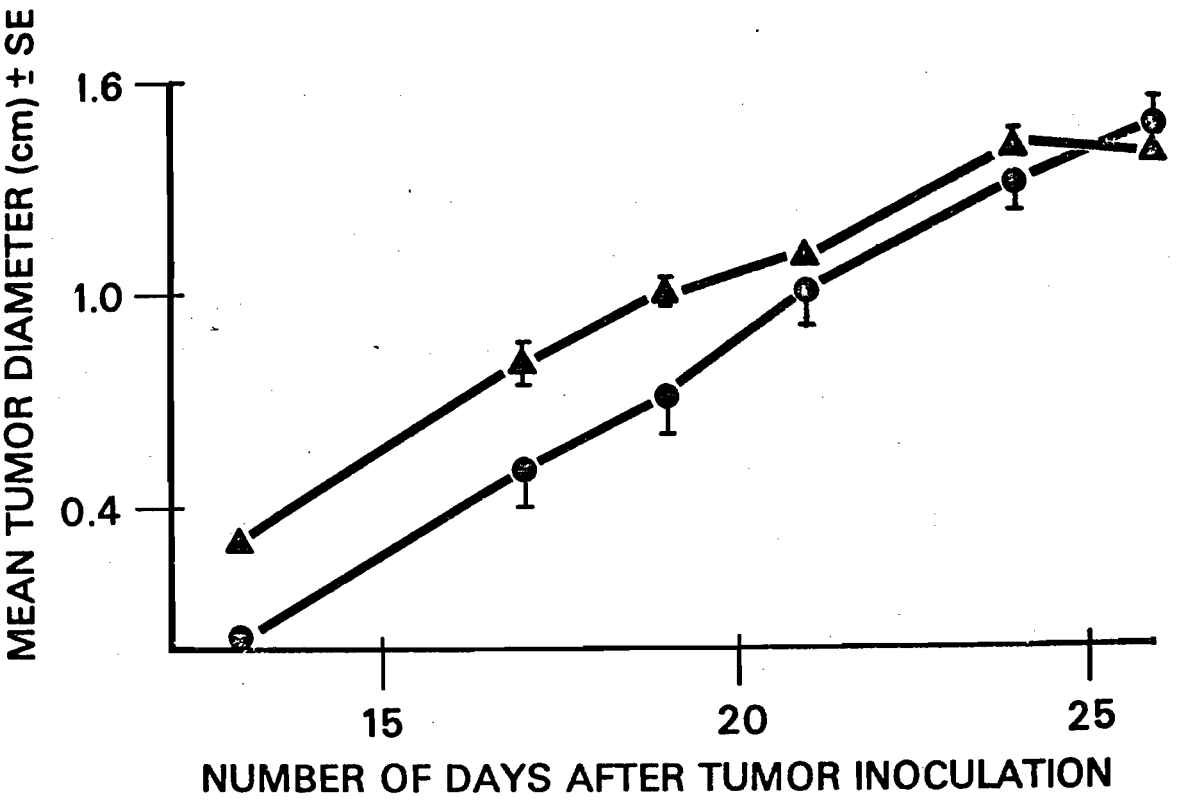
In a third experiment with the same tumor, 5×10^5 tumor cells were injected i.p. into 6 suppressed and 8 NRS-treated mice. An ascites eventually developed in all mice. Mice were palpated and their weights measured on alternate days. Judged on the basis of these measurements, tumor incidence in the control group was 100% within 10 days following tumor inoculation. Two of the suppressed mice developed tumors at approximately the same time, while the remaining 4 were tumor free until 4-7 days later. The difference in the latent period was also reflected in the survival rate

FIGURE 3.13: GROWTH RATE OF THE TUMOR MCA-3 IN IMMUNOSUPPRESSED AND NRS-TREATED MICE No.1.



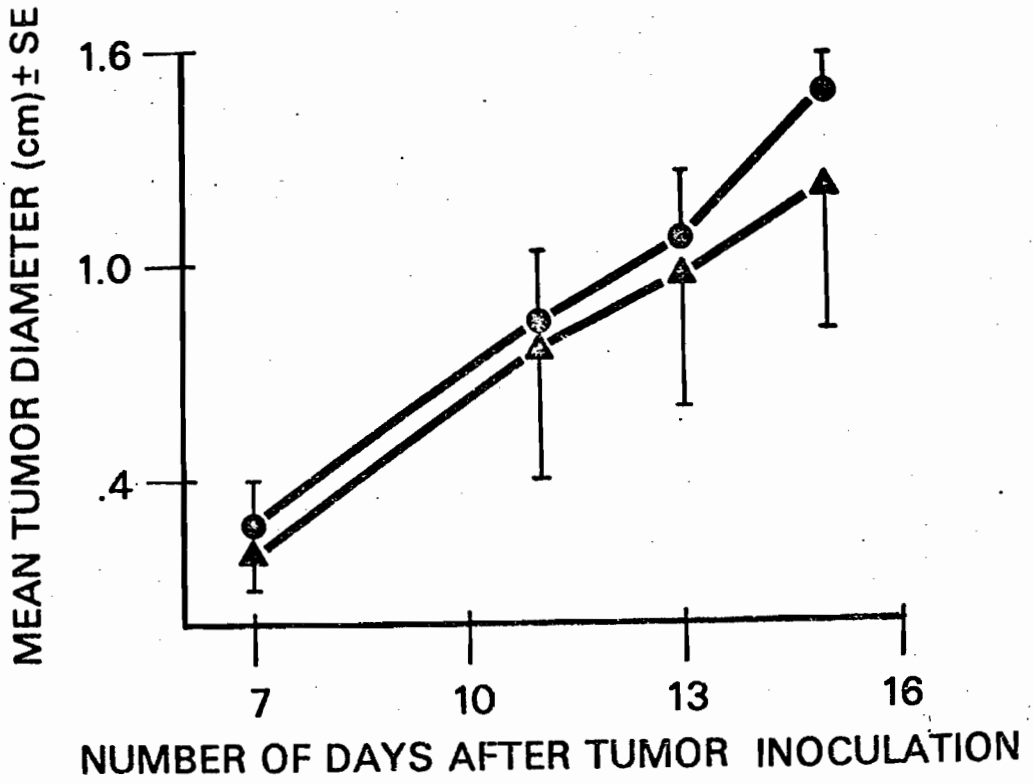
Thirteen week old males, 6 treated with anti-IgM (●——●) and 6 with NRS (▲——▲) were injected s.c. with 5 x 10^5 MCA-3 cells. All the mice developed tumors by day 13.

FIGURE 3.14: GROWTH RATE OF THE TUMOR MCA-3 IN IMMUNOSUPPRESSED AND NRS-TREATED MICE No.2



Thirteen week old males, 4 treated with anti-IgM (●——●) and 7 with NRS (▲——▲) were injected s.c. with 1 x 10^5 MCA-3 cells. All the mice developed tumors. They were palpable by day 13 in the group treated with NRS and by day 17 in the group treated with anti-IgM.

FIGURE 3.15: GROWTH RATE OF EL-4 IN IMMUNOSUPPRESSED AND NRS-TREATED MICE.



Nine week old females, 6 treated with anti-IgM (●——●) and 6 with NRS (▲——▲) were injected with 5 x 10^5 EL-4 cells. All tumors were palpable by day 11.

of tumor-bearing mice, as demonstrated in Table 3.3. It can be seen that suppressed mice had a significantly longer ($P<0.01$) mean survival time than their NRS-treated counterparts.

Rapid elimination of radiolabelled EL-4 from the circulation of immunosuppressed mice: The following experiment was undertaken in an effort to elucidate the mechanism which increases the resistance of immunosuppressed mice to intraperitoneally injected EL-4.

7 suppressed and 8 NRS-treated mice were injected i.v. with 7.5×10^5 ^{125}IUDR-labelled EL-4 cells. 30 minutes and 6 hours later, 3 or 4 mice of each group were sacrificied and the level of ^{125}I in their spleens, livers and lungs determined.

Results are shown in Table 3.4. They indicate that between the first and the sixth hour following the injection of the tumor a marked reduction occured in the levels of ^{125}I detectable in the various organs. This reduction however, was significantly higher ($P = 0.005 - 0.025$) in the organs removed from immunosuppressed mice, suggesting a faster elimination of tumor cells from the circulation of these mice.

3C. The Hybrid Resistance of the Immunocompetent and Immunosuppressed Mice.

The increased rate of elimination of EL-4 cells from the circulation of suppressed mice raised the possibility that this elimination was mediated by the so called "hybrid resistance" mechanism of rejection of parental grafts (13), and that this mechanism was superior in B-lymphocyte depleted animals. To test this possibility, lethally irradiated, immunosuppressed and control B6C3F_1 mice were injected with BM cells from either the parental strain C57BL/6 or the syngeneic F_1 strain. Repopulation of the spleens was ascertained 5 days later by the i.v. injection of the DNA-seeking isotope ^{125}IUDR followed by the removal, several hours later, of the spleens and count of their ^{125}I uptake.

The results are shown in Table 3.5. They indicate that B-lymphocyte-deprived mice had a greatly increased resistance to the parental BM. The results obtained with BM derived from the F_1 strain indicate that the spleens of the immunosuppressed mice could support the homing and proliferation of syngeneic hemopoietic cells as effeciently as spleens of the immunocompetent controls.

Table 3.3 . Survival of Anti-IgM and NRS-treated Mice Following the Intraperitoneal Injection of EL-4 Cells

	Treatment of Mice	
	NRS	Anti-IgM
Survival of individual mice (days)	13,14,15,15,18,18,18,18	18,18,20,20,24,27
Mean survival time (days) ±S.E.	16±2.1	21±3.6

[a] 9 week old females were injected with 5×10^5 EL-4 cells

Table 3.4. The Recovery of ^{125}IUDR in Different Organs After the Injection of Radiolabelled EL-4 Cells into Anti-IgM and NRS-Treated Mice

Time After Tumor Injection	% of the Total Injected Isotope ±S.E. Recovered In:					
	Spleens		Lungs		Livers	
	30 minutes	6 hours	30 minutes	6 hours	30 minutes	6 hours
NRS-treated mice	0.8±0.1	0.47±0.1	37±7	1.1±0.4	11±1.6	1.4±0.2
Anti-IgM-treated mice	0.27±0.04	0.27±0.04	21±6	0.27±0.05	14±1	0.8±0.01

10-16 week old females were injected i.v. with 7.5 x 10^5 ^{125}IUDR-labelled EL-4 cells.

TABLE 3.5. THE RESISTANCE OF B-LYMPHOCYTE DEPRIVED AND CONTROL-F_1 MICE TO PARENTAL MARROW GRAFTS.

Recipients	Source and dose of marrow cells injected				
	C57BL/6		$B6C3F_1$		Nil
	5×10^6	10×10^6	1×10^6	5×10^6	
Anti IgM-treated $B6C3F_1$	357 ± 92	400 ± 83	6089 ± 878	9352 ± 512	128 ± 11
NRS-treated B6C3F1	2660 455	4436 571	N.D.	N.D.	N.D.
Non-treated $B6C3F_1$	1832 ± 414	3849 ± 915	5095 ± 653	11,083 ± 1645	529 ± 82
Non-treated C57BL/6	5490 ± 412	7579 ± 639	N.D.	N.D.	197 ± 23

LEGEND TO TABLE 3.5.

B6C3F_1 and C57BL/6 female recipients were lethally irradiated using a ^{60}Co-unit and a dose of 925 rads. They were then injected i.v. with either parental (C57BL/6) or F_1 (control) bone marrow cells as indicated. Five days later, all the recipients were injected i.p. with 0.1 ml of a 10^{-6}M solution of FUDR followed 1 hour later by 0.5µci of ^{125}IUDR. Eight hours later spleens were removed and their ^{125}IUDR-uptake monitored in a gamma-counter. Results are expressed as the mean c.p.m. ± SE of ^{125}IUDR in the spleens removed from 5 animals in each category.

SUMMARY

Tumor incidence, progression and metastasis were studied in anti-IgM and NRS-treated mice, using 4 MCA-induced sarcomas and the chemically induced leukemia EL-4. The following observations were made:

1. The immunosuppressed mice had a heightened resistance to 3 out of 4 sarcomas tested, namely to tumors T-10, MCA-2 and MCA-3. This heightened resistance was manifest in either one or more of the parameters studied, namely in a decreased tumor incidence, a slower progression of local tumors or, where applicable, a reduction in tumor metastasis.

When injected with 5×10^5 tumor cells a characteristic profile of tumor growth was obtained with all three tumors. Thus, tumors in both anti-IgM and NRS-treated mice appeared at approximately the same time and their size, in the two groups, was not significantly different during the first week of tumor growth. However, thereafter tumors of NRS-treated (or normal) mice grew at a significantly faster rate resulting in the characteristic Y shaped curves (see Figure 3.6 and 3.12). The possible mechanisms responsible for the different modes of tumor progression will be discussed later in light of the results described in chapter 4.

It is interesting to note in this context that in one experiment, when an inoculum of 5×10^4 cells of tumor MCA-3 was injected (Figure 3.13) these kinetics of tumor growth were not observed. Thus a difference in mean tumor size of suppressed and normal mice, which was initially observed, could no longer be detected in the later phases of the experiment.

Of all tumors tested only T-10 metastasized readily to the lung. When pulmonary metastasis of this tumor was analyzed, it was consistantly more limited in the lungs of suppressed mice, even in an experiment where no difference was found in the rate of growth of the local tumors. These results suggest that a common host protective mechanism may be operating in the inhibition of both the growth and the dissemination of a local T-10 tumor.

This heightened resistance to tumor T-10 was not sex restricted and could be demonstrated with the tumor lines which were carried either *in vivo* or *in vitro*.

2. The chemically induced tumor MCA-1 grew equally well in the suppressed and the NRS-treated or non-treated mice. Similarly leukemia EL-4 when injected s.c. gave rise to local tumors which progressed at comparable rates in suppressed and normal mice.

3. When tumor EL-4 was inoculated i.p., a marked difference was observed between tumor development in the study groups, which resulted in a significantly higher survival rate of immunosuppressed mice. It should be noted that, unlike the syngeneic MCA-induced tumors, EL-4 is a semisyngeneic tumor derived from the parental strain C57BL/6.It is possible that the observed resistance to the tumor in the peritoneum was due to a mechanism which operates preferentially in this anatomic site and which is directed against parental determinants. Clearly one possibility to be considered is a hybrid resistance mechanism similar to the one demonstrated in hybrid mice against their parental BM. The results obtained with ^{125}I-labelled EL-4 cells which were injected i.v. support the notion of a spontaneous rapid mechanism of tumor elimination, which is operative in both normal and immunosuppressed mice, but is superior in the latter. Furthermore hybrid resistance studies carried out with immunosuppressed, NRS-treated and non-treated lethally irradiated mice, confirmed that the suppressed mice can reject a parental hemopoietic graft more effeciently than the immunocompetent controls.

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CHAPTER 4

IN VITRO STUDIES

INTRODUCTORY COMMENTS:

The general aim of the experiments described in this chapter was to gain an understanding of the mechanism(s) responsible for the heightened resistance to tumors observed in the immunosuppressed mice. To this end an *in vitro* study of cell mediated immune mechanisms in normal and suppressed mice was undertaken.

The first part of the chapter describes the lytic activity of effector cells from normal and suppressed mice against ^{125}IUDR-labelled tumor cells. The assays were designed to compare the specific cytotoxic activity which can be induced in these mice by tumor inoculation and to characterize the killer cell activity observed. This assay was selected on the basis of preliminary experiments. In these experiments, killer cells could easily be detected by this method in the spleens of DBA/2 mice, following their inoculation i.p. with the (allogeneic) tumor T-10. The assay gave reproducible results, low levels of spontaneous isotope release, and a high consistancy among duplicate samples.

In the second part of the chapter the spontaneous cytotoxic reactivity of spleen cells from immunosuppressed and normal mice against the allogeneic tumor YAC and other targets was compared, by the widely used ^{51}C-release assay.

MATERIALS AND METHODS

A. The ^{125}IUDR-Release Assay of Cell-Mediated Cytotoxicity

Labelling of target cells 5×10^5 cultured tumor cells in 5 ml RPMI containing 20% FCS, were seeded in tissue culture dishes (Falcon, 60 x 15 mm) one day before labelling and incubated at 37°C in a humid, 5% CO_2 atmosphere. On the day of assay, 2 µci/ml ^{125}IUDR and 10% FUDR 10^{-4}M were added to the cells and incubation was continued for four hours (1). Cells were then trypsinized as already described (p.86) and were washed three times in RPMI-FCS before use.

Preparation of lymphocytes Spleen, lymph node or peritoneal cells were used as the source of killer cells. Spleens were removed aseptically and the cells were released by repeated flushing with RPMI over a stainless steel mesh filter using a 25 g x 5/8 inch needle. The remaining cells were released by gentle teasing of the spleen over the filter. Cells were washed once by a ten minute centrifugation at 1,000 rpm in an IEC PR-6000 centrifuge and the pellet was treated for five minutes at 37°C with ACK (0.155 M NH_4Cl, 0.1 mM Na_2 EDTA, 0.01 M $KHCO_3$ in distilled water) to remove red blood cells (2). The treatment was stopped by dilution of the cells in cold RPMI-FCS followed by two washes. The number of viable cells was determined by a trypan blue exclusion count and the cell concentration was adjusted by the addition of RPMI with 20% FCS. Unless otherwise stated, spleen suspensions used were a pool of cells from 2-4 spleens.

Lymph nodes were placed on a mesh filter and gently teased to release lymphocytes. Cells were washed once and resuspended in RPMI with 20% FCS before assay.

Peritoneal cells were collected as described by Takasugi and Klein (3). 0.2 ml of mineral oil was injected i.p. into test mice 2-3 days prior to assay to increase the number of macrophages and lymphocytes in the peritoneum. Mice were sacrificed and their skin dissected and pulled back to expose the abdominal wall. Four ml RPMI were injected into the peritoneal cavity and the fluid manipulated to get a good suspension of cells in medium. Cells were collected by aspiration with a Pasteur pipette through the side of the abdomen. Clotting was prevented by the addition of 100 units of heparin to the suspension. Cells were washed twice and resuspended in RMPI and 20% FCS.

