REDUCED NATURAL ANTIBODY AND INCREASED TUMOR SUSCEPTIBILITY IN XID-BEARING B-CELL DEFICIENT MICE

BY

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TABLE OF CONTENTS

ABSTRACT

Evidence that natural antibodies contribute to the resistance against small tumor foci *in vivo* has been restricted to correlative studies and Winn-type assays. The low levels of IgG3 and IgM in the unstimulated serum of CBA/N mice and male (CBA/N x CBA/J) F1 mice associated with the xid-mutation has provided a genetic model to further test the hypothesis that natural antibodies are important mediators in host natural resistance. RI-28, a radiation induced leukemia of the CBA/H strain, was used to test the tumor incidence exhibited by these mice. The tumor susceptibility of threshold sc inocula in xid-bearing CBA/N and F1 male mice was significantly higher than that of the CBA/J and F1 female mice. The differences in tumorigenicity displayed by the xid and normal mice was inversely related to their levels of anti-RI-28 natural antibodies. This observation was further substantiated by comparing the NAb binding activity and tumor fate in individual (CBA/N x CBA/J) F1 mice. Tumors appeared in only 26.3% of the animals exhibiting high NAb activity compared to a tumor incidence of 77.3% in the animals with low NAb activity. Natural killer cells and macrophages have been implicated as mediators of tumor surveillance but no correspondence could be demonstrated between the activity of these effector cells and the differences in tumor incidence. The cytolysis by NK

cells and activated macrophages as well as the levels of anti-tumor NAb were tested whereby each of the mediators was isolated from the same individual F1 mouse. No consistent correlations could be demonstrated between the cellular effectors, therefore the differences in tumor susceptibility were probably not due to deficiencies in NK or macrophage activity. The inverse relationship between anti-TI-28 NAb levels and tumor incidence provide the first genetic evidence that NAb is an important mediator in tumor surveillance.

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LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
Con A	Concanavalin A
EDTA	Ethylenediamine tetraacetate
E:T	Effector to target cell ratio
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothyocyanate
FM	Fisher's medium
HBSS	Hank's Balanced salt solution
[^{131}I]dUrd	[^{131}I]-5-iodo-2'-deoxyuridine
IFN	Interferon
Ig	Immunoglobulin
ip	Intraperitoneal
iv	Intravenous
LPS	Lipopolysaccharide
MLR	Mixed lymphocyte reaction
Mls	Minor lymphocyte stimulating locus
NAb	Natural antibody
NK	Natural killer
NKr	Natural killer resistant
NKs	Natural killer sensitive
NR	Natural resistance
PBS	Phosphate buffered saline
PC	Phosphocholine
poly (I-C)	Polyriboinosinic-polyribocytidilic acid

RBC	Red blood cells
RPMI	Roswell Park Memorial Inst. 1640 medium
RT	Room temperature
sc	Subcutaneous
SRBC	Sheep red blood cells
TD	Thymic dependent
TI	Thymic independent
TNP	Trinitrophenyl
xid	X-linked immune deficiency

INTRODUCTION

Tumor Progression

In 1935, while studying tar and virus induced papillomas in rabbits, Rous and Beard concluded that malignancy was the result of a variety of influences which occur as the cancerous state develops (1). They suggested that both types of tumors, namely, tar or virus induced, arose from a single type of epidermal cell yet the resulting tumor cells had a variety of forms due to cellular alterations of that cell type. In subsequent studies, Rous and Kidd did a point by point analysis of the tar and virus papillomas (2). They found that the two distinct tumors had similar neoplastic effects and both exhibited the same limited diversity. These early studies led researchers to speculate that all tumor development followed a progressive series of necessary changes.

L. Foulds defined tumor progression as a series of permanent, irreversible changes occurring in one or more of the characteristics of the tumor (3). It had been shown that two separate elements could be distinguished in carcinogenesis, namely, tumor initiation and tumor promotion (4). Initiating agents caused normal cells to become latent tumor cells and this process was found to be irreversible. Promoting agents stimulated proliferation by producing

conditions which were favorable for cell growth in general and this process was reversible.

Other studies demonstrated that serial transplantation of tumors into normal mice resulted in tumor cells which were more aggressive than the original cells and that this progression was not dependent on the action of the carcinogen (5-7). As a result of these and his own studies, Foulds suggested that neoplasms develop and progress by the acquisition of new properties which are expressed in their behaviour patterns (Foulds 8, 9).

Tumor progression is a process of genetic and epigenetic development and these steps occur before the tumor becomes biologically or cytologically evident. Variants are generated in the preneoplastic cells such that they possess essential properties which allow them to proliferate. For example, these cells may develop variant characteristics which render them independent from local growth factors. The acquisition of these new, inheritable properties may be influenced by various environmental and host factors. The continuous operation of selective forces produce tumor populations with reduced heterogeneity and this variation and progression proceeds in the direction of freedom from growth regulation mechanisms <u>in vivo.</u> The changes that occur in the neoplasia may be the result of either genetic or epigenetic phenomena (10).

In this report a threshold tumor inoculum model is used to simulate the evolution of a spontaneous tumor. Threshold inoculum models have been used to support the tumor progression theory in that cloned tumors develop variants during passage in vivo (11, 12). By using a small number of tumor cells this should allow the growing foci to come in contact with specific and non-specific host immune mechanisms in a way that is similar to a spontaneously arising nascent tumor.

Natural Immunity:

This laboratory has been primarily interested in the early stages of neoplasia and thus has focused on natural immune systems which may play a role in tumor surveillance. The theory of immune surveillance was first proposed by Ehrlich in 1909 and it was later reformulated in 1959 by Thomas (13, 14). Foley provided correlative evidence for this theory by showing that mouse tumors could be immunogenic for their host (15). These early studies led researchers to search for the underlying mechanisms that were responsible.

Induced, antigen-specific immunity and natural immunity are thought to be the two major immune mechanisms that play a role in the defense against arising tumors (16). Induced cell-mediated immunity requires the expression of strong specific tumor-associated antigens which are recognized by T

cells. These cells may be quite effective in mediating antitumor responses, but some sort of priming is necessary. Since athymic and euthymic mice do not exhibit a higher incidence of spontaneous and carcinogen-induced neoplasms, the importance of these T cells is questionable (17, 19). Therefore, researchers began to focus on naturally occurring processes as the relevant mechanisms in tumor surveillance.

The thymus-independent processes which may be involved in anti-tumor resistance include natural killer (NK) cells, activated macrophages, and natural antibodies (NAb). The presence of these natural resistance mechanisms has been demonstrated in both in vivo and in vitro studies (20-27). These effector cells as well as NAb do not require induction for function and they can react against a broader range of cell surface markers compared to thymus-dependent mechanisms. This natural immunity may play an important role in the hosts' first line of defense against tumors, or it may contribute to the generation of more resistant and aggressive tumor variants.

Natural Effector Mechanisms

(i) NK Cells

Interest in natural killer (NK) cells came about as a result of the discovery of the autosomal recessive nu gene and its associated T lymphocyte defect in homozygote mice (28, 29). Researchers had been focusing their attention on

T cells as the major cellular immune mechanism in surveillance and cytolytic activity against growing tumors. The search for another mechanism began when Rygaard and Povlson showed that homozygous nu/nu (nude) mice had a very low incidence of spontaneous tumors (18). Additional evidence for the presence of another tumor surveillance effector was indicated when nude mice failed to exhibit an increased incidence of malignant neoplasia (carcinogen induced) compared to heterozygous littermates or normal mice (30-32).

Natural killer cells are lymphocytes that are capable of lysing tumor targets without being specifically induced. These cells stem from bone marrow and are found predominantly in the spleen or peripheral blood. They are neither T- or B-cells, are nonadherent and lack the characteristics of phagocytic monocytes (33-36). In rodents, the expression of NK cells is age-related and the cytolytic activity reaches a maximum level during the second and third months of life (35, 37). The NK cell is under genetic control and high reactivity is dominant over low reactivity in F1 crosses (38). The human counterpart to these cells is thought to be the large granular lymphocyte (LGL) which differs from the murine NK cell in a number of ways. The LGL exhibit T-cell properties such as sensitivity to anti-T-serum and these cells express Fc and complement receptors (39, 40).

A number of host factors have been shown to influence

the level of NK activity. For example, agents which stimulate the production of interferon increase NK activity, enhance the range of sensitive targets and modulate the morphology of NK cells (41, 42). Herberman demonstrated that macrophages were a necessary component for the interferon enhanced NK activity (40). Macrophages can also mediate the suppression of NK cells (43). A regulatory function for T cells has also been proposed since nude mice display a wider range of specificities and show less restricted age-dependence than conventional mice (44).

Over the past two decades, evidence has been accumulating which indicates that NK cells play an important role in natural resistance against tumors. Kiessling reported that normal mice had killer cells capable of rapid, cytolytic, specific activity against in vitro grown mouse Moloney leukemia cells (37). They also demonstrated that "high" NK strains were more resistant to a small inoculum of NK sensitive (NKs) tumors than were "low" NK animals (45). Further to this, Warner et al demonstrated that tumor cell lines which were susceptible to in vitro NK cytolysis showed a reduced growth rate in syngeneic nude mice (47). Talmadge et al found that when beige mice (which are NK deficient) were injected with a NKs tumor, they developed tumors which grew more rapidly and displayed more metastases than the same tumor injected into normally NK-constituted (+/bg) mice (46). Karre and colleagues demonstrated that beige mice

exhibited faster growing tumors and died earlier when challenged with threshold s.c. inocula of 3 different syngeneic leukemias compared to normal heterozygotes (+/bg). They also showed that bg/bg mice exhibited depressed splenic killing of leukemia cells *in vitro* (20). Though this evidence is indirect, it does provide evidence for the importance of NK cells in natural resistance to tumors.