Cytotoxicity assay The ^{125}IUDR-release assay was always carried out in RPMI with 20% FCS. 5×10^3 labelled tumor cells in 0.1 ml medium were seeded in flat-bottom wells of a Falcon Microtest II tissue culture plate. Lymphocytes in 0.1 ml medium were added at varying effector: target cell (E:T) ratios. Each effector (E) and target (T) combination was plated in quadruplicate. Mixtures were incubated at 37°C in a humid 5% CO_2 incubator on a rocker platform. Rocking was carried out for 16 hours at 7 cycles/minute. It was then stopped and incubation continued for two more hours. 0.05 ml aliquots of the supernatants were then collected and ^{125}I counted in a gamma counter model LKB.

specific release was calculated as:

$$\frac{^{125}\text{I released into the supernatant - spontaneous release}}{\text{Maximum release - spontaneous release}}$$

Spontaneous release was calculated from wells containing target cells and medium only. It normally ranged from 10-20% of the maximum release. Addition of non-labelled (cold) target cells to the wells did not alter the level of spontaneous release. Maximum release was determined by treating target cells with 0.1% SDS.

B. The ^{51}Cr-release Assay of Cell-Mediated Cytotoxicity

Labelling of target cells Cultures of YAC-1 were always fed with fresh medium one day before labelling (for feeding procedure see p. 87). 5×10^6 cells were harvested on the day of assay and centrifuged for 10 minutes at 1,000 rpm in an IEC International Clinical centrifuge. The pellet was resuspended in 0.2 ml of a 100 µci/ml $Na_2{}^{51}CrO_4$ solution in saline. One hour incubation at 37°C in a Dubnoff metabolic shaking incubator followed. Cells were then washed three times in RPMI with 5% FCS and adjusted to a concentration of 10^5 viable cells/ml. In all assays, viability of target cells exceeded 90%.

Preparation of effector cells BM cells were collected from left and right femur bones by flushing the bones with 0.5 ml HBSS using a 5 g x 5/8 inch needle. Clumps were dispersed by repeated flushing and the suspension was passed through a stainless steel mesh. The cells were washed once. The nucleated cells were counted and, after a second wash, the volume of the suspension was adjusted to contain the desired number of cells. Spleen cells were prepared as already described. The assay medium was RPMI-FCS.

Cytotoxicity assay Effector cells in 0.1 ml medium were added to flat-bottom microtitre plates and serial dilutions were made to give a range of 2×10^5 - 3.2×10^6 effector cells/well. 1×10^4 viable target cells in 0.1 ml medium were then added to each well. Effector and target cells were incubated for 4-5 hours at 37°C in a humid 5% CO_2 incubator. Mixtures were agitated at 7 cycles/minute on a rocker platform. At the end of the incubation, the plates were centrifuged in an IEC model PR-2 centrifuge for 10 minutes at 1,000 rpm to pellet the cells. Aliquots of 0.05 ml of the supernatants were collected from each well and a count of ^{51}Cr taken. Each effector and target cell combination was assayed in triplicate and the mean and standard deviation of each combination calculated. The cytotoxic reactivity of effector cells was expressed either as % specific release or in lytic units.

Specific release was calculated as:

$$\frac{^{51}Cr \text{ released into the supernatant - spontaneous release}}{\text{total radioactivity - spontaneous release}}$$

The spontaneous release of ^{51}Cr was that detected in wells containing labelled target cells and medium only. It normally ranged between 5 - 10% of the total radioactivity incorporated. Total radioactivity was determined from 0.1 ml aliquots of the target cell suspension.

A lytic unit was defined as the number of effector cells required to yield a specific release of 20% of the total isotope incorporated.

C. Characterization of the Killer Cell

Characterization of the killer cell was based on fractionation procedures aimed at the selective removal of a specific cell population from the heterogenous effector cell preparation. An increase or decrease in the ability of the fractionated cells to lyse target cells was then indicative of the relevance of the removed population to the lytic reaction.

Antibody and Complement-Mediated Lysis of θ-Bearing Lymphocytes. Rabbit anti-mouse T-cell sera were purchased initially from Litton Bionetics, and later from Cedarlane Lab. The sera were tested before use for cytotoxicity with rabbit complement and the optimal serum dilution determined.

The rabbit complement used was pretested and batches were selected on the basis of their relatively low toxicity to spleen cells. Where stated, the complement was absorbed twice before use, with spleen cells of B6C3 F1 mice. Each absorption was for 30 minutes at $4^{o}C$, with packed spleen cells collected from five 8-week old mice.

For the lysis of θ-bearing cells, 50×10^{6} spleen cells from normal or anti-IgM-treated mice were centrifuged and the pellets were resuspended in 1 ml of RPMI containing 5% FCS and a 1:10 dilution of the antiserum. Incubation was for 45 minutes at room temperature. Cells were washed once before

the addition to the pellet of a 1:10 dilution of (rabbit low tox.) complement in 1 ml RPMI + 5% FCS. Cells were suspended and incubated at 37°C for 30 minutes. They were then washed three times before use in assay. Non-treated cells, or cells incubated with complement only, were used as controls.

Rabbit Anti-Ig Serum and Complement Mediated Lysis of Ig-Bearing Cells.

Class-specific rabbit anti-mouse immunoglobulin sera were prepared in our laboratory, in the following manner: specific precipitates in agar were prepared using normal mouse sera with class-specific goat anti-mouse immunoglobulins (Meloy Labs). The precipitates were washed and injected s.c. into rabbits together with Freund's Complete Adjuvant. Three injections were given at two week intervals. Seven weeks after the first injection, rabbits were boosted by an i.v. injection of 0.5 ml of the purified corresponding immunoglobulin (Litton Bionetics). The rabbits were bled one week later. The serum preparations were precipitated twice with ammonium sulfate, first at 50% and then at 33% saturation. They were then dialyzed against saline, centrifuged (100,000 g, 30 minutes, Beckman Model L Ultracentrifuge) and stored at -80°C.

The different antisera were pooled and heated (30 minutes at 56°C) in order to inactivate complement. They were then absorbed twice with an equal volume of packed C57BL/6 liver cells using a 30 minute incubation at room temperature . This was followed by absorption with a 1:10 volume of C57BL/6 thymus cells (4). After each absorption, the cells were spun down for 10 minutes at 4,000 rpm at 4°C in an IEC PR-6000 centrifuge. The absorbed sera were stored at -80°C in an Ultra Cool Revco freezer. A Commercial rabbit anti-mouse Ig serum purchased from Cedarlane Laboratories was similarly absorbed. The specificity of the antiserum was determined using a complement mediated cytotoxicity assay. Serum preparations which were not cytotoxic to spleen cells of anti IgM-treated mice or to normal thymus cells, but lysed 40-50% of normal spleen cells,were used for the selective removal of Ig-bearing lymphocytes.

The procedure used for the removal of Ig-bearing cells from spleen suspensions was essentially as described above for the complement-mediated removal of θ-bearing lymphocytes

Removal of Nylon Wool Adherent Cells

Preparation of the wool. Nylon wool from LP-1 leukopak leukocytes filter was removed and washed as described by Julius et al. (5). The wool was soaked for a week at 37°C in a beaker containing doubly distilled water. (Beakers were presoaked in normal saline for two hours at 37°C and rinsed two times in glass distilled water before use). Three changes of water were made during the week. The nylon was then wrung out and dried for two days in a 37°C incubator. 1.2 gm of nylon wool was then packed into 12 ml of a 20 ml plastic syringe, wrapped in paper, and sterilized.

Cell fractionation (6). Nylon wool was saturated with PBS containing 10% FCS (PBS-FCS). Syringes were plugged with a rubber stopper, placed in a 5% CO_2 incubator and incubated for one hour at 37°C. 1 - 2 x 10^8 spleen cells in PBS-FCS were then layered on the column. Cells were allowed to sink, were overlayed with 5 ml PBS-FCS, and were incubated at 37°C for 45 minutes. Non-adherent cells were eluted with 40 ml PBS-FCS, washed once, and resuspended in RPMI-FCS. Adherent cells were recovered by teasing the wool with sterile forceps in a glass petri dish containing PBS-FCS.

Removal of Phagocytic Cells by Carbonyl Iron and Magnet (7).

3 x 10^6 spleen cells in 3 ml RPMI-FCS were mixed with 0.4 gm carbonyl iron particles, which had been presoaked in 70% ethanol and washed in RPMI. The mixture was agitated at 37°C for 35 minutes.

The bulk of the iron particles was removed with a large magnet. Cells were then passed through a 20 ml plastic syringe to which small magnet bars were attached, for the removal of the remaining particles (8). Control cells were incubated without iron particles and similarly treated. An analysis of the ability of the depleted population to phagocytose latex particles was always carried out.

Removal of Fc-Receptor-Bearing Cells

Fc-receptor-bearing cells were removed on SRBC (sheep red blood cells) anti SRBC monolayers as described by Kedar et al. (9).

Preparation of SRBC anti-SRBC monolayers. Tissue culture dishes were incubated for one hour at room temperature with 3 ml of a 50 µg/ml solution of poly-L-lysine (PLL) in PBS. They were rinsed by repeated flushing with PBS.

SRBC were washed three times with PBS before use. A 1.5% suspension of packed cells in PBS was then prepared and 3 ml layered on PLL-treated dishes. Plates were incubated for one hour at room temperature. Non-adhering RBC were removed by repeated gentle flushing with PBS. Plates with a confluent homogenous monolayer were selected for the assay.

For fixation, plates were further incubated for 10 minutes with 0.2% glutaraldehyde in PBS and then thoroughly washed and incubated for another 10 minutes with 0.1 M glycine in PBS. Both incubations were at room temperature. Monolayers were again washed, covered with sterile PBS, and stored until use.

Non-fixed monolayers were used within 24 hours of preparation, whereas fixed monolayers were kept at $4^{\circ}C$ for up to three days, before use.

Binding of serum to monolayers and cell fractionation. A hyperimmune anti-SRBC serum prepared in Swiss mice was a kind gift of Dr.R. Murgita (Dept. of Microbiology and Immunology, McGill University, Montreal, Quebec). It was heat inactivated (30 min at $56^{\circ}C$) and its hemaglutinin titre was determined (the serum was positive in a dilution of 1:2048). Heat inactivated normal mouse serum absorbed with SRBC was used in control monolayers.

On the day of the assay, the monolayers were rinsed with PBS and overlayed with 3 ml of a 1:50 dilution of either anti-SRBC serum or control serum. After 45 minutes at $37^{\circ}C$, the serum was decanted and the monolayers were washed with PBS. Plates were then incubated for 20 minutes at room temperature with 3 ml MEM with 10% FCS (MEM-FCS).

2×10^7 spleen cells in 2 ml MEM-FCS were added to each monolayer. The plates were incubated at $37^{\circ}C$ for 30 minutes in a 5% CO_2 incubator on a rocker platform (5 cycles/minute) and then for an additional 30 minutes without rocking. Supernatants were collected and monolayers repeatedly

rinsed with MEM-FCS to remove non-adherent cells. The cells collected were pooled, washed once, resuspended in RPMI-FCS, and used in the subsequent assays.

Release of the cells adherent to the monolayer. Fc-receptor-bearing cells were released from non-fixed monolayers by either one of the following methods:

1. Monolayers were covered with ACK medium and incubated for 2 minutes at room temperature in order to lyse red blood cells.
2. Plates were incubated at 37°C on a rocker platform with 2 ml of a 36 µg/ml Protein A solution in PBS (10) for either 1 or 2 hours.

The recovered cells were washed twice before their resuspension in RPMI-FCS and use.

D. Identification of the Fractionated Spleen Cell Populations.

Stimulation of cultured cells with Concanavalin A (11). Spleen cells were incubated in flat-bottom wells of micro test II tissue culture plates at a concentration of 2×10^6 cells/0.1 ml. The medium was RPMI supplemented with 10% FCS (batch pretested for optimal stimulation of spleen cells), 2 mM glutamine, 1% Hepes, and 0.001% gentamicin. 1 ml of a 0.75 µg/ml solution of Con A in the same medium was added for stimulation. Control wells received medium only. Plates were incubated at 37°C in a 5% CO_2 humid incubator for 48 hours at which time 2 µCi ^3H Thymidine in 0.1 ml RPMI were added to each well for a further incubation of 18 hours. The reaction was stopped by freezing the plates.

For determination of ^3H Thymidine uptake, plates were thawed and cells harvested onto glass fibre filters with a Mash II cell harvester and continuous flushing with water. The cell extracts on glass fibre filters were dried, the filters were placed in a mixture of toluene and Solimix I and the isotope was counted using a Packard liquid scintillation counter.

Analysis of phagocytic cells by the latex particles uptake assay (12) 5×10^6 cells in RPMI-FCS were incubated with $1 - 2 \times 10^9$ latex particles at 37°C for 45 minutes. The mixture was agitated in a Dubnoff metabolic shaking incubator. Free latex particles were removed by low speed (500-600 rpm) centrifugations (IEC International centrifuge) of cells suspended in RPMI. A cell count was taken in trypan blue and the percentage of viable cells to which latex particles were attached was recorded.

Surface immunolabelling of B and T lymphocytes. The method used to label cell surface determinants of B and T-lymphocytes was that described by Lala et al. (13). 2×10^6 spleen cells were incubated at 4°C for 30 minutes with either 1:20 dilution of rabbit anti-mouse IgM serum (Litton Bionetics) or 1:10 dilution of rabbit anti-mouse brain serum (a kind gift of Dr. A. Ahmed, Bethesda, Maryland). The cells were then layered on a discontinuous FCS gradient (50, 75, and 100% FCS in MEM) and centrifuged for seven minutes at 400 g (Sorvall GLC-2,) at 4°C. Cells in the pellet were resuspended in MEM and layered on a second FCS gradient for an additional wash.

Cell pellets were then resuspended in 0.1 ml MEM and incubated for 30 minutes at 4°C with 0.1 ml of ^{125}I-labelled protein A (40 µCi/ml). The final concentration of protein A in the mixture was 1 µg/ml. (Iodinated protein A was a kind gift from Dr. G. Osmond and Associates, Dept. of Anatomy, McGill University, Montreal, Quebec). Two more washes through discontinuous FCS gradients followed to remove free protein A molecules. Pellets were resuspended in MEM and the cell suspensions layered on 100% FCS in a 6 x 50 mm glass tube, for a final four minute centrifugation at 1,800 rpm (Clay Adams Safety Head), the cells were resuspended in minute amounts of FCS and smeared on gelatin-coated microscope slides. Fixation was for four minutes in absolute methanol. Slides were

processed for radioautography as described by Kopriwa et al. (14).

After a three day exposure, the slides were stained with McNeal tetrachrome and the number of silver grains overlaying the cells was counted under oil immersion. Only cells with six or more overlaying particles were considered positive.

Cold Target Inhibition Assay for Killer Cell Specificity. The assay used in this study was a modification of the one described by Koren et al. (15). To assay inhibition of labelled target cell lysis by tumors growing in suspension, several concentrations of the latter in 0.05 ml RPMI-FCS were seeded into microtiter wells. To each well & 10^5 spleen cells in 0.05 ml medium, were added and the mixtures were incubated at 37°C for 30 minutes. To study inhibition by adherent cells, they were seeded into the wells and incubated at 37°C for either 4 or 18 hours prior to the addition of spleen cells, to allow the regeneration of surface antigens after trypsinization (16). As was the case with suspension tumors, adherent cells were incubated with spleen cells for 30 minutes. This incubation was followed in both assays by the addition to each well of 0.1 ml RPMI-FCS containing 10^4-labelled target cells. The incubation was then continued for 5 additional hours and the specific release determined as previously described.

% inhibition was calculated as:

$$\frac{\text{Specific release without inhibitor} - \text{specific release in the presence of inhibitor}}{\text{specific release without inhibitor}}$$

Only "cold" target cells which gave a dose-dependent inhibition of isotope release were considered to have specifically competed for the killer cells.

The Effect of Preincubation of Spleen Cells with Serum on their Ability to Lyse ^{51}Cr-Labelled YAC. The effect of serum from anti-IgM or NRS inoculated mice, as well as that of anti-IgM serum and NRS on spleen cell cytoxicity

to ^{51}Cr-labelled YAC was ascertained. Sera in different dilutions were either added to the effector and target mixtures for the duration of the cytotoxicity assay, or added to the effector cell preparation for a one hour incubation at 37°C prior to the addition of target cells. In the latter procedure, serum was removed by three washes of effector cells in RPMI-FCS, after wich they were seeded into the wells, and mixed with the labelled target cells. The ^{51}Cr-release assay then proceeded as already described.

RESULTS

4.A. Killer Cell Activity Measured by an ^{125}IUDR-Release Assay.

4.A.1. The Cytotoxic Response

Cytotoxicity of effector cells from normal mice to the tumor T-10. In order to analyse the cytotoxic reactivity which can be induced in normal mice by the growing tumor T-10, spleen, lymph node, and peritoneal exudate cells were removed from mice at various intervals following the s.c. injection of 2.5×10^5 T-10 cells. The assay was designed to test and compare cytotoxic cells prior to the appearance of a local tumor and during its growth.

The results shown in table 4.1 indicate that at no time following the injection of tumor cells was a significant cytotoxic activity detectable in either the spleens or the regional (inguinal) lymph nodes of the host mice. A high cytotoxic activity was demonstrable using peritoneal exudate cells of mice which either were or were not injected with the tumor.

Cytotoxic activity of spleen cells from normal and immunosuppressed mice following the inoculation of T-10. The anti-tumor reactivity of splenocytes from suppressed and normal mice was compared at various time intervals following the s.c. injection of T-10 cells.

Results shown in table 4.2 demonstrate that spleen cells from immunosuppressed mice were significantly more cytotoxic to ^{125}IUDR-labelled T-10 than spleen cells from normal mice ($P = 0.0005 - 0.05$) at every interval assayed except day 3.

Repeated assays with spleen cells from suppressed, normal or NRS-treated, tumor bearing mice have consistantly confirmed this observation. The results of a representative assay are illustrated in Figure 4.1. They demonstrate, again, that a significantly higher level ($P = 0.0025 - 0.025$ at E:T ratio of 100:1 - 400:1) of specific isotope release could be obtained with spleen cells of immunosuppressed mice. They further indicate that the specific release increased proportionally to the concentration of effector cells, giving a linear dose response curve at effector:target ratios of 50:1 to 200:1.

Table 4.1. Cytotoxicity of Effector Cells From Normal Mice Following Inoculation of Tumor T10[a]. (Part 1)

Number of Days After Tumor Inoculation[b]	Spleen Cells From:[c] Tumor Inoculated		Spleen Cells From:[c] Non-inoculated		Regional Lymph Node Cells From: Tumor Inoculated		Regional Lymph Node Cells From: Non-inoculated		Maximum Release Spontaneous Release (cpm)±SE
	100:1[e]	200:1	100:1	200:1	100:1	200:1	100:1	200:1	
3	5	11[d]	5	7	3	ND	0	0	19,870±98 2,230±49
6	3	4	4	7	1	2	1	2	10,717±272 552±22
10	3	3	3	4	1	2	1		13,058±230 580±26
13	1	7	6	12	0	1	2		2,414±43 292±10
17	4	8	2	3	0		0		9,697±138 450±17
26	1	3	1	4	0		0		25,428±590 622±34

[a]The results are expressed as % specific release of ^{125}IUDR following an 18 hour incubation of effector and target cells at the specified ratios.