(ii) Activated Macrophage

The concept of macrophages being involved in antitumor immunity was suggested by Gorer in 1956 (48). Later studies demonstrated that macrophages could lyse tumor targets *in vitro* in response to lymphokines (49). Some of the lymphokines include specific Macrophage Arming Factor and Macrophage Activating Factor which are capable of rendering macrophages specifically or non-specifically cytotoxic to tumor targets (50-53). It is also well known that macrophages can be activated *in vivo* by non-specific stimulants such as endotoxin, peptone, *Corynebacterium* and *Mycobacterium butyricum* (54-56, 23). After injection of these stimulants, macrophages are capable of lysing syngeneic, allogeneic and xenogeneic tumor cells *in vitro.*

Macrophages may participate in host defense against tumors in one of two ways. They may mediate the processing and presentation of cellular antigens or they may cause tumor cell lysis directly. Evans showed that macrophages

infiltrate the tumor site in large numbers and Alexander demonstrated that macrophages isolated from tumor-bearing mice can inhibit tumor growth *in vitro* (57, 58). A number of investigators have shown that tumor growth *in vivo* could be accelerated by treating animals with agents that are inhibitory to macrophages (23, 59, 60). Conversely, agents which stimulate macrophages such as *C.parvum*, *M.butyricum* and proteose peptone, decreased the tumor frequency of small tumor inocula, inhibited tumor growth and reduced metastases (23, 61, 62). In addition, Alexander et al found an inverse correlation between the number of macrophages in the tumor and metastases (63). This is just some of the evidence which indicates the macrophages may inhibit tumor growth as well as limit the metastatic spread of tumors.

(iii) Natural Antibody

Antibodies which exist in the serum of animals which have not been intentionally immunized are defined as natural antibodies. The antigenic reactivity of NAb is extensive and includes antigens found on normal cell surfaces. For example, NAb have been found which react with structural virion proteins of human and murine retroviruses (64, 65), human DNA virus antigens (66), H-2 associated antigens (67), a large array of normal cell surfaces [e.g. brain, fibroblasts, erythrocytes, lymphocytes (68-70)], and finally

tumor cells such as plasma cell tumors (71), leukemias (72, 73), and lymphomas (74). These are true antibodies although there is evidence which suggests that the binding affinity of NAb may be lower than that of induced antibodies (75, 76).

It has been proposed that NAb production occurs as a result of stimulation from external environmental antigens and endogenous antigens. It has been well documented that gut microorganisms and viruses can stimulate NAb production (77, 78). For example, antigenic challenge from viruses might occur as a result of horizontal transmission of contagious virus or vertical transmission by integrated proviral DNA. The presence of extensive autoantibodies suggests that auto-reactive B cell clones are not eliminated during fetal life and instead there exists an active regulatory mechanism which controls these cells.

In this report the focus is on antitumor NAb, therefore a brief description of some of the properties of these antibodies is necessary. Martin and Martin found that mice had NAb which were directed against type C viral envelope antigens and this was interesting since type C viruses were frequently found in murine tumors (79). They suggested that these antitumor NAb could assist in the immunological recognition of neoplasia. Wolosin and Greenberg tested several different tumor cell lines and they found that these cells acquired immunoglobulin (Ig) very rapidly after implantation

into normal syngeneic mice. They demonstrated a high correlation between the amount of Ig bound in vivo and the amount of Ig bound to the same cells after in vitro incubation with normal syngeneic serum. These findings suggested to them that the Ig acquired in vivo represented NAb binding (80). Since the capacity to reject syngeneic tumors occurs only if a small inoculum is used, it seems reasonable to suggest that an immune surveillance system must act quickly before the tumor reaches a critical mass (81). The acquisition of NAb occurs very quickly and this supports the idea that NAb may play a role in tumor surveillance at an early stage in tumor development.

Studies of the genetics of natural antitumor antibody have found that high serum NAb levels are inherited recessively (67, 82). This differs from NK cells since high NK cell activity is codominantly inherited; therefore, the NK receptor is probably not passively acquired NAb. NK cell activity declines in older animals, whereas the levels of NAb remain relatively constant. Also, beige mice express normal levels of NAb despite their NK cell deficit. Despite these differences in the genetics of NAb and NK cells, Chow, Wolosin and Greenberg demonstrated some common features in the regulation of these activities. For example, interferon inducers or microbial products such as proteose peptone and lipopolysaccharide can increase the levels of NAb and NK cells (82). Interestingly, if NKr tumor cells are treated

with interferon (IFN) directly, the reactivity of tumor cells to serum NAb increases *in vitro* and the *in vivo*, i.p. acquisition of NAb also increases. That is, the host anti-tumor resistance may be enhanced by IFN by increasing tumor reactivity to antibody (83).

A number of studies have provided correlative evidence which suggests that NAb contributes to host-mediated natural resistance (NR) against neoplasia. For example, Gronberg et al found that YAC lymphoma which had been grown *in vivo* bound low levels of NAb and was resistant to NK cell cytolysis and after 2 to 3 weeks of growth *in vitro* the YAC cell line had become NKs but remained resistant to NAb. They selected YAC variants for low NK sensitivity and the degree of NAb able to absorb to these cells varied considerably. Tunicamycin treatment decreased the sensitivity to NAb lysis but had no effect on NK cell cytolysis therefore they suggested that the target structure recognized by NAb included asparagine-linked saccharides (198). Chow et al found that animals treated with adjuvant, which was known to increase NAb levels, caused the mice to be more resistant to small sc tumor inocula. They also demonstrated in a Winn-type assay that tumor cells coated with NAb were less tumorigenic than control cells (25).

Finally, several reports have indicated that *in vivo* grown tumor cells display a decreased sensitivity to NR as demonstrated by [131I]-5-iodo-2'-deoxyuridine elimination

assays, NAb and complement lysis, NK cell cytolysis and hypotonic lysis (12, 199-201). For example, L5178Y-F9 grown in vivo in syngeneic DBA/2 mice were not eliminated as rapidly as the in vitro grown cells in the [131I]-dUrd elimination assay and the in vivo L5178Y-F9 exhibited a range of susceptibility to Nab and complement (12). L5178Y-F9 grown ip or sc or removed from spleen, brain and lungs after intravenous (iv) inoculation displayed a reduced sensitivity to complement-mediated NAb lysis. L5178Y-F9 passaged sc also bound less NAb consistent with growth in vivo under the selective pressure of NAb mediated defense mechanisms (200). This supports the hypothesis that NAb can contribute to antitumor defense including the metastatic spread of tumors. The decreases in susceptibility to NR were shown to be time-dependent and the in vivo grown tumor cell lines exhibited an increased tumorigenicity (199). Finally, Brown et al demonstrated that in vivo grown L5178Y-F9 and SL2-5 lymphoma had reduced sensitivity to hypotonic lysis and bound less NAb measured through fluorescence activated cell sorting (201).

NAb has the capacity to exert its antitumor effects in one of two ways. The Ig might combine with complement to lyse tumor cells or they may activate nonimmune lymphocytes or macrophages to become killer cells in antibody-dependent cellular cytotoxicity (ADCC) reactions (84). Unfortunately, antitumor antibodies have also been shown to protect tumor

cells and enhance their growth. For example, Hellstrom and Hellstrom demonstrated that sera from mice whose tumors had regressed were capable of tumor cell lysis *in vitro* whereas sera from tumor bearing animals was not cytotoxic (85, 86). These serum blocking factors were found in a number of other cell systems and proposals were made that they may be antigen-antibody complexes which bind to either the target or the killer cell (87, 88). Antitumor antibodies may also induce the release of tumor antigens which may block cell-mediated cytotoxicity by binding to receptors on those cells (88). These detrimental blocking antibodies are usually reactive with specific tumor associated antigens. Since NAb are reactive with a wide spectrum of antigens other than just these specific tumor antigens it is likely that NAb assists in the lysis of tumors rather than enhancing the growth of tumors (16).

(iv) Interaction between Effector Mechanisms

Antitumor immunity is obviously very complex, therefore it would be unrealistic to believe that one effector population was more important than another. Many reports have indicated that these effectors work together either by direct contact or through communication via lymphokines. This interaction among effector cell populations could either result in an active antitumor response or result in suppression of the antitumor response (16).

The following is a brief summary of some of the evidence which indicates that antitumor processes can be enhanced or require more than one of the effector systems previously described:

(a) Matthews et al found that a hyperimmune antitumor serum could suppress the outgrowth of tumor and that this effect could be enhanced by i.p. injection of C.parvum which is known to increase intraperitoneal macrophage numbers (89).

(b) Haskill demonstrated that macrophages isolated from a mammary adenocarcinoma had little cytotoxic effect unless in the presence of antibody (90).