[b]7 week old males were injected s.c. with 2.5 x 10^5 T10 cells.

[c]Harvesting of all effector cells was as described in Materials and Methods.

[d]S.E. of quadruplicates exceeded 5% of the mean in only 3 of all tests performed.

[e]Effector:target cell ratio.

Table 4.1. Cytotoxicity of Peritoneal Exudate Cells From Normal Mice Following Inoculation of Tumor T-10[a.b]. (Part 2).

Number of Days After Tumor Inoculation[b]	Peritoneal Exudate Cells From: Tumor Inoculated 200:1	Peritoneal Exudate Cells From: Non-inoculated 200:1	Maximum Release Spontaneous Release (cpm)±SE
6	44	42	10,717±272 552±22
10	21	26	13,058±230 580±26
13	60	60	2,414±43 292±10
17	21	16	9,697±138 450±17

[a]The results are expressed as % specific release of ^{125}IUDR following an 18 hour incubation of effector and target cells at the specified ratios.

[b]For details of the experiments see legend to Table 4.1 part 1.

Table 4.2. Cytotoxicity of Spleen Cells from Suppressed and Normal Mice Following the Injection of Tumor T-10[a]

Mice	Number of days after tumor inoculation[b]				
	3	10	15	22	29
Anti-IgM treated, tumor-bearing	1	9	16	10	10
Normal, tumor-bearing	5	1	7	2	4
Mean tumor diameter[c] (cm)	-	0.2	0.6	tumor regressed	0.75
Uninjected controls	1	1	9	4	3
maximum release (cpm)	40,925±2,276	16,747±430	7,339±124	21,841±682	23,947±398
spontaneous release (cpm)	648±27	1,193±42	1,327±78	1,895±43	3,487±104

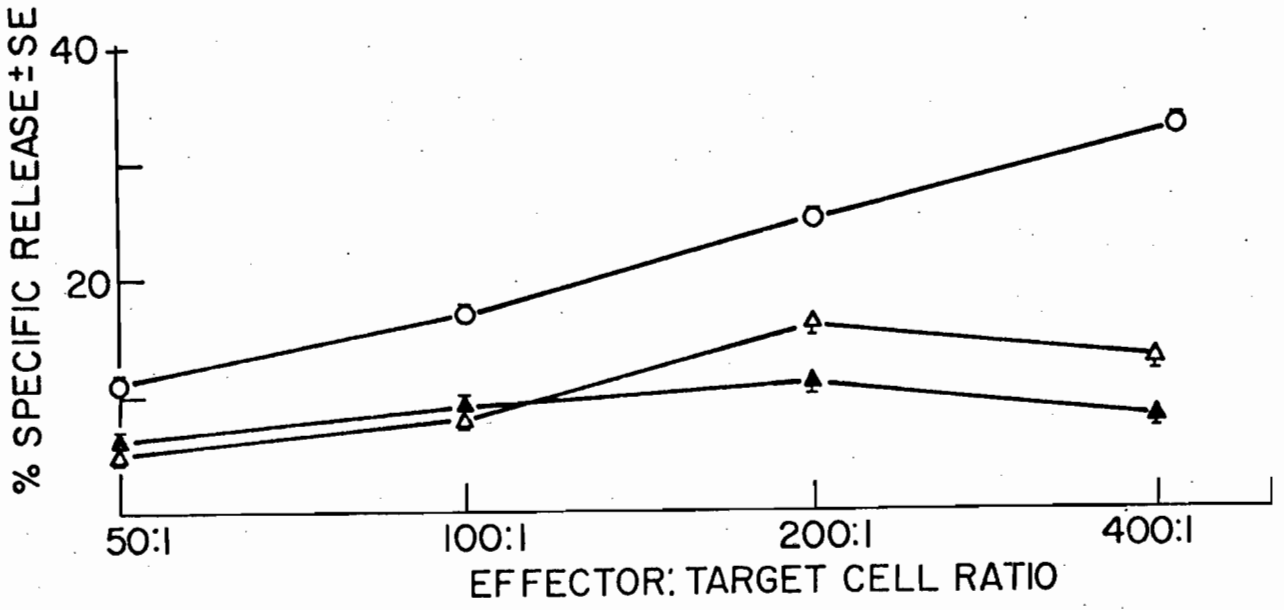
[a]Results expressed as % specific release of ^{125}IUDR following a 16-48 hour incubation of spleen and T-10 target cells at a ratio of 500:1. The S.E. of the quadruplicate samples did not exceed 5% of the mean.

[b]Animals were injected s.c. with 5 x 10^5 T-10 cells

[c]The assays were carried out with pools of spleen cells derived from 3 mice of each group. The mean tumor diamter recorded refers both to normal and anti-IgM-treated mice. The individual mice were selected at each point of the study to have tumors of a comparable size

[d]The S.E. of the quadruplicate samples did not exceed 5% of the mean,

FIGURE 4.1: CYTOTOXICITY OF SPLEEN CELLS FROM SUPPRESSED AND NORMALS MICE TO THE TUMOR T-10.



Six weeks old suppressed (o——o) and normal (Δ——Δ) mice were injected s.c. with 1 x 10^6 T-10 cells. Seven days later 3 mice of each group were sacrificed their spleens pooled and assayed. Spleens of a third group of normal mice which were not injected with the tumor, were also assayed. (▲——▲)

The level of ^{125}IUDR release following a prolonged incubation period: The effect of an increase in the incubation period given to effector and target cells mixtures, on the levels of isotope release was studied.

Results shown in table 4.3 demonstrate that close to maximal levels of release (95%) could be obtained with splenocytes of immunosuppressed mice if incubation was allowed to proceed for 40 hours. The extended incubation however, failed to increase the level of release caused by spleens of normal, tumor-inoculated mice, above the level which was obtained using splenocytes of normal, non inoculated animals.

It should be noted that spontaneous release of ^{125}IUDR increased from 9% of the total label after 18 hours to 25% following a 40 hour incubation. For this reason subsequent assays were usually restricted to incubation periods of 16-20 hours.

Cytotoxic reactivity of regional lymph node cells. The cytotoxicity of inguinal lymph node cells from suppressed and normal mice injected in the hind leg with T-10 was studied. Cytotoxicity of spleen cells derived from the same donors was assayed simultaneously.

Results shown in table 4.4 are representative of results obtained on several occasions. They demonstrate that the lymph nodes of both suppressed and normal mice had no detectable cytotoxicity against T-10, at a time when such a reactivity was displayed by spleen cells.

Note that, in agreement with the data described above, the lysis obtained with splenocytes of suppressed mice was significantly higher than that obtained with spleen cells of normal mice ($P < 0.0005$).

The specificity of the cytotoxic reaction. The specificity of the cytotoxic response detected in the spleens of suppressed mice following the inoculation of T-10 was studied using the B-16 melanoma and the C3H/HeJ mammary adenocarcinoma as non specific targets.

The results shown in table 4.5 are representative of several assays performed with B-16, and those shown in table 4.6 describe the cytotoxic

Table 4.3. Effect of Prolonged Incubation of the Effector and Target Cell Mixture on the Level of ^{125}IUDR Release[a]

Animals	Incubation Period	
	16 hours	40 hours
Anti-IgM treated, tumor-bearing[b]	12	95
Normal, tumor-bearing	6	38
Uninjected controls	5	40

Maximum release: 9987 ± 180 (cpm)

Spontaneous release: 16 hr: 902 ± 14 (cpm)
40 hr: 2439 ± 73 (cpm)

[a]Results are expressed as % specific release of ^{125}IUDR following 16 and 40 hour incubation of spleen and T-10 target cells at a ratio of 250:1. S.E. ranged from 1-9% of the mean.

[b]The assay was carried out 7 days following a s.c. injection of 1×10^6 T-10 cells.

Table 4.4. Comparison of Cytotoxicity to T10 of Spleen and Regional Lymph Node Cells of mice injected with T-10[a]

Mice	Source of Effector Cells	
	Spleen[b]	Regional lymph nodes
Anti-IgM-treated,[b] injected with T10	830±96 [a]	200±14
Normal, injected[b] with T10	380±28	288±23
Uninjected controls	330±42	180±17

Maximum release: 3,610±622

Spontaneous release: 303±34

[a]The results are expressed as the specific release of ^{125}IUDR in cpm after an 18 hour incubation of spleen and target cells at a ratio of 100:1. This form of presentation was chosen because of the very low counts detected in the supernatants.

[b]The assay was carried out with tissues from mice bearing small tumors, 30 days after the s.c. injection of 1 x 10^6 T-10 cells.

Table 4.5. The Cytotoxicity of Spleen Cells to the B-16 Melanoma Following the Injection of Tumor T-10[a]

Mice	Spleen Cells	
	Non-treated	Treated with anti-θ and complement
Anti-IgM treated, injected with T-10[b]	15	55
Normal, injected with T-10	3	9
Non-injected controls	1	2

Maximum release: 16,580 ± 144 (cpm)

Spontaneous release: 3,184 ± 90 (cpm)

[a]Results are expressed as % specific release of ^{125}IUDR after an 18 hour incubation of spleen and B-16 melanoma cells at a ratio of 250:1. S.E. was 0.3-5% of the mean.

[b]The assay was carried out 9 days after the s.c. injection of 1×10^6 T-10 cells.

Table 4.6. Cytotoxicity of Spleen Cells to Tumor C_3H/HeJ After the Injection of T-10[a]

Mice	Number of Days After Tumor Inoculation				
	3	10	15	29	33
Anti-IgM-treated tumor-bearing	7	20	45	52	32
Normal, tumor-bearing	9	0	0	0	0
Uninjected controls	0	0	1	0	0
Maximum release (cpm)	8,251±174	14,356±44	9,166±45	5,742±159	22,618±2,890
Spontaneous release (cpm)	2,968±50	6,711±119	5,152±159	3,733±80	11,123±358

[a]Results are expressed as %specific release of ^{125}IUDR after an 18 hour incubation of spleen cells and tumor cells at a ratio of 500:1. S.E. was 1-6% of the mean.

response obtained with tumor C3H/HeJ. They indicate that killer cells present in the spleens of suppressed mice could lyse target cells other than the inoculated tumor T-10.

The results further demonstrate that the non-specific reactivity was significantly higher in spleens of suppressed mice than in spleens of normal mice ($P < 0.0025$).

Effect of the inoculation of tumor T-10 on the reactivity of spleen cell in vitro. The possible role of tumor T-10 cells injected in vivo, in promoting the cytotoxic reactivity of spleen cells detectable in vitro, was investigated by comparing killer activity of splenocytes from suppressed and normal mice, before and after the s.c. injection of T-10.

Results shown in Figure 4.2 indicate that the injection of 1×10^6 tumor cells 9 days prior to the assay of spleen cells did not significantly alter the cytotoxic response mediated by the splenocytes.

The effect of the removal of Ig-bearing lymphocytes from normal spleen cells on the cytotoxicity. The aim of the following experiment was to determine whether the low levels of cytotoxicity obtained with spleen cells of normal mice were related to the presence of B-lymphocytes in the suspension.

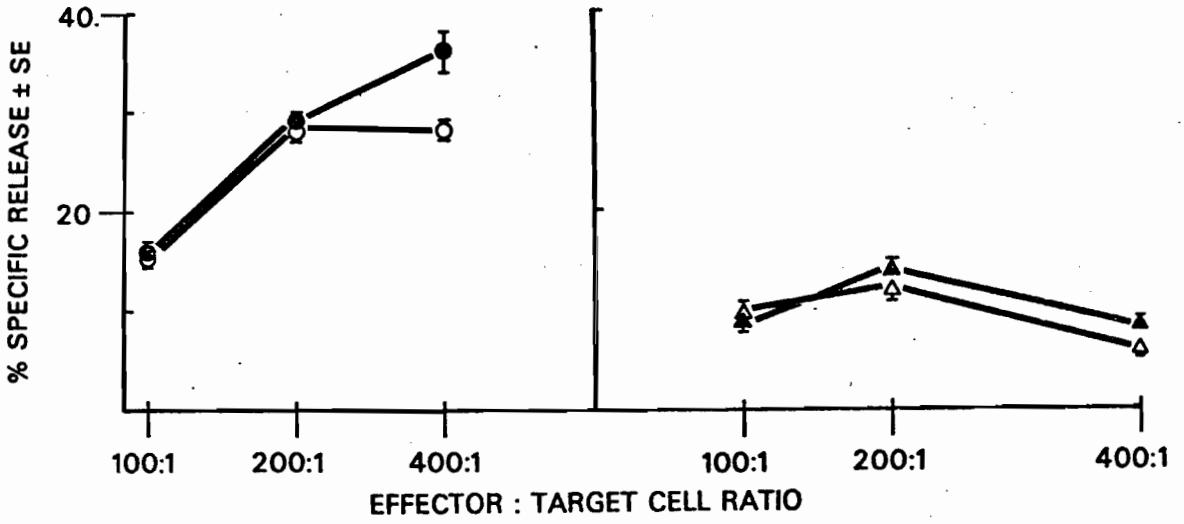
Normal spleen suspensions were depleted of B-lymphocytes by treatment with rabbit anti-mouse Ig serum and rabbit complement.The treatment resulted in the depletion of 40% of the cells. Spleen suspensions from suppressed mice treated in the same manner served as controls.

Results shown in Table 4.7 demonstrate a failure to significantly enrich killer cell activity against T-10 by this procedure.

4.A.2.Characterization of the Killer Cell.

The role of T-cells. Spleen cells from immunosuppressed mice were treated with rabbit anti mouse T-cell serum and rabbit complement, in a 2 step complement mediated cytotoxicity assay designed to selectively lyse T-cells. The treatment resulted in the depletion of 70-85% of the cells. The surviving cells failed to respond to a stimulatory dose of Concanavalin A

FIGURE 4.2: THE EFFECT OF THE INJECTION OF TUMOR T-10 ON SPLEEN CELL CYTOTOXICITY.



Six-eight week old suppressed and normal males were injected s.c. with 1×10^6 T-10 cells. Their spleen cells were assayed for their cytoxicity 9 days later together with spleen cells of mice which were not injected with tumor.

Anti-IgM treated mice
●——● - injected with T-10.
o——o - non injected.

Normal mice
▲——▲ - injected with T-10.
Δ——Δ - non injected.

Table 4.7. The Effect of Pretreatment of Spleen Cells with Anti-Ig Serum and Complement on Their Ability to Lyse T10 cells[a].

Animals	Treatment	
	Nil	Rabbit Anti-Mouse Ig Serum + Rabbit Complement
Anti-IgM-treated injected with T10[b]	16	26
Anti-IgM-treated non-injected	23	25
Normal injected with T10	8	11
Normal non-injected	8	9

[a]Results are expressed as % specific release of ^{125}IUDR following an 18 hour incubation of effector and target cells at a ratio of 200:1. S.E. was 2-6% of the mean.

[b]The assay was carried out 8 days after the injection of 1 x 10^6 T-10 cells.

in a 40 hour culture assay, as demonstrated in Table 4.8.

The results shown in Table 4.9 indicate that the killer cells could not be removed from the spleens of immunosuppressed mice by the selective depletion of T-cells. Moreover, they demonstrate,that the fractionation procedure resulted in a considerable enrichement of killer cell activity by a factor of 1.6 to 3.6.

Results shown in Table 4.5 further demonstrate that following the removal of T-cells a similar enrichement in lytic activity against target B-16 could also be obtained.

A similar treatment of spleen cells from normal mice resulted in the depletion of 40-50% of the splenocytes and a corresponding elimination of the Con A-responsive population (Table 4.8). It failed however to increase killer cell activity in 3 out of 4 experiments performed, as shown in Table 4.10.

The role of phagocytic cells. Spleen cell suspensions were treated with carbonyl iron and magnetism in an attempt to selectively remove phagocytic splenocytes. The treatment resulted in a loss of 20-25% of the cells. The ability of the remaining cells to ingest latex particles was assayed.

The results shown in Table 4.11 demonstrate that the treatment was successful in eliminating 88% of the strongly phagocytic (> 10 particles/cell) splenocytes. The cytotoxicity of the fractionated population was assayed. The results shown in Table 4.12 indicate that a reduction of 20-30% in the lytic activity of the splenocytes occured following the removal of phagocytic cells.

Table 4.8: Stimulation by Concanavalin A of Spleen Cells Treated with Anti-T Cell Serum and Complement[a]

Source of Spleen Cells	Non-treated Cells		Cells treated with anti-T cell Serum and Complement	
	Medium Only	Con A	Medium Only	Con A
Anti-IgM-treated, tumor-bearing mice	25,189	38,622	360	126
Normal, tumor-bearing mice	28,227	34,836	4,637	5,228
Uninjected controls	28,071	71,420	6,587	8,552

[a]Results are expressed as cpm of ^{3}H-Thymidine taken up by the spleen cells after a 40 hour incubation with or without Concanavalin A and a 16 hour pulse with ^{3}H-Thymidine. S.E. of the triplicates in each category did not exceed 8% of the mean.

Table 4.9.: Effect of Pretreatment of Spleen Cells Cells From Suppressed Mice with Anti-T-Cell Serum and Complement on Their Ability to Lyse T-10 Cells

Experiment Number	% Specific Release of ^{125}IUDR[a]		Mean Tumor Diameter	Maximum release Spontaneous release (cpm)±SE
	Non-Treated Spleen Cells[b]	Spleen Cells Treated With anti-θ and Complement		
1	8	22	not measureable	5,721±22 377±24
2A	23	34	mice not injected with T10	7,422±467
2B	20	32	not measureable	1,580±66
3[c]	28	100	0.6 cm	14,462±902 7,394±265

Results are expressed as % specific release of ^{125}IUDR following an 18-20 hour incubation of effector nd target cells at a ratio of 200:1. S.E. was 1-5% of the mean

Spleen cells were assayed 9-12 days after the s.c. injection of 2.5 x 10^5 - 1 x 10^6 T10 cells.

Effector and target cell incubation was for 42 hours.

Table 4.10 : Effect of Pretreatment of Spleen Cells from Normal Mice with Anti-T Cell Serum and Complement on Their Ability to Lyse T-10 Cells[a]

Experiment Number[b]	Non-treated Spleen Cells	Spleen Cells Treated with Anti T-Cell Serum and Complement
1	6	7
2A	11	9
2B	20	13
3	0	26

[a]Results are expressed as % specific release of ^{125}IUDR following an 18-20 hour incubation of effector and target cells at ratio of 200:1. S.E. was 1-5% of the mean.