(c) Greenberg, Shen and Medley found that different tumor cells could be killed by non-immune spleen cells in the presence of antibody but the effectiveness of the killing varied markedly. They also found that an allo-antibody-coated SL2 lymphoma was sensitive to lysis by non-phagocytic spleen cells. In addition, they demonstrated that the growth of alloantibody-coated P-815-Y mastocytoma could be inhibited in the presence or normal spleen cells and antibody, yet these cells were relatively resistant to cytolysis (91).

(d) Tumor-activated human NK cells were found to rapidly release factors which trigger a respiratory burst in monocytes. A preformed macrophage-activating factor

was released when LGL were incubated with a NKs tumor cell or heat-killed Staphylococcus aureus and this factor enhanced the intracellular killing of S.aureus by rat and human alveolar macrophages (92, 93).

(e) Urban and Schreiber demonstrated that tumors could develop macrophage resistance after passage in vivo but this selection was dependent on the presence of functional tumor-specific T cells (94).

The preceding reports indicate than an antitumor response involves communication between T cells, macrophages, NK cells and antibody. This network probably also requires as yet unidentified cell types and factors.

X-linked Immunodeficiency (xid) in Mice

An abnormality was discovered in the CBA/N mouse strain when it was shown that the mice had an X-linked deficiency which affected their ability to respond to polysaccharide antigens (95, 96). Studies of the CBA/N mice have identified the gene(s) responsible for the immune deficiency and this gene(s) has been named xid. The CBA/N (also known as CBA/HN) subline was derived from the CBA/Harwell line. In 1963 a homozygous lethal autosomal recessive gene resulting in foam cell reticulosis (fm+) was found in the offspring of a few breeding pairs of CBA/H mice (97). The CBA/N mice were derived from fm+/fm- heterozygotes by mating fm-/fm-offspring. In 1968 the subline was almost lost, but one pregnant

female survived and all subsequent CBA/N mice are derived from her litter (98). Hemizygous (xid/Y) males and homozygous (xid/xid) females were found to be unresponsive to certain T-cell independent antigens and these mice exhibited reduced responses to T-cell dependent antigens. These mice have reduced serum IgM and IgG3 levels and they fail to respond to B cell mitogens. The B cells of xid-bearing animals lack several differentiation antigens usually found on normal B cells. Conversely, the T-cell, macrophage and NK cell activity of xid mice appear to function normally (99). The following discussion will summarize these properties of xid-bearing animals.

(i) Thymic-independent Immune Responses

Thymic-independent antigens (TI) are those in which antibodies are produced without T-cell help. These TI antigens are large polymeric molecules which are slowly degraded and persist a long time in vivo (100-102). Adult CBA/N mice expressing the xid defect were found to respond differently to various TI antigens. Mosier et al characterized TI antigens as type 1 (TI-1) or type 2 (TI-2) by their ability to induce an antibody response in CBA/N mice (103, 104). If a TI antigen can elicit an immune response in CBA/N mice, then it is considered to be TI-1, and if no response can be detected, then the antigen is classified as TI-2. The antigens trinitrophenyl-conjugated lipopolysaccharide (TNP-LPS) and TNP-Brucella abortus (TNP-BA) are TI-1

antigens since CBA/N mice can respond immunologically to them (105-107). The response of xid spleen cells to TI-1 antigens results in the production of IgG2, IgG3 and IgM antibodies and the presence of macrophages or T-cells is not required since depletion of these cell types from in vitro cultures has no effect on the immune response. On the other hand, the TI-2 antigens, such as TNP-Ficoll, do not elicit an immune response in CBA/N mice. Responses to these TI-2 antigens by non-xid mice are dependent on the presence of macrophages and small numbers of T-cells and the antibodies produced are predominantly of the IgG3 and IgM class (108-111).

(ii) Thymic-Dependent Immune Responses

Thymic-dependent (TD) antigens such as sheep red blood cells (SRBC), and TNP keyhole limpet hemocyanin (TNP-KLH) require T-cell help in order to elicit an immune response in mice. B-cell deficient mice can develop measurable primary responses to TD antigens such as SRBC but the levels of IgM and IgG compared to normal mice are only 10-50% and 1-10% respectively. Optimal concentrations of SRBC had to be used in order to measure an immune response from xid-bearing (CBA/N x DBA/2) F1 male mice. When these mice, normal F1 females and immune deficient F1 males, were challenged again with SRBC, the titers of their sera were comparable (112). Stein demonstrated that after primary challenge with DNP-KLH, the sera of F1 females had an antigen-binding capacity

that was greater than the F1 male sera. After secondary challenge with the TD antigen, the binding capacity of the male and female sera was the same (113). This data suggests that some component of the primary response of the xid-bearing F1 male mice is impaired. Greenstein demonstrated that (CBA/N x B10) F1 male B cells were unresponsive to erythrocyte bound antigens in the presence of nonspecific T-cell helper activity but if the T-cells were first primed with RBC then an immune response was exhibited by the B cells from xid-bearing mice (114). In addition, Boswell et al found that helper T-cells and antigen-presenting accessory cells from the F1 male mice were functionally as competent as cells from normal F1 females (115).