[b]For details of the experiments see legend to Table 4.9.

Table 4.11. Uptake of Latex Particles by Splenocytes Depleted of Iron-Ingesting Cells.

Treatment of Spleen Cells	Number of Spleen Cells with: 0	1-10	>10	Latex Particles[a]
nil	163	10	27	
30 min incubation at 37° without carbonyl iron	177	9	24	
30 min incubation with carbonyl iron and passage over a magnet	191	6	3	

[a]200 spleen cells were counted in a hemocytometer.

Table 4.12: The Effect of the Removal of Phagocytic Cells on the Ability of Spleen Cells from Anti-IgM-treated Mice to Lyse T10 Cells.

Experiment No.	% Specific Release of ^{125}IUDR[a]		Maximum released spontaneous release (cpm)±SE
	Non-treated cells	Cells treated with carbonyl iron and magnet	
1	12	8	14,954±80 1,900±142
2[b]	23	17	80,600±7,000 10,973±564
3	54	44	11,646±458 4,233±22

[a]The assay was carried out at a killer to target cell ratio of 200:1, the incubation was for 18 hours, except for experiment 3, where it was prolonged to 24 hours. S.E. was 2-8% of the mean.

[b]Mice were injected s.c. with 10^6 T10 cells 9 days prior to assay. Other animals in the study were not injected with the tumor.

4.B. Cytotoxic Activity of Effector Cells from Anti-IgM and NRS-Treated Mice Measured by the Lysis of ^{51}Cr-Labelled YAC.

The results described so far, in this chapter, suggested that a killer cell population with some of the characteristics of the mouse NK cell, may be responsible for the enhanced cytotoxicity of spleen cells from immunosuppressed mice to T-10 and other tumors. The experiments to be described in the remaining part of the chapter were designed to test this possibility and to further characterize the killer cell. YAC, a lymphoma of A/Sn origin which is widely used as target for NK, served as target in many of the experiments and a short term ^{51}Cr-release assay was employed.

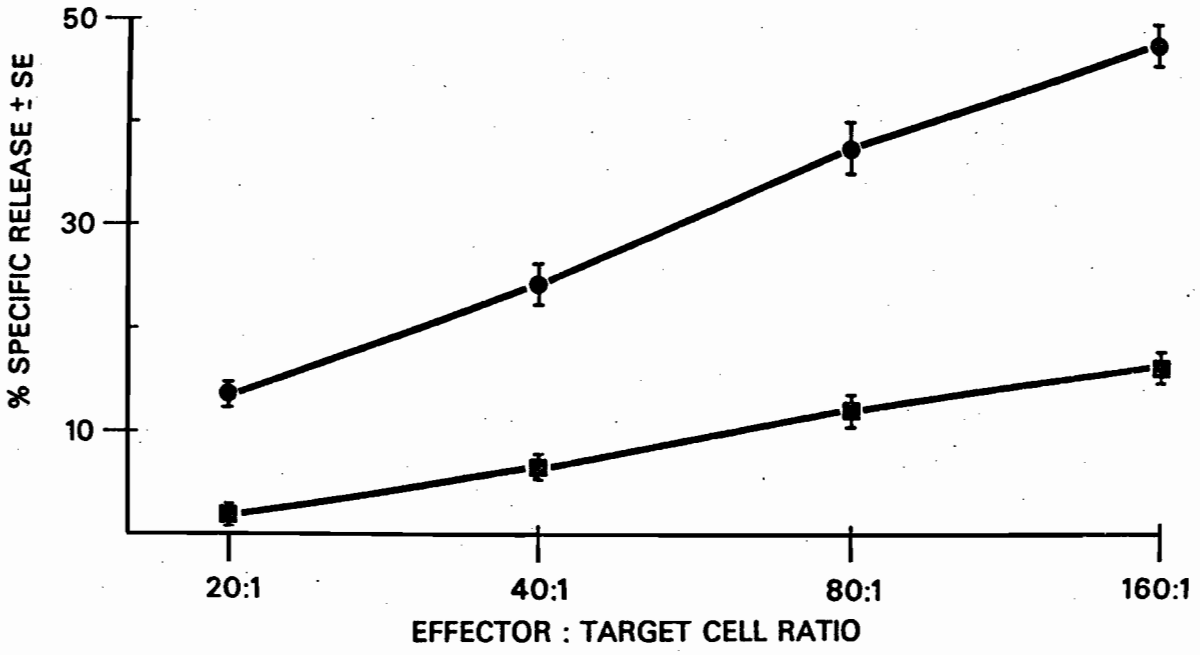
4.B.1. The Cytotoxic Reaction

Cytotoxicity of spleen cells to YAC. Spleen cells from 7-10 week old anti-IgM and NRS-treated mice were assayed for their ability to lyse ^{51}Cr-labelled YAC cells. The results shown in Figure 4.3 demonstrate that target cell lysis by splenocytes from the immunosuppressed mice was 3-4 fold higher than that by splenocytes of NRS-treated controls.

The number of lytic units/spleen was calculated by extrapulation of the linear part of the curves in Figure 4.3. The results, shown in Table 4.1 3, support the findings illustrated in the figure and demonstrate that although there was a reduction in the overall number of nucleated cells in the spleens of suppressed mice, their ability to lyse YAC cells was enhanced. The results further suggest that this enhancement was not due entirely to a relative enrichment - by depletion of B cells - of a killer cell population which was equally represented in the spleens of both groups. Instead it seems that the enhancement reflected a true increase in the killer cell reactivity.

The cytotoxicity of bone narrow (BM) cells. The cytotoxicity of BM cells from suppressed and NRS-treated mice against YAC cells was examined. The results shown in Figure 4.4 and Table 4.1 4 demonstrate that, BM cells from anti-IgM treated mice, similarly to their spleen cells, had an enhanced lytic activity against YAC.

FIGURE 4.3: CYTOTOXICITY OF SPLEEN CELLS FROM ANTI-IgM AND NRS-TREATED MICE TO ^{51}Cr LABELLED YAC.



2×10^5 - 1.6×10^6 Spleen cells from either anti-IgM or NRS-treated mice were incubated for 5 hours at 37^o in microtiter wells with 1×10^4 ^{51}Cr labelled-YAC cells. The isotope released into the supernatants was then counted.

Results are expressed as a mean of 11 experiments in which mice ranged in age from 7-10 weeks.

●——● spleens from anti-IgM treated mice.

■——■ spleens from NRS-treated mice.

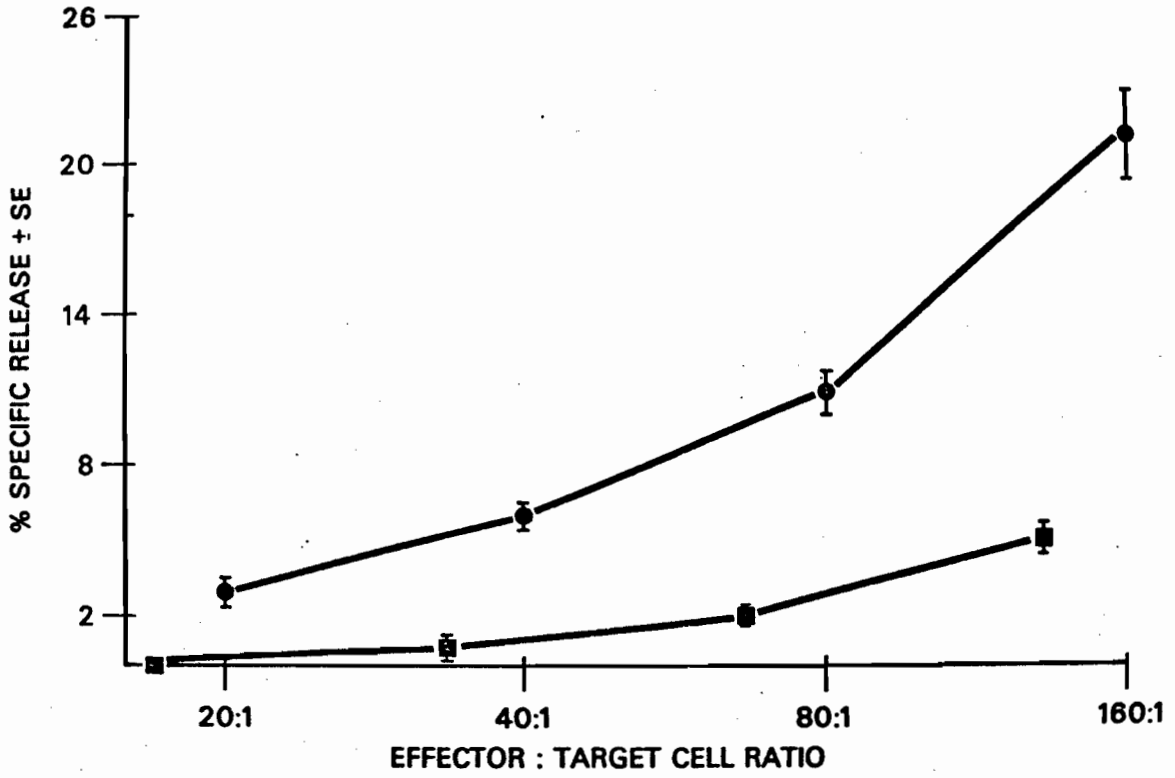
TABLE 4.13. Cytotoxicity of Spleen Cells to ^{51}Cr-Labelled YAC Expressed in Lytic Units.

Animals	Number of spleen cells/lytic unit ($x10^{-6}$)[a]	mean number of nucleated cells/spleen[b] ($x10^{-6}$)	lytic units/ spleen
Anti IgM-treated	0.33	37	112
NRS-treated	2.4	140	59

a. One lytic unit was defined as the number of spleen cells which was required for a specific release of 20%.

b. The number was calculated on the basis of cell yields obtained in 11 experiments described in legend to Fig. 4.3.

FIGURE 4.4: CYTOTOXICITY OF BONE MARROW CELLS FROM IMMUNOSUPPRESSED AND NORMAL MICE TO ^{51}Cr-LABELLED YAC.



Pools of BM cells were prepared from the femur bones of 12 week: old suppressed and 3 age-matched NRS-treated mice. Their cytotoxicity to ^{51}Cr-labelled YAC was assayed as described in the legend to Figure 3.

●——● - BM cells from anti-IgM treated mice.

■——■ - BM cells from NRS-treated mice.

TABLE 4.14. Cytotoxicity of Bone Marrow Cells (BM) to ^{51}Cr-Labelled YAC Expressed in Lytic Units[a].

Animals	Number of BM cells/ lytic unit ($x10^{-6}$)	Mean of nucleated BM cells animal ($x10^{-6}$)	Lytic units/ animal
Anti IgM-treated	1.5	25	17
NRS-treated	4.9	34	7

a. For definition see legend to Table 4.13.

b. Calculated on the basis of yields obtained in the experiment described in the legend to Fig. 4.4.

The influence of the sex of donor mice on spleen cell cytotoxicity. The cytotoxicity of spleen cells from male and female anti-IgM treated mice to YAC target cells was compared. Results shown in Figure 4.5 indicate that regardless of the sex of spleen donors, high and comparable levels of lysis could be obtained.

The effect of age on spleen cell cytotoxicity. Spleen cells from 7, 12, and 17 week old anti-IgM and NRS-treated mice were assayed. The results shown in Figure 4.6 represent a mean of 3 such experiments. They indicate a difference in the effect of age on the cytotoxicity of spleen cells from these study groups. Thus, whereas the cytotoxic activity of normal spleen cells was considerably higher at 7 weeks of age than at 12 and 17 weeks ($0.05<P<0.10$ at 12 weeks and $P<0.02$ at 17 weeks), no such decline in cytotoxicity was observed with spleen cells from anti-IgM-treated mice. The hightened cytotoxic reactivity displayed by the latter at 7 weeks of age was maintained and could still be demonstrated when the mice were 17 weeks old.

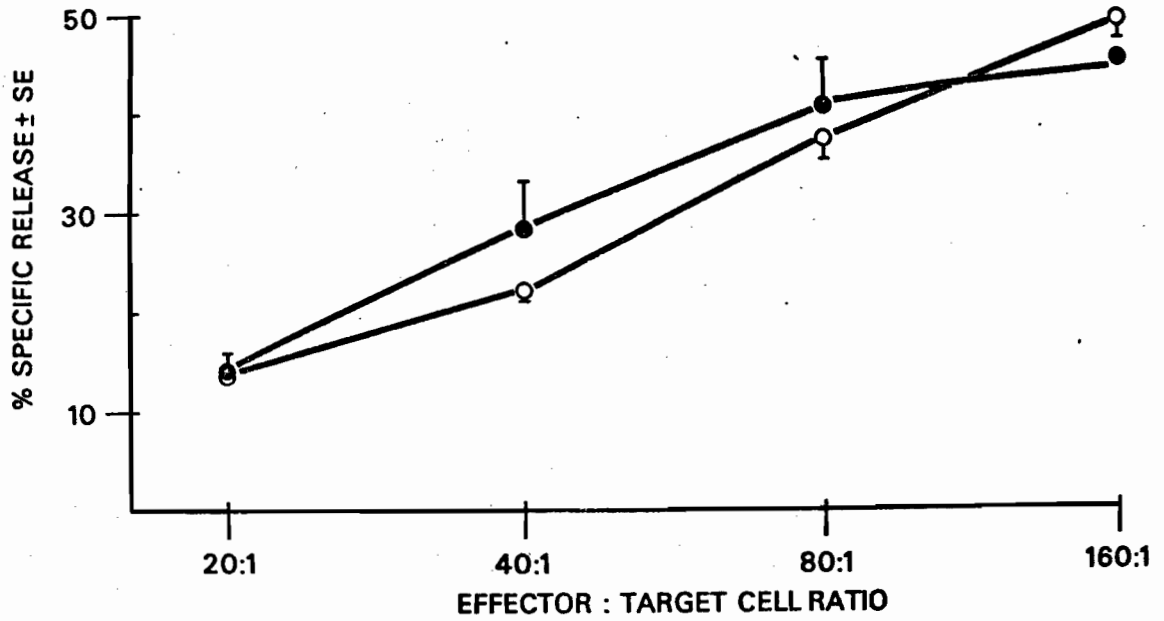
The effect of tumor inoculation on spleen cell cytotoxicity. The cytotoxicity of spleen cells from mice inoculated with tumor was compared to this of non-inoculated mice. 1×10^6 T-10 cells were injected s.c. into anti-Ig^M and NRS-treated mice 8 days prior to assay of their splenocytes.

The results shown in Figure 4.7 indicate that the inoculation of mice with tumor did not significantly modify the cytotoxic activity of their spleen cells as compared with the activity of spleen cells from age and sex matched non-injected controls.

4.B.2. Characterization of the Killer Cell.

The effect of the selective removal of B-lymphocytes from normal spleens on the cytotoxicity. The aim of the following two experiments was to ascertain whether the selective removal of B-cells from normal splenocytes would result in an increase in their cytotoxicity to the level

FIGURE 4.5: THE EFFECT OF THE SEX OF DONOR MICE ON THE CYTOTOXICITY OF THE SPLEEN CELLS.

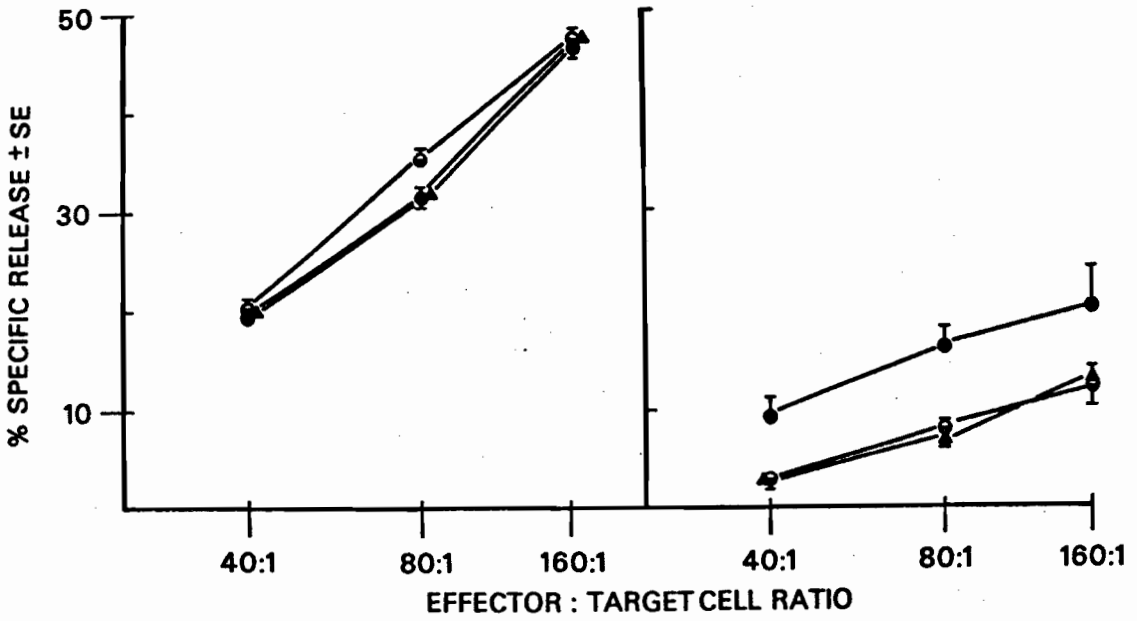


Results are expressed as the mean of 4 experiments with spleen cells from 7-10 weeks old males and 5 experiments with age matched females.

●——● - male spleens

o——o -female spleens

FIGURE 4.6: THE EFFECT OF AGE ON THE CYTOTOXICITY OF SPLEEN CELLS.



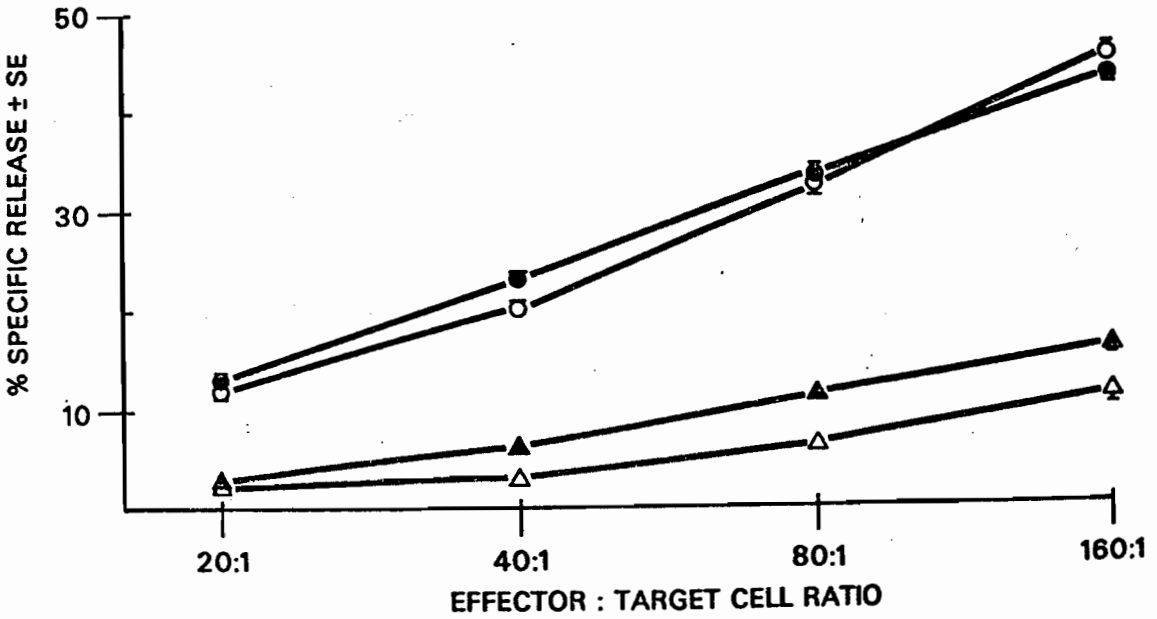
Spleen cells from 7, 12 and 17 week old mice were assayed. The left side of the figure describes results obtained with spleen cells from anti-IgM treated mice whereas the right side describes these obtained with NRS-treated mice. Each point on the curves represents the mean of 3 experiments.

●——● - 7 week old mice.

●——● - 12 week old mice.

▲——▲ - 17 week old mice.

FIGURE 47 : THE EFFECT OF TUMOR INOCULATION ON THE CYTOTOXICITY OF SPLEEN CELLS.



Spleen cell suspensions, were each a pool of 2 spleens derived from 8 weeks old mice treated in one of the following manners.

●——● - anti-IgM treated mice inoculated with T-10.

o——o - anti-IgM treated mice not inoculated.

▲——▲ - NRS-treated mice inoculated with T-10.

Δ——Δ - NRS-treated mice not inoculated.

observed with spleen cells of immunosuppressed mice.

Two methods widely used for the depletion of B-lymphocytes were employed, namely the treatment of splenocytes with anti-Ig serum and complement, or their fractionation on nylon wool columns.

Results obtained using the first approach are shown in Figure 4.8. They indicate a failure to increase the cytotoxicity of normal splenocytes by the lysis of Ig-bearing cells in a complement mediated cytotoxicity assay. They further demonstrate that this treatment did not have an adverse effect on the ability of splenocytes from immunosuppressed mice to lyse YAC targets.

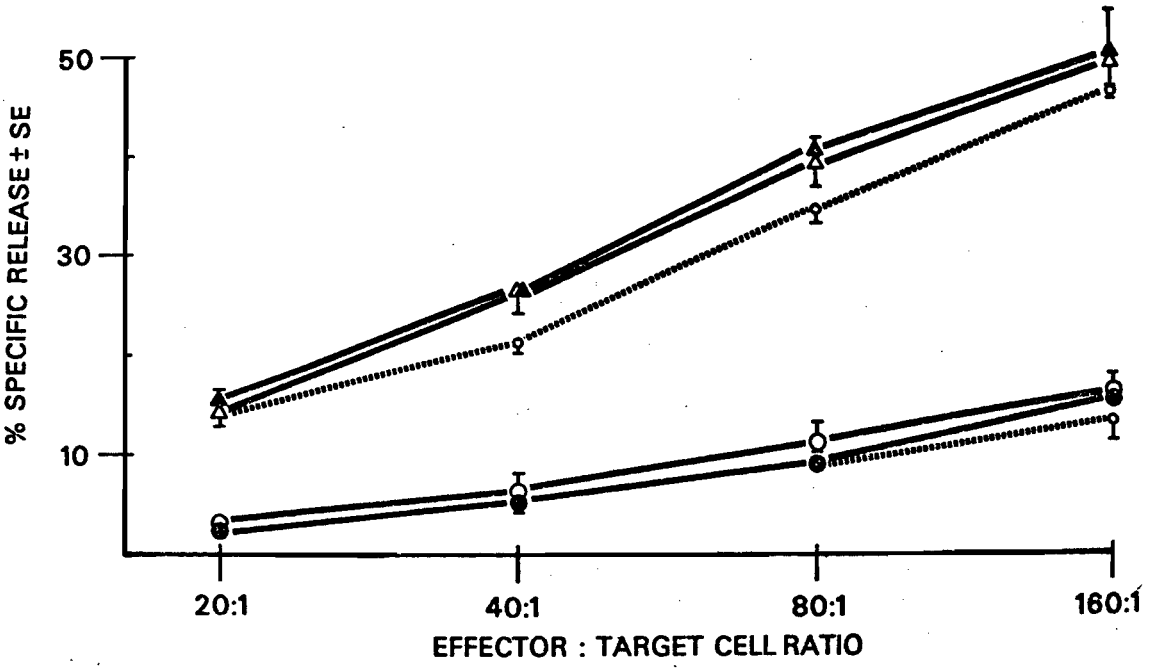
Following the fractionation of spleen cells by passage through nylon wool, 75-85% of the splenocytes from immunosuppressed mice and 40-50% of these from normal mice could be recovered in the non adherent fraction. The majority (80%) of the adherent cells could then be released off the wool by teasing.

T and B-lymphocyte contents of the adherent and non-adherent fractions was analysed by radioautography, using hyperimmune antisera directed against IgM or θ antigens and ^{125}I-labelled protein A. The cells shown in Figure 4.9 are representative of the cells seen in the smears. Heavily labelled and non-labelled, small and medium-size lymphocytes are shown.

Results of the radioautographical analysis are described in Table 4.15. They indicate that the majority of IgM-bearing cells (77%) bound to and were recoverable from the wool whereas the majority of T-cells (83%) could be found in the non adherent fraction.

The cytotoxicity of the non adherent cells to YAC targets was assayed. The results shown in Figure 4.10 demonstrate that the depletion of B-cells by nylon wool columns was effective in increasing anti-YAC reactivity of normal spleen cells. It failed however to elevate this activity to the levels attained by splenocytes from immunosuppressed mice. The results further demonstrate that the killer cell population which resided in the spleens of either suppressed or normal mice was non-adherent to nylon wool.

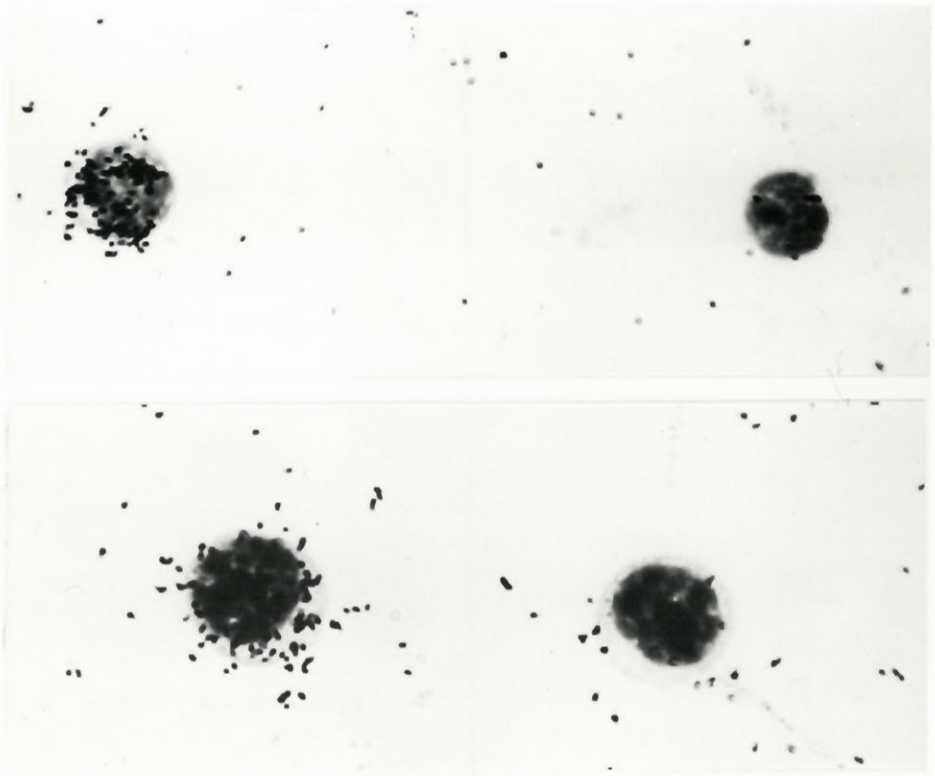
FIGURE 4.8: THE EFFECT OF RABBIT ANTI-MOUSE-Ig SERUM AND COMPLEMENT TREATMENT ON SPLEEN CELLS CYTOTOXICITY.



The results are expressed as the mean of 4 experiments in which 8-12 week old mice were used as spleen donors.

Spleen cells from suppressed mice	▲——▲	- treated with anti-Ig serum and complement.
	Δ——Δ	- treated with complement only.
Spleen cells from NRS-treated controls	●——●	- treated with anti-Ig serum and complement.
	o——o	- treated with complement only.
	o,,,,o	- non treated suspensions.

FIGURE 4.9. RADIOAUTOGRAPHY OF SPLEEN CELLS FRACTIONATED BY NYLON WOOL COLUMNS AND LABELLED WITH SPECIFIC ANTISERA AND ^{125}I-PROTEIN A.



Spleen cells which were either adherent or non-adherent to nylon wool were incubated with either anti-T-cell or anti-IgM serum. This was followed by an incubation with ^{125}I-labelled Protein A, after which the cells were smeared, processed for radioautography, and stained with McNeal Tetrachrome. The cells shown are representative of the heavily labelled (left) and non-labelled, small (top) and medium-sized lymphocytes, observed.

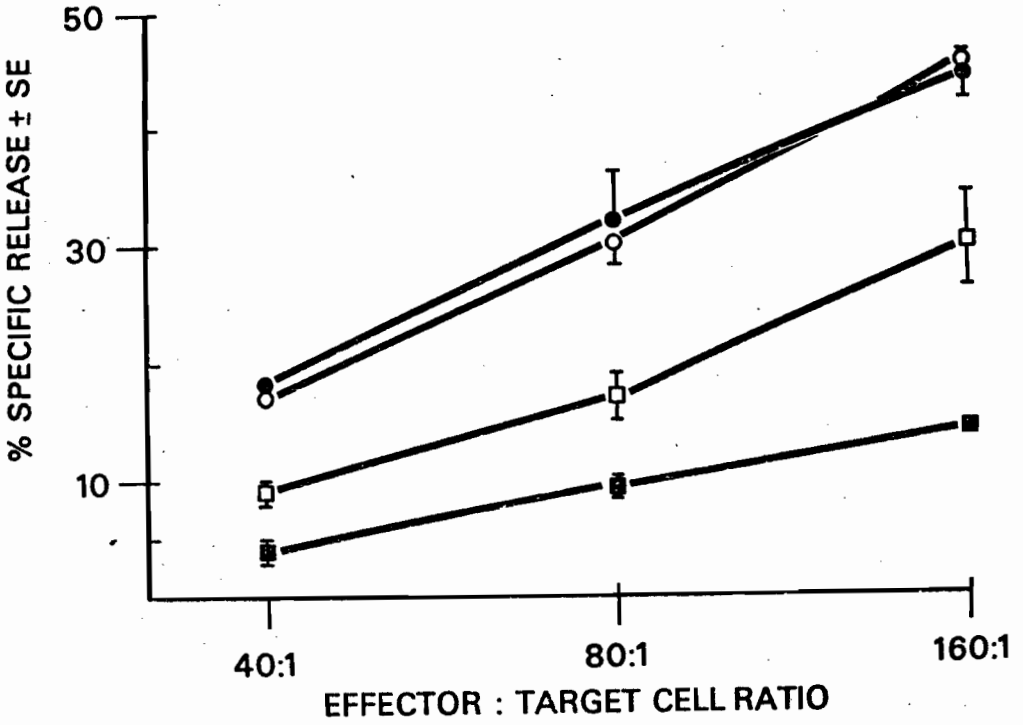
Table 4.15 : Surface Labelling of Spleen Cells with Specific Antiserum and ^{125}I Protein A Before and After Fractionation on a Nylon Wool Column[a]

Spleen Suspension Tested	Antiserum Used for Labelling	Number of Cells[b] With: 0-5 grains	6-10 grains	>10 grains
1) non-fractionated	anti-T cell serum	90	22	88
2) nylon wool adherent	anti-T cell serum	168	8	24
3) non-adherent	anti-T cell serum	43	17	141
4) non-fractionated	anti-IgM serum	88	15	97
5) nylon wool adherent	anti-IgM serum	30	7	163
6) non-adherent	anti-IgM serum	75	5	20

[a] 100×10^6 normal spleen cells were fractionated. 35% of the cells were then recovered in the non-adherent fraction, whereas 30% could be recovered from the wool.

[b] 200 cells were counted per slide with the exception of slide #6, where only 100 cells were enumerated.

FIGURE 4.10: CYTOTOXICITY OF NYLON WOOL-FRACTIONATED SPLEEN CELLS.



Results are expressed as the mean of 2 experiments carried out with splenocytes of 9 and 12 week old mice.

Splenocytes of anti-IgM treated mice.
- ●——● - non-fractionated.
- o——o - non-adherent to nylon wool.

Splenocytes of NRS-treated mice.
- ■——■ - non-fractionated
- □——□ - non-adherent to nylon wool.

The role of Fc-receptor-bearing cells in the cytotoxic response. The following experiment was designed to examine the role played by Fc-receptor bearing cells in the lysis of YAC. Spleen cells from suppressed mice were incubated on monolayers of SRBC (sheep red blood cells) which had been pretreated with either mouse anti-SRBC serum or with control serum derived from normal (non-immunized) mice. The cytotoxicity of the non-adherent cells was then assayed.

This procedure resulted in the depletion of 10-20% of the cells incubated on the anti-SRBC coated monolayers, while, monolayers which were pretreated with the normal serum (control monolayers) failed to bind a detectable number of splenocytes.

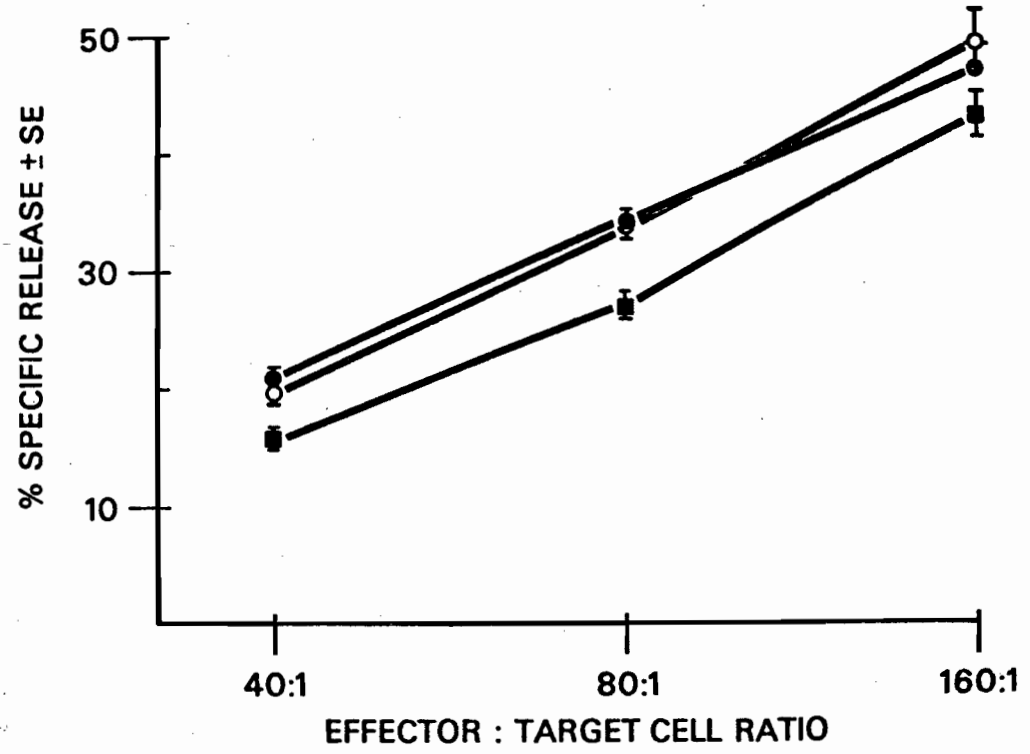
The results illustrated in Figure 4.11 indicate that the incubation of spleen cells on control monolayers did not affect their ability to lyse YAC cells. However, a low (10-20%) but significant reduction (P=0.01) in this ability did occur after the depletion of F_c-receptor bearing cells.

An attempt to ascertain the cytotoxic activity of monolayer-bound cells followed. The cells (5% of input) were released by an incubation of the monolayers with Protein A and their cytotoxicity compared to that of non-adherent or non-fractionated preparations.

The results shown in Figure 4.12 demonstrate that protein A was indeed effective in releasing killer cells from the monolayers and that these cells were more cytotoxic ($P = 0.001 - 0.05$ at a natio of 40:1 effector:target cells) to YAC than the non-fractionated or non-adherent cells.

The specificity of the killer cell as determined by the cold target inhibition assay. The previous experiments demonstrated that spleen cells from immunosuppressed mice had an elevated cytotoxic response against both T-10 and YAC. In the proceeding experiment the cold target inhibition assay was employed to ascertain whether a common killer cell was responsible for this killing and whether it was triggered by the recognition of surface receptors, shared by both tumors. In this assay, only one tumor was radioactively labelled. The ability of the other(s) (non-labelled) tumor(s) to

FIGURE 4.11: CYTOTOXICITY OF SPLEEN CELLS FRACTIONATED ON MONOLAYERS OF SRBC COATED WITH ANTI-SRBC SERUM.