(iii) Immune Responses to Infectious Agents

CBA/N and xid-bearing mice appear to have an altered immunity to naturally occurring infectious agents. O'Brien et al found that CBA/N mice were more susceptible to Salmonella typhimurium and Listeria monocytogenes than normal mice (116). In another study, O'Brien et al demonstrated that the transfer of normal immune serum to xid F1 males protected them from a lethal challenge of S.typhimurium, therefore, it was suggested that xid B cells are the major cause of the increased susceptibility of these mice (117). Other infectious agents, such as malaria, have been tested in xid mice. Hunter found that after malaria infection the polyclonal IgM and IgG responses of (CBA x DBA/2) F1 male

mice were delayed and the sera levels of those immunoglobulins were lower than normal F1 female mice. He also demonstrated that specific IgM antiplasmodial antibodies remained depressed during the infection, whereas IgG antiplasmodial antibodies approached normal levels after some time (118).

Similarly, xid-bearing mice fail to produce antiphosphocholine (PC) antibody in response to the PC in the capsules of Ascaris suum or the C5 variant of S.pneumoniae (119, 120). In normal BALB/c mice, anti-PC antibodies are predominantly of the T15 idiotype and IgG3 and IgM are the major immunoglobulins produced (121). The xid-bearing (CBA/N x BALB/c) F1 male mice fail to make these predominant antibodies in response to PC antigens such as PC-KLH or PC-LPS (122, 123). Kenny et al suggest that the T15 idiotype dominance may be controlled by Lyb-5+ B cells since these cells are deficient in xid mice (124).

(iv) Serum Immunoglobulin Levels

In 1974 Amsbaugh showed that the serum IgM levels of CBA/N mice were only 20% of normal and the administration of Boivin-LPS or phenol-LPS resulted in large increases in those levels (125). Further studies of serum Ig isotypes revealed that IgG3 was also low at about 15% of normal, but that this isotype did not increase after LPS stimulation. The levels of IgG1, IgG2a, IgG2b, and IgA in immune-defective (CBA/N x DBA/2) F1 male mice were found to be similar to levels measured in normal F1 females (121).

(v) Functional properties of T-cells, macrophage, NK cells and Accessory cells in xid mice

Scher et al found that B-cell deficient xid mice were able to reject allogeneic skin grafts and they were able to kill allogeneic tumor cells as well as normal mice in in vitro T-lymphocyte mediated cytotoxicity tests. Also the T-cell mitogen responses to ConA or phytohemagglutinin were equivalent when xid-bearing (CBA/N x DBA/2) F1 male mice were compared to normal F1 female mice (126). As was mentioned previously, xid-bearing mice have a low primary response to TD antigens but Boswell demonstrated by adoptive transfer studies that the abnormality was not due to T helper cells or accessory cell presentation (115). Similarly, Scher et al found that activated T cells from xid mice were as competent as normal activated T cells from F1 females in adoptive transfer experiments (112). Many of the abnormalities exhibited by xid mice appear to be intrinsic to the B cells and not due to T-cell abnormalities.

Macrophages are known to release lymphocyte-activating factor (LAF) and prostaglandins when exposed to LPS. Rosenstreich demonstrated that macrophages derived from both (CBA/N x DBA/2) F1 male and female mice produced equivalent amounts of these factors in response to various concentrations of LPS (127). The antigen-presenting functions of macrophages have also been tested by using preparations of

macrophage enriched, spleen adherent cells. These preparations may contain nonphagocytic accessory cells as well. Boswell compared the ability of normal (DBA/2 x CBA/N) F1 male and immune defective (CBA/N x DBA/2) F1 male spleen adherent cells to present TNP-keyhole limpet hemocyanin or TNP-Ficoll to normal (adherent cell depleted) spleen cells and he found them to be equivalent (128).

Lastly, a number of researchers have tested the ability of immune defective NK cells to lyse tumor cells or chicken RBC in unprimed in vitro cytotoxicity assays. The NK cells from normal and xid-bearing mice displayed equivalent cytolytic activity (126, 129, 130).

These findings indicate that the X-linked defect found in CBA/N mice and F1 male mice has little or no effect on T-cells, macrophages, NK cells or accessory cells. The xid gene(s) affects the B cells of these animals and in particular, those B cells responsible for the production of IgM and IgG3 antibodies.