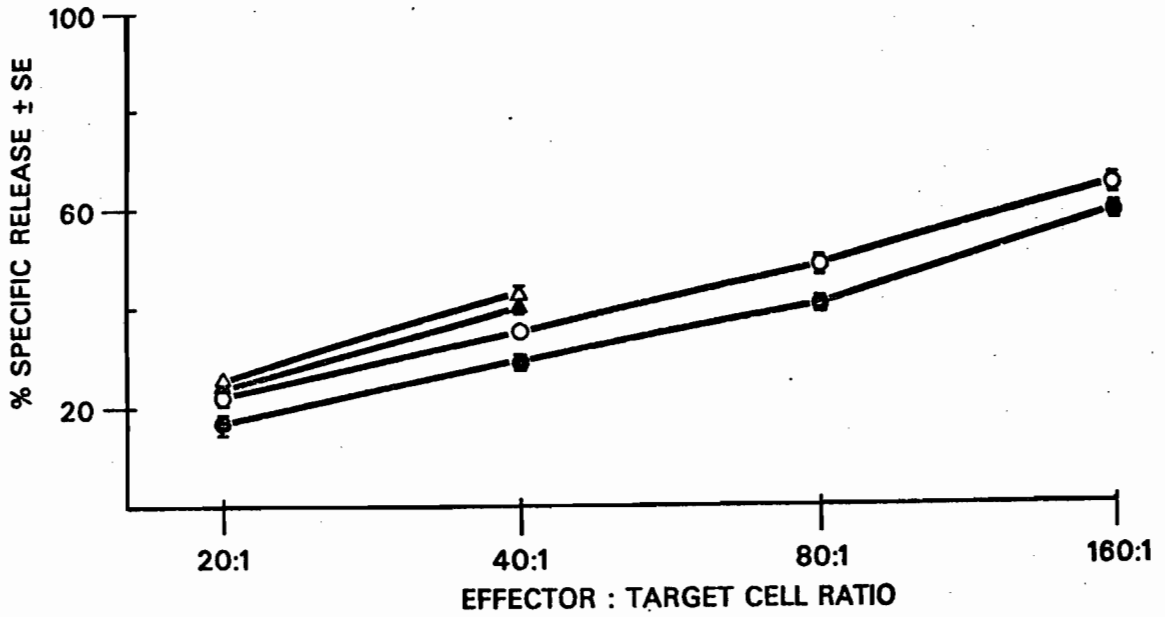
Results are expressed as the mean of 3 experiments in which spleen cells were derived from 7-12 weeks old mice.

●——● - non fractionated spleen cells.

o——o - cells incubated for 1 hour at 37°C on SRBC monolayers overlayed with normal serum from $B6C3F_1$ mice.

■——■ - cells incubated for 1 hour at 37°C on SRBC monolayers overlayed with swiss mouse anti SRBC hyperimmune serum.

FIGURE 4.12: CYTOTOXICITY OF SPLEEN CELLS, RELEASED OFF SRBC-ANTI-SRBC MONOLAYERS BY PROTEIN A.



Results were obtained with a pool of cells prepared from the spleens of 6-9 week old anti-IgM treated mice.

o——o - non fractionated cells.

●——● - cells fractionated by 1 hour incubation at 37°C on SRBC anti-SRBC monolayers.

▲——▲ - cells released off SRBC-anti-SRBC monolayers by a 1 hour incubation with 36 μg/ml Protein A.

Δ——Δ - cells released off SRBC-anti-SRBC monolayers by a 2 hour incubation with 36 μg/ml Protein A.

compete for killer cells and block lysis of the labelled target was determined.

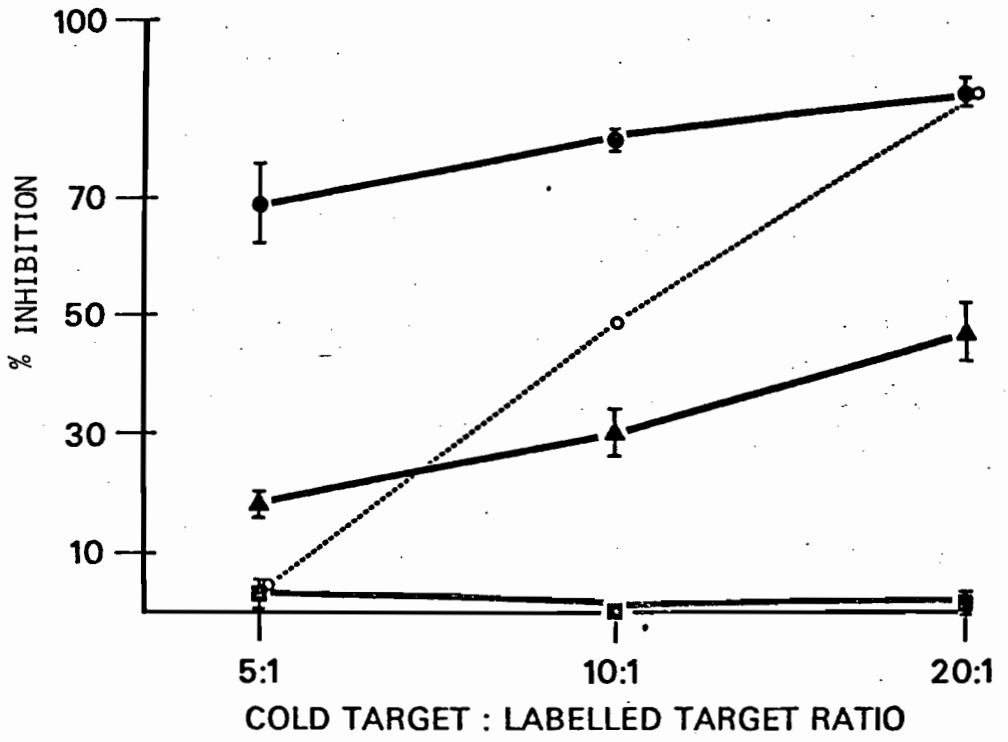
The results shown in Figure 4.13 indicate the following:

1) Non labelled T-10 cells could inhibit the lysis of ^{51}Cr-labelled YAC.
2) Non labelled YAC cells could inhibit the lysis of $^{125}IUDR$ labelled T-10.
3) In both instances the inhibition was proportional to the concentration of the competing target.
4) Tumor P815-X2 could not inhibit the lysis of ^{51}Cr-labelled YAC.

In a second experiment the chemically induced tumors MCA-1, MCA-2 and MCA-3, as well as leukemia EL-4 were tested for their ability to compete for killer cells with ^{51}Cr-labelled YAC.

Results shown in Figure 4.14 indicate that all the tumors tested could block lysis of YAC. The tumors however, can be devided into 2 groups on the basis of the kinetics of their inhibition. Thus, the inhibition mediated by MCA-2 and MCA-3, similarly to that mediated by T-10, was reproducible from one assay to another, was proportional to their concentration in the reaction mixture and gave a linear dose response curve. The inhibition mediated by tumors EL-4 and MCA-1 however, varied from one assay to another, and at inhibitor: target ratio of 5:1 ranged from 32-66% in the case of MCA-1 and from 20-62% in the case of EL-4. Furthermore the inhibition mediated by these tumors was not proportional to their concentration and gave either an irregular (EL-4) or a flat (MCA-1) dose response curve. It should be noted that because of the great variations obtained in the levels of inhibition, the results of only 1 assay out of 4 performed with EL-4 and MCA-1 are shown in Figure 4.14.

FIGURE 4.13: COLD TARGET INHIBITION ASSAY Nol.



Results are expressed as the mean of 3 experiments in which 10-15 week old anti-IgM treated mice were used.

The ratio of spleen cells: ^{51}Cr labelled YAC cells in all assays was 80:1.

The mean specific release of ^{51}Cr obtained (in these assays) in the absence of competing targets was 38%.

In the $^{125}IUDR$-release assay the ratio of spleen cells: $^{125}IUDR$ labelled T-10 was 200:1 and the specific release in the absence of the competing target was 21%.

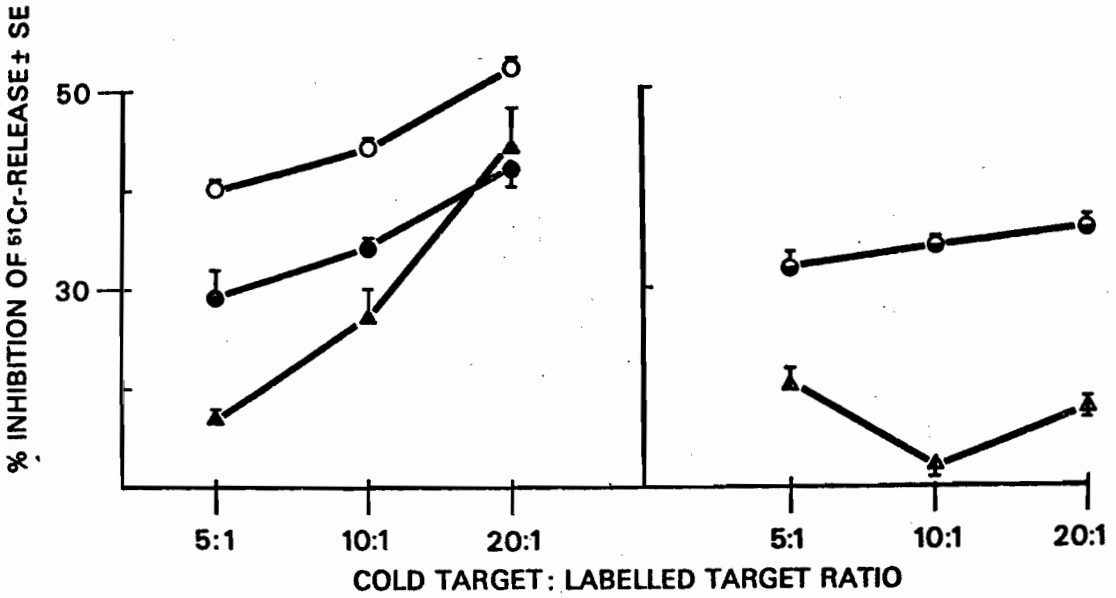
The following tumors were used as cold targets.

●——● - YAC
▲——▲ - T-10
■——■ - P-815

In an assay with ^{51}Cr-labelled YAC.

o....o - YAC in an assay with $^{125}IUDR$-labelled T-10.

FIGURE 4.14: COLD TARGET INHIBITION ASSAY No2.



Results on the right side of the figure are expressed as a mean of 2-4 experiments/competing tumor. Results on the left side are expressed as the inhibition obtained in one of 4 assays performed.
Assay conditions were as described in the legend to Figure 4.12.
The following tumors were used as competitors:

o——o - MCA -2
●——● - MCA -3
▲——▲ - T-IO
o——o - MCA -1
▲——▲ - EL-4

The cytotoxicity of spleen cells to ^{51}Cr-labelled EL-4. The ability of spleen cells from either NRS or anti-IgM-treated mice to lyse EL-4 cells was examined in a ^{51}Cr-release assay. Results shown in Figure 4.15 demonstrate that cells from both sources were inefficient in lysing EL-4, under conditions which allowed high levels of lysis of YAC.

Isolation of a killer cell-enriched population. On the basis of the results described in Figure 4.10 and table 4.9, an attempt was made to isolate the killer cell population by a 2-step fractionation procedure aimed at the selective removal of nylon wool-adherent and Θ-positive cells.

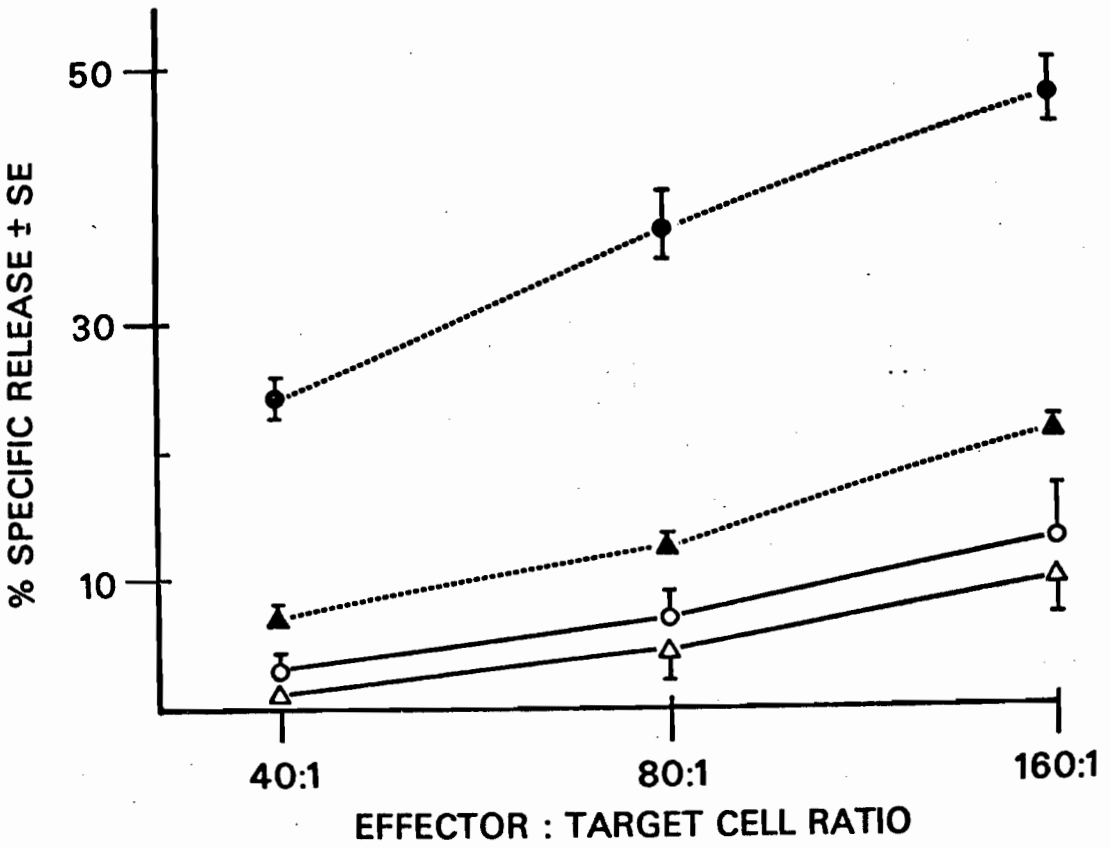
Spleen cells from suppressed mice were passed through a nylon wool column and the non-adherent fraction treated with anti-T cell serum and complement. The combined procedure resulted in the eleimination of 90-95% of the viable cells present in the original suspension. The lytic activity of the remaining 5-10% is illustrated in Figure 4.16. It demonstrates a marked enrichment (x4) of the killer cells in this fraction as compared to non-adherent spleen cells treated with complement only.

Smears were prepared of the killer cell-enriched preparation and the cells stained with McNeal's Tetrachrome. The results, shown in Figure 4.17, revealed a preponderance of small lymphocytes (a) and some, mostly immature, granulocytes (b).

The cytotoxicity of a mixture of spleen cells derived from suppressed and NRS-treated mice. Spleen cells from anti-IgM and NRS-treated mice were mixed at various ratios and the cytotoxicity of the mixtures assayed. This was done in an effort to determine whether suppressor cells, capable of inhibiting killer cell activity, were present in the spleens of NRS-treated mice.

The results shown in Figure 4.18 indicate that normal spleen cells could not suppress the cytotoxic response of spleen cells from anti-IgM treated mice, at any of the ratios used. Moreover an examination of the results reveals that the actual levels of specific release obtained by the mixtures, slighly exceeded the expected values, calculated from the known reactivities of the individual suspension.

FIGURE 4.15: THE CYTOTOXICITY OF SPLEEN CELLS TO ^{51}Cr-LABELLED EL-4.



Results are expressed as the mean of 2 experiments in which spleens of 8 and 11 week old mice were used.

Reaction mixtures were as follows:

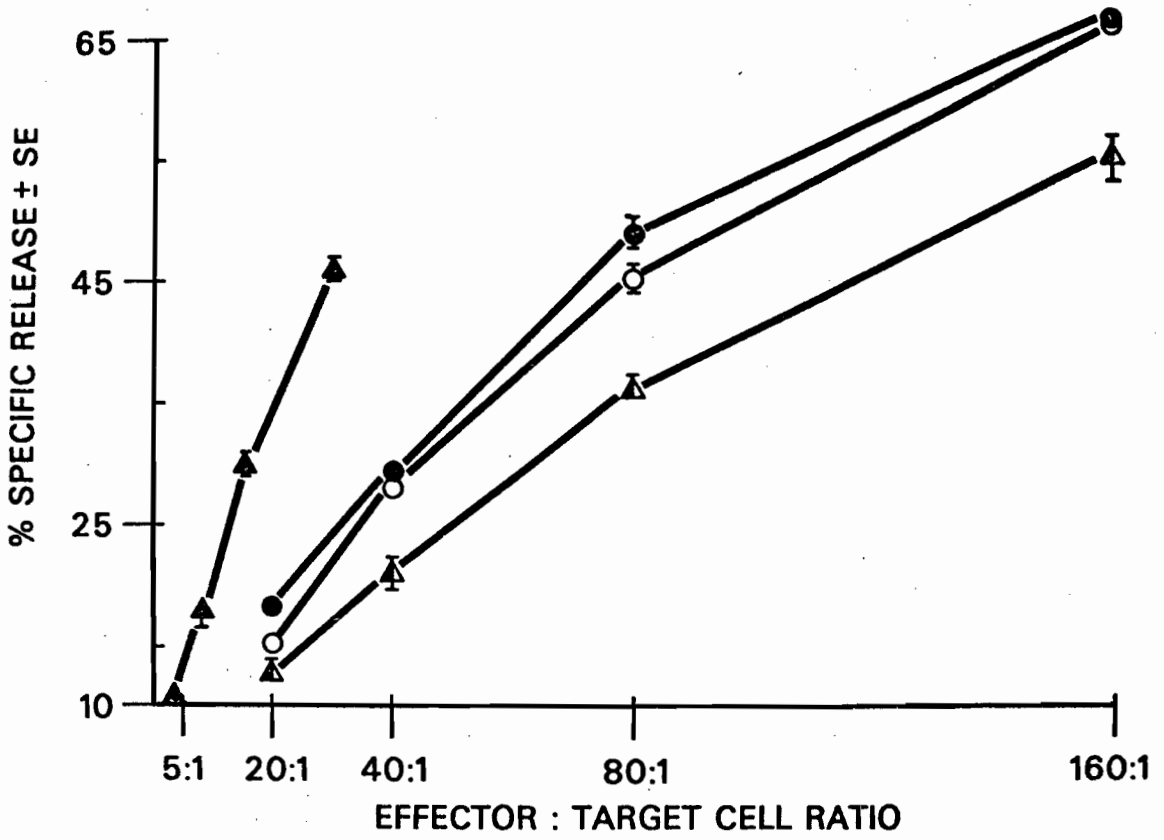
o——o - Tumor El-4 + spleen cells from suppressed mice.