(vi) B cells of xid mice

The B cells of xid-bearing mice display a variety of abnormalities, some of which have already been discussed. These immune deficient mice do not respond to TI-2 antigens, display reduced primary responses to TD antigens, have an increased susceptibility to infectious agents and exhibit reduced serum IgG3 and IgM levels. The B cells from adult CBA/N mice resemble the B cells from normal neonatal mice

and some of these similarities will be discussed.

Mitogens such as LPS and polyriboinosinic-polyribocytidilic acid (poly I-C) or anti-Ig antibodies are known to directly stimulate proliferation in B cells and not T cells. It has been demonstrated that xid-bearing mice exhibit greatly reduced responses to the above mitogens. Suppressor T cells were ruled out since (CBA/N x DBA/2) F1 male immune defective spleen cells depleted of T cells by anti-Thy antibody and complement treatment displayed the same reduced response (126, 105). Similarly, cultures of B cells from xid mice did not exhibit proliferation when exposed to anti-K and anti- antibodies and this lack of response was shown to be T-cell independent (131-134).

Metcalf et al were interested in the susceptibility of xid mice to tolerance induction when exposed to tolerogen *in vitro.* It was known that IgM producing adult bone marrow B cells were reduced in their system and that both IgM- and IgG-producing neonatal B cells were reduced. The splenic B cells of xid-bearing mice were found to resemble neonatal B cells and not adult bone marrow B cells (135, 136).

The *in vitro* B cell colony formation of CBA/N mice was examined by Kincade. He found that these mice lacked the ability to form B cell colonies and that this inability was not due to a lack of granulocyte-macrophage progenitors or multipotential stem cells in the bone marrow of these mice. He concluded that xid mice lacked progenitor cells which

represent a lineage of functionally specialized B cells (137).

Since xid mice appear to lack a subpopulation of B cells a number of researchers have examined the surface determinants of the B cells from these animals. Surface immunoglobulin isotypes, I-region-associated antigens, minor lymphocyte-stimulating determinants, complement receptors and Lyb antigens have all been characterized for xid mice.

The splenic B cells of immune defective mice exhibit a much higher to surface Ig ratio than B cells from normal mice. This high ratio was not from a high incidence of + - cells which are the predominant type of cells found in the spleens of neonatal (1 to 4 days old) normal mice. Rather, the cells from xid mice have a relatively high density of on their B cells, thus resembling the B cells of developing immature (2 to 3 weeks old) normal mice (138).

Huber found that the B cells of xid-bearing mice lack the surface Ia antigen Ia.W39 which is an I-A specific determinant present on 50% of normal Lyb-3+ B cells (139).

The mixed lymphocyte reaction (MLR) is under the genetic control of the major histocompatibility complex and the minor lymphocyte-stimulating (Mls) locus and these Mls-determined markers are present on B cells and not T cells. It has been demonstrated that CBA/N mice fail to induce an Mls-determined MLR and this inability appeared to be due to

the lack of these determinants on the B cells of CBA/N mice (140, 141).

Mond et al studied the presence of complement receptor on the spleen cells of xid-bearing F1 males and normal F1 females. They found that only 10% of the B cells from adult male F1 mice were CR+ compared to 50% CR+ in F1 females (reviewed in 142).

Finally, several studies have detected determinants which are found on normal adult B cells but not immune-defective B cells. That is, B cells from xid mice lack the cell surface determinants Lyb-3, Lyb-5 and Lyb-7. It is possible that the antisera used to detect Lyb-5 and Lyb-7 determinants were directed against MIs-encoded antigens but it was shown that the genes coding for these determinants segregated independently (143-145).

The absence of the Lyb-5 determinant on xid B cells is not absolute since a small percentage of splenic B cells are Lyb-5+ (146). It was predicted that normal spleen cells also have a subpopulation of B cells which express the Lyb 5- phenotype. By using anti-Lyb 5 antisera to kill the Lyb 5+ cells in normal spleen cell preparations, the remaining Lyb 5- cells could be analyzed. These normal Lyb 5- cells were unable to stimulate MIs-encoded MLR, failed to respond to TNP-Ficoll but made excellent responses to TNP-Brucella abortus and therefore resemble the B cells from adult xid-bearing and normal neonatal mice (144, 140, 115, 147).

These findings suggest that the absence of Lyb 5+ B cells in xid mice is responsible for the above immune defects.