Δ——Δ - Tumor El-4 + spleen cells from NRS-treated mice.

●·····● - Tumor YAC + spleen cells from suppressed mice.

▲·····▲ - Tumor YAC + spleen cells from NRS-treated mice.

FIGURE 4.16: THE PREPARATION OF A KILLER CELL-ENRICHED FRACTION OF SPLEEN CELLS.



Results were obtained with a pool of cells prepared from the spleens of 5-9 week old anti-IgM treated mice.

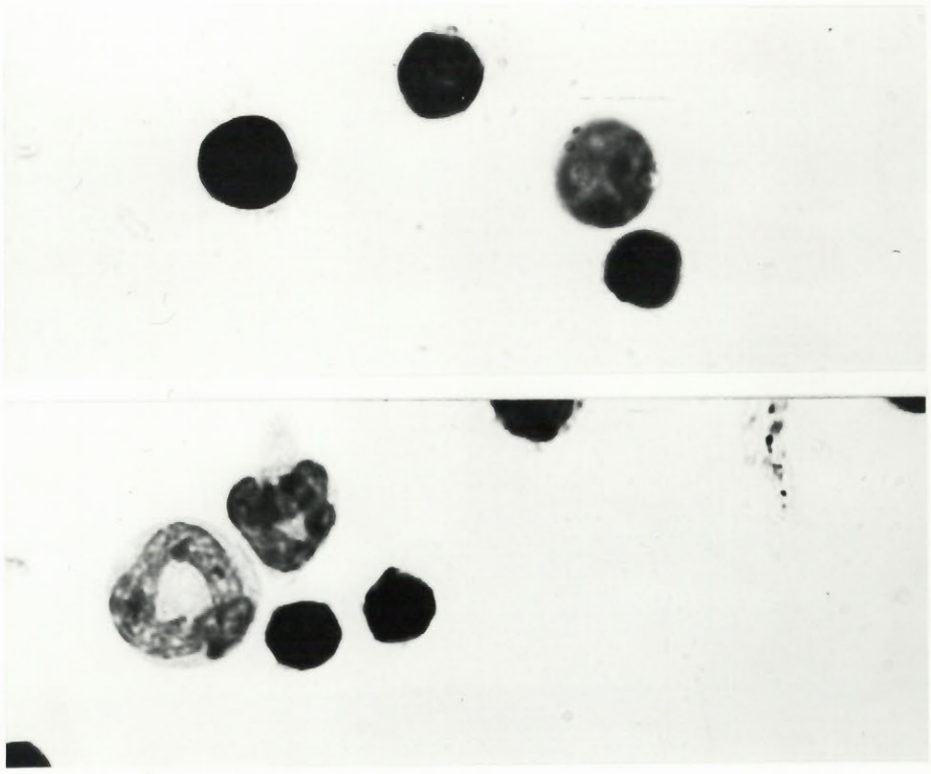
o——o : non fractionated cells.

●——● : cells non adherent to nylon wool.

▲——▲ : cells non adherent to nylon wool, treated with rabbit complement.

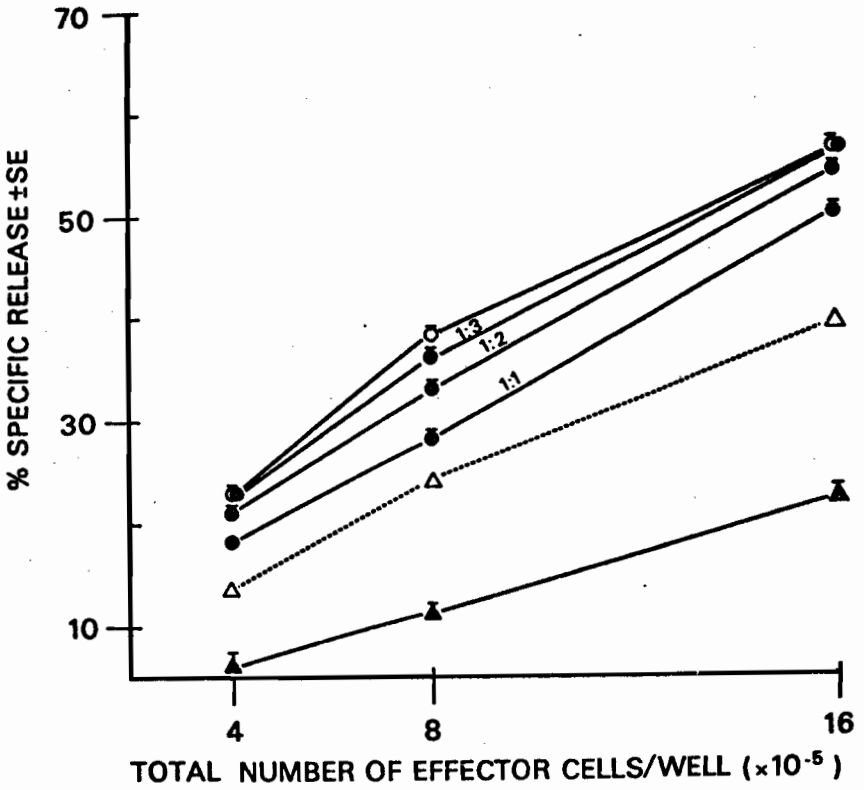
▲——▲ : cells non adherent to nylon wool, treated with rabbit anti-T cell serum and complement.

FIGURE 4.17. A KILLER CELL-ENRICHED FRACTION OF SPLENOCYTES.



Spleen cells were passed through a nylon wool column and the non-adherent cells treated with anti-T cell serum and complement as described in Materials and Methods. The remaining cells were smeared and stained with McNeal Tetrachrome. The two major cell populations observed were small lymphocytes (top) and granulocytes (mostly immature, bottom). Dead T cells (dark) were not removed. Magnification was X1740.

FIGURE 4.18: CYTOTOXICITY OF MIXTURES OF SPLEEN CELLS FROM ANTI-IgM AND NRS-TREATED MICE.



Results were obtained in an assay with spleens from 8-week old mice. The numbers on the graphs represent the ratio of spleen cells from NRS-treated mice: spleen cells from suppressed mice.

●——● - spleen cells from suppressed mice only.

▲——▲ - spleen cells from NRS-treated mice only.

Δ''''Δ - expected cytotoxicity of the above, mixed at a ratio of 1:1.

The effect of serum from anti-IgM and NRS-treated mice on the cytotoxicity of spleen cells. The aim of the following experiment was to ascertain whether serum components played a role in mediating or regulating the cytotoxic responses of spleen cells, from either suppressed or NRS-treated mice. Sera collected from suppressed or NRS-treated mice were added to reaction mixtures containing ^{51}Cr-labelled YAC and spleen cells from either NRS-treated or suppressed mice respectively.

Results shown in Table 4.1 6 demonstrate that serum from anti-IgM treated mice as well as rabbit anti-mouse IgM serum, at the dilutions specified, failed to modify the lysis mediated by normal spleen cells. Similarly serum from NRS-treated mice had no effect on the level of lysis mediated by splenocytes of suppressed mice.

Table 4.16: The Effect of Serum from Anti-IgM and NRS-treated Mice on the Lysis of ^{51}Cr Labelled YAC by Spleen Cells[9]

Source of Spleen Cells	Nil	Mouse Serum Added[b] / Serum Dilution								Rabbit anti-mouse IgM	
		#1		#2		#3		#4			
		10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-2}	10^{-3}
NRS-treated mice	10	9	10	13	12	12	11			12	11
Anti-IgM-treated mice	47							45	47		

[a] Results are expressed as % specific release of ^{51}Cr after a 4 hour incubation of effector and target cells at a ratio of 80:1.

[b] Sera #1, #2, and #3 were collected from 3 anti- IgM-treated mice. Preparation #4 was a pool of sera from 2 NRS-treated mice. Sera in the dilutions specified were added to the mixture of effector and target cells prior to incubation.

SUMMARY

The Cytotoxic Response Measured by the ^{125}IUDR-Release Assay.

The ^{125}IUDR-release assay was used to measure the *in vitro* cytotoxic reactivity of effector cells from suppressed and normal mice against the tumor T-10. It was found that spleen but not lymph node cells from immunosuppressed mice had a heightened lytic activity against the tumor. Although a high reactivity could also be detected using peritoneal exudate cells, it was probably due to the intraperitoneal injection of mineral oil prior to assay. This is suggested by the high levels of lysis obtained with peritoneal cells of normal mice which were not presensitized with the tumor.

It was further observed that the heightened reactivity of spleen cells was not specific to T-10 and did not require the injection of tumor cells *in vivo*. Such an injection did not normally alter spleen cell cytotoxicity. However, both increases and decreases in the cytotoxic response *in vitro*, were occasionally observed following the injection of the tumor cells. The variables which determined the effects of tumor injection are not clear at present.

Attempts to characterize the cytotoxic response revealed that it was not mediated by T cells and that the majority of the killer cells were not phagocytic. Thus, the selective removal of T cells from spleen suspensions resulted in an enrichment of the killer population, whereas the removal of phagocytic cells caused a reduction of only 20-30% in the cytotoxic response. It is possible that some phagocytic cells can mediate cell lysis or that they function as amplifier or accessory cells in the response. Further support to the latter notion is lent by the finding (not shown) that following nylon wool fractionation of spleen cells, a reduction occurs in their ability to lyse T-10. However, nylon wool adherent spleen cells exhibit a poor cytotoxic response against the tumor.

The Cytotoxic Response Measured by the ^{51}Cr-release assay.

The spontaneous cytotoxicity of effector cells from anti-IgM and NRS-treated F_1 mice to the allogeneic tumor YAC was studied. Using a 4 hour ^{51}Cr-release assay, it was found that spleen cells from either male or female suppressed mice were highly cytotoxic to this target, significantly more so than spleen cells from control mice. It was also found that this heightened cytotoxicity did not require prior contact with a tumor. Bone marrow cells from the suppressed mice were also found to be more cytotoxic to YAC than their controls. The overall levels of kill attained by BM cells however, were lower than those found with the spleen cells.

When the effect of aging on the cytotoxicity of the spleen cells was studied, it was found that the lytic activity of normal spleen cells, peaked before they reached 12 weeks of age and declined thereafter. The activity of splenocytes from suppressed mice however, was more stable and the high level of cytotoxicity was maintained even when mice were 17 weeks old.

Several lines of evidence suggest that this enhanced activity represents a true increase in either the number of killer cells or the lytic potential of individual cells, rather than a mere relative enrichment of a killer population by the removal of B-cells from anti-IgM treated mice. Thus, when expressed in lytic units per spleen, splenocytes of suppressed mice showed a 2-fold enrichment of lytic activity over their controls. Additionally, the selective removal of the majority of B-cells from normal spleen preparations failed to elevate their cytotoxicity to levels attained by spleens of B lymphocyte-deprived mice.

A characterization of the killer cell population revealed that they were non-adherent to nylon wool and insensitive to treatment by anti-T cell serum and complement. In addition, they were found to constitute a mixed population of which only a minority was Fc-receptor-positive.

A suspension of spleen cells which was prepared by selective fractionation procedures to highly enrich the killer cells, consisted of small and medium-size lymphocytes, as well as some granulocytes.

By employing the cold target inhibition assay it was found that, in addition to YAC, the killer cell could recognize, and was inhibited by, the tumors T-10, MCA-2 and MCA-3, but not by the tumor P815-X2. Tumors EL-4 and MCA-1 could also inhibit killer cell activity against YAC. Their inhibition, however, had the characteristics of a non-specific interference, i.e. irregular dose response curves and great variations in the levels of inhibition obtained in different assays (17). Using a ^{51}Cr-release assay it was additionally found that the killer cells of either anti-IgM or NRS-treated mice could not lyse EL-4 targets.

Finally, the addition of spleen cells, or serum, from NRS-treated mice to spleen cells of suppressed mice failed to reduce the cytotoxicity of the latter, implying the absence of suppressive cells or serum factors which could act at the efferent end of the response. At the same time, serum from anti-IgM treated mice, as well as rabbit anti-mouse IgM serum failed to modify the cytotoxic response of normal spleen cells, thus excluding the possibility that these sera could participate in an ADCC-type response against YAC.

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CHAPTER 5

THE PRODUCTION OF LARGE VOLUMES OF ANTI-TUMOR ANTIBODIES

MATERIALS AND METHODS

The Production of Anti-Tumor Antibodies.

A modification of the method described by Tung et al. (1) was used for the production of large amounts of anti-T-10 antibodies in individual syngeneic mice.

Immunization B6C3 F1 female mice which either rejected a small s.c. inoculum of T-10 or were immunized by the excision of a small tumor were chosen for the production of antibodies. They were repeatedly boosted s.c. at different sites with increasing doses (0.5 - 5 $x10^6$) of viable tumor cells. Alternatively, they were injected i.p. with irradiated tumor cells. Irradiation was with a ^{60}cobalt source at a dose of 12,000 rads. The immunization was at two week intervals.

Production of the Ascites Ascites were produced by i.p. injections of the immunized mice with 0.2 ml of Complete Freund's Adjuvant (CFA) in an emulsion, at a 9:1 ratio, with saline. Injections of the adjuvant were initiated five weeks after the immunization procedure and eleven days after the rejection by the mice of a s.c. challenge of 1 x 10^6 viable T-10 cells. Two weeks were allowed between the first and second injection. Thereafter CFA injections were continued at weekly intervals.

Most animals produced an ascites after three injections of the adjuvant. They were then tapped twice a week by inserting a sterile 20 g x 1,5 inch needle into the abdominal cavity and allowing the fluid to drain. One to eight ml of fluid were collected at each tapping for a total of 10 - 50 ml/mouse. The fluid was centrifuged immediately after

tapping (4,000 rpm at 4°C in an IEC PR-6000 centrifuge) and supernatants stored at -20°C. Before assay for anti-tumor antibody, ascites were thawed out and centrifuged at 20,000 rpm in a Beckman Model L Ultra centrifuge to remove fibrin clots. Complement was inactivated by a 30-minute incubation in a 56°C water bath. Several tappings from the same animals were pooled.

B. Protein A Assay for Anti-Tumor Antibody

The assay used was an adaption for adherent tumor cells, of the procedure described by Dorval et al. (2). A T-10 culture was trypsinized and cells were collected one day before assay 1×10^5 cells in 0.2 ml RPMI-FCS were then seeded into flat-bottom wells (Falcon Microtest II tissue culture plates) and incubated at 37°C for 20 hours in a 5% CO_2 incubator. The medium was discarded from the wells and 50 µl of several dilutions of the test ascites in RPMI were added to the cells. Each dilution was assayed in quadruplicate. Cells and ascites were incubated for 30 minutes at room temperature. The cells were then washed three times with RPMI by filling the wells with medium, briefly agitating the plates on a Mini Shaker and inverting them onto an adsorbant pad.

50 µl of ^{125}I-labelled protein A with a specific activity of 8 µci/mg (courtesy of Dr. G. Dorval, The Royal Victorial Hospital, Montreal, Quebec) inRPMI with 2% ovalbumin, were added to each well. Incubation was for 30 minut at room temperature and was followed by three washes of the wells witl RPMI containing 5% FCS. 1 ml of 1% SDS was then added to each well, and the wells rinsed several times with distilled water. The respective SDS extracts and washes were pooled and counted in the gamma counter.

Negative controls consisted of ascites collected from non-immunized mice. A rabbit anti-mouse lymphocyte serum (courtesy of Dr. P. Kongshavn, Montreal General Hospital, Montreal, Quebec) was used as a positive control for the efficiency of the assay.

C. Absorption of the Ascites with Tumor Cells.

Tumor monolayers were prepared one day before use by plating 2×10^5 trypsynized tumor cells suspended in 3 ml RPMI-FCS into tissue culture dishes and incubating them for 18 hours at 37°C in a 5% CO_2 incubator. The medium was then decanted before the addition of 1 ml of the ascites diluted in RPMI, for absorption. Ascites fluid and cells were incubated at 4°C for 30 minutes. The fluid was collected and centrifuged once, at 4°C and 4,000 rpm, in an IEC PR-6000 centrifuge. Where stated the absorption was repeated on a fresh monolayer. The fluid was again collected, centrifuged, and tested for anti-tumor antibodies, as described.

RESULTS

The Measurement and Characterization of Anti-T-10 Antibodies in the Immune Ascites.

The production of immune ascites-general observations. Following two or three i.p. injections of an emulsion of Complete Freund's Adjuvant (CFA) in saline, all treated mice developed an ascites, which was tapped twice weekly. One to eight ml of fluid were collected at each tapping. This resulted in the accumulation of 10-50 ml of cell-free fluid per mouse. During the experiment, which extended over a period of 9 months, 40% of the treated mice died. Autopsies revealed the formation of internal adhesions which were wide spread, affecting the intestines, spleen, liver and kidneys.

The detection of antibodies in the ascites. Anti T-10 antibodies were measured by a radioimmunoassay using ^{125}I-labelled protein A. Monolayers of the tumor were first treated with pools of ascitic fluid which were collected from individual mice following the 4th s.c. injection of viable tumor cells ant 4th i.p. injection of CFA. Cell-bound antibodies were then monitored by the addition of ^{125}I-labelled protein A which binds to the Fc portion of IgG molecules.

The results of 2 experiments are shown in Table 5.1. They indicate that the concentration of T-10-bound antibodies in the immune ascites, greatly exceeded that of the control non-immune ascites.

The specificity of the antibodies. The specificity of the T-10-binding antibodies which were detected in the ascites was determined by pre-absorption of the ascites with monolayers of either T-10 or the syngeneic tumors MA and MCA-1.

The results shown in Table 5.2 indicate that anti-T-10 antibodies could, as expected, be absorbed out with T-10 cells. However, the results also indicate that the antibodies were not specific for this tumor and could be absorbed out by both tumors MA and MCA-1.

Table 5.1. Detection of Anti-T10 Antibodies in Ascites Fluid Using ^{125}I-labelled Protein A[a]

	Sample Tested[c]	Total Volume Collected/Animal (ml)	Cell Bound ^{125}I (cpm)[b] ± S.E. Dilution Tested 0	1:5	1:10	Cell Bound Protein A (x 10^6 µg) 0
Expt. #1	Immune ascites #1	11	910±265	825±34	556±57	50
	Immune ascites #2	55	600±10 (657±60)[e]	329±51	273±28	32.5
	Immune ascites #3	20	700±24 (798±24)	490±32	400±34	38
	Control (non-immune ascites)		153±14	155±10	149±12	8
	Rabbit anti-mouse lymphocyte serum[f]				3,987±211	218 (1:10 dilution)
Expt. #2	Immune ascites #2	40	531±140	372±15	294±10	29
	Immune ascites #3		1,026±48	923±27	862±31	56
	Immune ascites #4		419±14	296±26	242±8	22.5
	Control ascites[d]		189±6	180±10	160±20	10
	Rabbit anti-mouse lymphocyte serum)				2,364±96	129 (1:10 dilution)

[a]Results are expressed as the total ^{125}I count (cpm) that was bound to 2 x 10^5 T10.

[b]Cells were first incubated for 30 minutes at 37°C with 0.05 ml of the ascites fluid. After several washings, a second incubation for 30 minutes at room temperature with ^{125}I-protein A and additional washings followed.

[c]Samples tested were each a pool of 6 tappings obtained from the same animal.

[d]Control ascites was induced in normal non-immunized mice by the same procedure that was used for immunized animals.

[e]Numbers in brackets indicate the ^{125}I bound to T10 cells normally maintained in vivo which were cultured 2 weeks before assay.

[f]Courtesy of Dr. P. Kongshavn, Montreal General Hospital, Montreal, Quebec.

Table 5.2. The Specificity of Anti-T10 Antibodies in Ascites Fluid[a]

Ascites Tested	Cell Bound ^{125}I (cpm) ±S.E.	
	Expt. 1	Expt. 2
Immune ascites[b]	710±25	574±47
Immune ascites absorbed once with T-10	398±25	380±24
Immune ascites absorbed twice with T-10[c]	318±16	N.D.
Immune ascites absorbed once with unrelated tumor[d]	368±13	500±11
Immune ascites absorbed twice with unrelated tumor	341±14	N.D.
Control ascites	191±9	77±4

[a] Results are expressed as total ^{125}I bound to 2 x 10^5 T10 cells (for procedure see legend to Table

[b] All assays were done with a 1:5 dilution of the ascites.

[c] Absorption was by incubation of ascites for 30 minutes at $4^{o}C$ on a monolayer of 5 x 10^5 tumor cells in a culture dish.

[d] Tumor MA was used in experiment 1 and tumor MCA-1 was used in experiment 2.

SUMMARY AND DISCUSSION

An ascites was induced in B6C3 F_1 female mice which were pre-immunized with the tumor T-10 and could reject an inoculum of 1×10^6 viable tumor cell injected in a permissive site. This was done in an attempt to obtain large volumes of anti-tumor antibodies which could then serve as a tool for the analysis of both tumor antigen, and antigen-antibody complexes, in the sera of tumor-bearing mice.

The levels and specificity of antibodies in the ascites were analyzed using radiolabelled protein A as a marker of cell-bound antibodies.

It was found that the immune ascites indeed contained anti T-10 antibodies. The levels of antibody varied from one ascites pool to another and reflected the levels detectable in the serum of the respective donor mice. (serum levels not shown) Absorption of the ascites with MCA-induced tumors other than T-10 revealed that the antibodies were not specific to T-10. However, the two tumors assayed varied in their degree of cross reactivity with T-10. Thus, absorption with the tumor MA was more efficient in removing anti-T-10 reactivity than absorption with the tumor MCA-1. This cross reactivity was not surprising in view of the fact that the tumor line used for immunization was propagated *in vitro*. Antisera raised against serially passaged tumor lines have been shown in the past to react with a wide range of tumor lines whose common characteristic was prolonged propagation in culture, *in vitro*. Virally induced antigens which are commonly expressed on cultured tumor lines, as well as growth medium components, have been suggested as the possible cross-reacting determinants on cultured cell lines (3,4,5).

Additional absorption studies with tumors of either viral or spontaneous origin, as well as with normal or fetal tissues, should be instructive in elucidating the target antigen for the antibodies in the immune ascites.

Preliminary attempts in which the ascitic fluid was used as a probe in serum absorption studies failed to detect tumor antigen in the serum of mice bearing T-10 tumors. However it is unclear, at present, whether this failure reflected the absence of tumor antigens or an inefficiency of the test.

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CHAPTER 6

DISCUSSION

DISCUSSION

The role played by B-lymphocytes and their products in host protection against tumors is a complex and intricate one and at present poorly understood. Reports available in the literature suggest that when antitumor antibodies, are formed, they can play different and opposing roles in relation to the growth of the tumor and either suppress, enhance, or exert no effect on its development. Evidence for the suppressive effects of antibodies is based mainly on *in vitro* studies in which sera from mice inoculated with tumor cells could be shown to lyse tumor targets in the presence of either complement or non-sensitized lymphocytes and macrophages (1, 2). Evidence for enhancement which is derived from both *in vivo* and *in vitro* studies, attributes it to the ability of humoral immune mechanisms to interfere with cell mediated cytotoxicity against tumors (3, 4). It is also possible that tumors vary in their sensitivity to antibody-mediated lysis, and that cells of the same tumor undergo changes in their susceptibility to lysis during tumor growth (5,6).

In this study, an attempt was made to determine the role of B-lymphocytes in host protection against chemically-induced tumors, *in vivo*. To this end, we studied tumor growth in mice which were selectively depleted of their B-lymphocytes (suppressed) by the continuous inoculation from birth, of rabbit anti-mouse IgM serum. We compared their resistance to tumor induction, transplantation and metastasis to that of NRS-treated and/or non treated, control animals.

The results obtained in the course of the study can be divided into two major groups. The first group was derived from *in vivo* studies, while the second is the product of experiments carried out *in vitro*.

In vivo, it was found that the suppressed mice had a heightened resistance to both primary and transplanted tumors. Thus, when 3-MCA was

used to induce intra-muscular tumors, the latent period which preceded the appearance of tumors was longer in the B-lymphocyte depleted group than in their immunocompetent controls. Similarly, when inoculated with syngeneic chemically induced tumor cells, 3 out of 5 tumors grew less well in the suppressed animals even when the tumor inoculum used was 5-10 fold higher than the minimal dose required for 100% take. The other tumors namely MCA-1 and EL-4 grew equally well after a s.c. injection into animals treated with either anti-IgM or NRS. However, when injected i.p., the tumor EL-4 also progressed at a slower rate in the suppressed mice,causing a significant increase in the mean survival time of these animals.

The increased resistance to 3-MCA induced tumors is best exemplified and was best studied with the metastasizing fibrosarcoma T-10. This resistance was manifest in all the parameters analyzed, namely, in a lower incidence of local tumors, a slower rate of tumor growth and a decreased incidence of pulmonary metastasis.

A direct effect of rabbit anti-IgM serum on this tumor was ruled out on the basis of both in vivo and in vitro studies. Thus, the cessation of anti-IgM injections 7 days prior to the inoculation of the tumor failed to modify the rate of growth of the tumors as compared to those growing in mice continuously injected with the antiserum. This was the case in spite of the fact that the discontinuation of serum injections led to the elimination of detectable levels of the antiserum from the circulation by the time tumor was inoculated. Furthermore, mice in a third group included in the same experiment, which were lethally irradiated and then treated with massive doses of anti-IgM, did not exhibit a heightened resistance to the tumor T-10 although anti-IgM was detectable in their circulation. Additionally in vitro, the anti-IgM serum failed to mediate either an ADCC-like, or a complement dependent, lysis of ^{51}Cr-labelled T-10 targets. (results not shown).

Physiological differences between normal and suppressed mice, such as loss of weight or infections in the latter, were also unlikely to be the cause of the slower rate of tumor growth, since the experiments were performed on young (8-12 week old) mice which appeared vigorous and healthy, showed no macroscopic evidence of infection in autopsies, and had a mean weight (monitored prior to experiment) similar to the control group.

The possibility that non-immune mechanisms affected by the continuous administration of the antiserum might have been responsible for the increased lost resistance to tumors could not be completely ruled out. However, as the known target of our treatment was the immune system, it was reasonable to assume that the modulation of this system was at the root of the heightened resistance which we observed.

Antibodies have previously been shown to block cell mediated immune responses against tumors either by binding to tumor cells (7) by forming antibody-antigen complexes (8) or by accelerating the release of tumor antigens into the circulation (6). Moreover B-cell themselves were reported to mediate suppression of cellular cytotoxic immune responses (9). In addition, it was conceivable that the depletion of a major population of lymphocytes resulted in the disruption of the lymphopoietic balance and brought about the enchancement of other immune population(s) or mechanism(s) relevant to host protection against tumors. The enhancement of the T-cell mediated DTH response which was observed in mice following the elimination of their B-lymphocytes by high doses of cyclophosphamide may be one example for such a mechanism.(10).

The injection of serum from suppressed or normal tumor-bearing mice to normal recepients prior to, or together with, the s.c. injection of T-10 cells gave inconclusive results since no difference could be detected between the rate of tumor growth in the recipients of normal serum and the recipients of the immunoglobulin - depleted serum.

In the <u>in vitro</u> study subsequently undertaken, cell mediated anti-tumor reactivity was compared in normal and suppressed mice following the injection

of tumor T-10. It was found that spleen cells from suppressed mice, but not those from normal mice, were cytotoxic to ^{125}IUDR-labelled T-10 target cells. The lack of a detectable cytotoxic response in spleens of normal mice was not surprising. It could in fact be predicted on the basis of numerous reports in the literature that lymphocytes from normal, tumor-bearing mice can give only weak cytotoxic responses against syngeneic chemically induced tumors following a primary challenge with the tumor in vivo (11). These negative findings served however to emphasize the significance of the cytotoxic response detected in spleens of suppressed mice. In the ensuing experiments it was found that this response was not specific to the tumor T-10 and was mediated by a θ-negative, non-phagocytic cell which is a natural resident of the spleens, but not of the lymph-nodes, of suppressed mice.

Further studies, using the NK-sensitive target YAC confirmed that the spleens (and to a lesser extent the BM) of suppressed mice were enriched by a killer cell population with many of the characteristics of the mouse NK cell (12). These characteristics included a non-adherence to nylon wool, a preferential localization in the spleen, an inability to lyse NK-resistant targets P815 and EL-4, and a short-term lytic event (completed in 4 hrs).

Cold target inhibition assays suggested that the lysis of the syngeneic fibrosarcoma T-10 and that of the allogeneic lymphoma YAC were indeed mediated by the same spontaneous killer cell. A microscopic analysis of a killer cell-enriched spleen-cell suspension indicated that the cell was probably a small to medium size lymphocyte, but did not exclude the possibility that granulocytes also played a role in the lysis.

The mechanism for the enhanced NK activity in the suppressed mice is presently unclear. We considered the possiblity that subclinical viral infections, which could not be eliminated due to the absence of a humoral immune response, caused elevated level of interferon to be continuously maintained in the circulation of suppressed mice. This in turn could maintain their high level of NK activity (13). The following three observations indicated that this was probably not the major mechanism

causing the enhanced NK response.

1. In a collaborative study with Dr. R.B. Stewart (Queen's University Kingston, Ontario, Canada), the levels of interferon in the serum of 6-12 week old suppressed and normal mice was ascertained. These levels which ranged from 0-800 units of interferon/ml serum were comparable in the two groups of animals.

2. Interferon-activated NK cells were reported in the past to loose their characteristic target selectivity and lyse tumor cells which are normally insensitive to NK (13). Splenocytes from suppressed mice however, retained their target specificity and could not lyse, or be blocked by, two NK-insensitive targets namely tumors P815 and EL-4.

3. In preliminary assays (not shown) spleen cells from suppressed and NRS-treated mice were incubated with interferon in vitro for periods of 1-2 hours. This resulted in an increase in their lytic activity against YAC which was comparable in both spleen populations. It seems unlikely therefore that there was a difference in their initial state of activation prior to the incubation.

A second plausible cause for the low level of NK activity detected in spleens of normal, relative to suppressed, mice could be the presence of suppressive cells or serum factors which were capable of inhibiting NK activity, in the circulation (and spleens) of the former group (14). Such a suppressive mechanism could actually explain the difference in the slopes of the dose response curves obtained with splenocytes from these mice (Figure 4.3). However, mixing experiments failed to support this interpretation and in fact suggested a slight synergistre effect upon mixing of spleen cells from suppressed and NRS-treated mice. This synergistic effect coupled with the finding that the removal of phagocytic cells from splenocytes of suppressed mice decreases their ability to lyse T-10 targets by 20%, may suggest that macrophages in the spleens of suppressed mice are capable of "amplifying" the activity of NK cells. Such an involvement of macrophages in the lytic reaction could also explain the different slopes of the dose response curves obtained with splenocytes of suppressed and normal mice.

A third possibility to be considered is that the elimination of a major population of lymphocytes from the BM of suppressed mice, by blocking one important pathway of differentiation and maturation, resulted in an increase in the relative availability of stem cells in their hemopoietic organs. This excess pool of stem cells could serve as a rich source of precursor cells for the differentiation pathway leading to mature NK cells. Studies on the "null" lymphocytes population in spleens of suppressed mice are now in progress in a collaborative study with the laboratory of Dr. G. Osmond (Department of Anatomy, McGill University, Montreal, Quebec). This study should be instructive in this respect.

It should be noted in this context that the athymic nu/nu mice were also reported to have an increased NK activity (12). Furthermore, in both the congenitatlly athymic and the B-lymphocyte depleted mice, NK activity was found to be more stable than in normal mice and less dependent on the age of the mouse. (Ref. 13 and Figure 4.6). These findings in the nude mice among others, prompted R.H. Herberman and his colleagues to suggest that NK cells are Fc-receptor positive pre-T-cells and can, under a thymic influence, mature into functional T cells (13). It is unclear whether this is also the case for all NK cells detected in the B-lymphocyte depleted mice. Fc-receptors could, under our conditions, be detected on only a small fraction (approx. 20%) of the NK cells. Furthermore, B-cell depleted mice although not defecient in T-cell functions were not reported to have an enhanced T-cell reactivity (15). Such an enhancement should have been expected if the findings presented in this study were to be interpreted on the basis of the Herberman model.

Other studies on the effect of B-lymphocyte depletion on NK activity range in their findings from a lack of a detectable effect (13) to a slightly enchanced activity (16). The reasons for the differences between these reports and our observations are not clear. They may be related to the protocol of immunosuppression, to the housing and environmental conditions in which mice were kept, or to differences in the assay system.

Of major importance to this study is the question of the relevance of the NK cell to host protection against tumors in vivo. As was already pointed out in the review of the literature (P. 34), direct evidence in support of an active role played by NK cells in the control of tumor growth is presently scarce. (13). In our system, the evidence which links the increased resistance to malignancy observed in B-lymphocyte depleted mice to their heightened NK response is based on several observations which were made both in vitro and in vivo.

In vitro, using the cold target inhibition assay to study the spectrum of specificities of the NK cells from suppressed mice, we found that tumors T-10, MCA-2, and MCA-3 could all block the lysis of ^{51}Cr-labelled YAC targets in a specific manner. On the other hand, tumors EL-4 and MCA-1 caused only a non specific interference of the lytic reaction. (For the distinction between specific and non specific inhibition (see p.128 and Ref. 17).

A close examination of the data on the growth of these tumors in suppressed and NRS-treated mice will reveal an interesting correlation between the in vitro and in vivo results. Thus, while suppressed mice showed a heightened resistance to the s.c. growth of the tumors T-10, MCA-2 and NCA-3, they were as susceptible as NRS-treated controls to the local growth of tumors MCA-1 and EL-4.

A similar correlation between the sensitivi:of a tumor to NK cells in vitro and their growth in vivo was also reported in studies with nude mice (12). In these studies it was interpreted as evidence for the host protective role played by NK cells in vivo. In other studies with the athymic mice a resistance to tumor induction and tumor transplantation reminiscent of the resistance found in the B-depleted mouse was also observed.(12,18). These suggests that a common mechanism of host protection against tumors may indeed be operating in the suppressed and athymic mice, and that in both cases it may be mediated by the enriched NK cells.

An even more convincing argument in favor of the *in vivo* relevance of the NK cells in suppressed mice, is probably provided by a series of observations made in both tumor-related and non-related studies with these mice. It was found, that the B-lymphocyte depleted mice displayed a heightened resistance to maternal BM, and had a higher mean survival time after the intraperitoneal injection of the maternal leukemia EL-4. Additionately it was found that, following the i.v. injection of EL-4, the suppressed mice could eliminate it from their circulation at a significantly higher rate than normal mice. Furthermore, in preliminary (not published) studies done in collaboration with Dr. P. Kongshavn (Department of Physiology, McGill University, Montreal, Quebec) it was found that suppressed mice had a heightened resistance to infection with the intra-cellular parasite *Listeria monocytogenes*.

It seems, therefore, that the suppressed mice have an enhanced natural resistance not only to syngeneic tumors but also to semisyngeneic (hemopoietic or tumor) grafts and to intracellular parasites. That these seemingly different immune phenomena are indeed only different manifestations of one wide-ranging natural resistance system has already been suggested by Cudkowicz and coworkers (14). They supported their claim by evidence of a striking parallelism in the factors which influence and regulate these mechanisms of immunity (ibid). The fact that in the suppressed mice all three measurable parameters of these natural immune system are elevated supports this claim. It also suggests that, similarly to the heightened resistance to semisyngeneic grafts and parasites which are evident *in vivo*, the heightened NK response, measurable only *in vitro*, also plays a significant host protective role *in vivo*.

The fact that similar findings were also reported for the nude mice (14) may suggest that the depletion of one central immune mechanism can bring about a "compensatory" enhancement of the natural defences available to the deprived animal.

The process of recruitment of NK cells to the local site of the tumor is poorly understood. The finding of NK cells in the tumor mass has only been reported by one laboratory (19). One could envisage however a mechanism of protection provided by circulating NK cells which, upon contact with disseminating tumor cells, release lytic enzymes carried to the tumor site via the circulation. The involvement of lytic enzymes in the NK mediated cytotoxicity was suggested on the basis of results obtained with the human NK system (20). This mode of protection could explain the decreased incidence of pulmonary metastasis observed in the suppressed mice. It may also shed light on the observation that significant differences in the mean size of tumors of suppressed and control mice are only detectable after the tumors in both groups reach a comparable minimal size. (see P.100). One could postulate that the stimulation of NK cells requires the presence of disseminating tumor cells in the circulation which in turn is dependent on the progression of the local tumor to a characteristic size.

Additional experiments are required in order to firmly establish the relevance of the heightened NK response of suppressed mice to their increased resistance to malignancy. One useful approach may be the treatment of suppressed mice with agents such as ^{89}Sr or β-estradiol which were shown to cause the destruction of the BM and consequently the elimination of NK cells (21,22).

On the basis of the results presented in this study, it is possible to conclude, however, that the clinically important processes of tumor induction and tumor metastasis may be controlled by common mechanisms. Among these protective mechanisms, natural killer cells may be playing a central role whereas humoral immune responses are probably of secondary importance.

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