In the early 1980's it was discovered that some B cells express the Ly-1 antigen which was previously thought to be restricted to T cells (181,182). In normal and xid-bearing mice the Ly 1+ B cells appear early during ontogeny and quickly reach adult levels but their frequency relative to other lymphocyte populations decreases with age. The frequency of Ly 1+ B cells coincides with the B cell subpopulation III (subpopulations I, II and III are designated according to the quantitative expression of IgM and IgD) in normal mice but in xid-bearing mice the B cells found in subpopulation III are comprised of mainly Ly 1- B cells (183). Hayakawa et al found that Ly 1+ B cells predominate in the peritoneum of normal mice but CBA/N and (CBA/N x BALB/c) F1 males exhibit undetectable peritoneal Ly 1+ B cells (184). Finally, Forster and Rajewsky suggest that high levels of serum IgM are produced by Ly 1+ peritoneal B cells since the transfer of adult peritoneal cells into newborn, allotype-congenic mice results in the production of serum IgM that is of donor origin (185). Therefore, low serum IgM levels found in xid-bearing mice may be a direct result of their lack of Ly 1+ peritoneal B cells. The human counterpart to murine Ly 1+ B cells are thought to be Leu-1+ B cells since these cells share functional and developmental similarities (186).

A Genetic Model for NAb Participation in Tumor Surveillance.

Evidence has accumulated which supports the hypothesis that NAb may play a role in the host first line of defense against small tumor foci (25, 74, 148). The X-linked immune deficiency in CBA/N mice provides a model for examining this hypothesis. The xid mutation is characterized by deficiencies in serum IgG3 and IgM levels accompanied by the presence of a subpopulation of mature B cells which are characterized by the absence of Lyb-3, 5, 7, and Ia.W39 determinants (reviewed in 142). These mice were found to possess low or undetectable levels of NAb against a wide range of tumor cell lines (149). Previous studies have indicated that the T-cells, macrophages, accessory cells and NK cells of these mice are unaffected by the xid mutation; therefore, the CBA/N and (CBA/N x CBA/J) F1 mice provide an *in vivo* model in which only the NAb effector mechanisms are defective (115, 126, 128-130).

A threshold, subcutaneous inocula of a radiation-induced T cell leukemia (R1-28) of the CBA/H strain was used to compare the tumor frequencies exhibited by CBA/N vs CBA/J mice and the hemizygous xid-bearing (CBA/N x CBA/J) F1 male mice vs normal heterozygous F1 female mice. The NAb serum levels of these mice were analyzed as well as the *in vitro* cytotoxic functions of their macrophage and NK cells.

MATERIALS AND METHODS

Mice

a) Source

Normal immune competent CBA/J mice (haplotype H-2^k) were obtained from the Jackson Laboratory, Bar Harbor, ME until they were unable to maintain promised delivery dates. Thereafter, all CBA/J stock were bred in the University of Manitoba breeding facility. The B cell deficient xid bearing CBA/N (haplotype H-2^k) breeding pairs were obtained from the National Institute of Health, Bethesda, Md., housed in 18 x 28.5 x 13 cm plastic mouse cages, and placed in laminar flow containment hoods. The CBA/J mice were placed in metal or plastic mouse cages and no hoods were necessary. All animals were allowed food and water ad libitum.

b) Breeding

CBA/N mice were bred by mating females homozygous for the xid defect with males hemizygous for the same, and CBA/J mice were bred by mating males and females of that inbred strain. The (CBA/N x CBA/J) Fl animals were bred by crossing xid homozygous CBA/N females with normal CBA/J males. All the resulting Fl male progeny will be xid hemizygous and B-cell deficient since the immune deficient marker is carried by the X chromosome and the female progeny will be heterozygous for the recessive xid and thereby display a normal phenotype.

CBA/N, CBA/J and (CBA/N x CBA/J) F1 mice were bred based on the methods outlined in a manual from the Institute of Animal Technicians (IAT #1). Briefly, random breeding was practiced in order to retain all the variables originally present in the colony and to keep them evenly distributed throughout. Brother sister or cousin matings were avoided when possible to decrease the level of inbreeding resulting in the generation of parallel lines which may lead to considerable genetic variation within a few generations. For experimental animals, two male mice and two or three female mice were placed in opaque plastic mouse cages and allowed to breed freely. After 1 - 2 weeks the females were examined and if pregnant they were removed to clean cages containing wood chips and a small piece of cotton batting. If a large number of litters were required, male breeders were removed after 4-5 days from the breeding pairs and used to fertilize additional female mice. The previously bred females were monitored for a maximum of three weeks and if not pregnant they were placed back into the breeding pool. If this scheme was unsuccessful it was of some use to place a larger number of male and female mice into a 25.5 x 47.5 x 16 cm rat cage and examine them after 1 - 2 weeks.

The pregnant females were removed to clean, separate cages and left undisturbed meaning that the change of cage, food and water was performed less frequently to minimize stress for the expectant mothers. Once the litters were born, a day or two was allowed to pass to ensure survival of the offspring, and then the cages were cleaned as usual. After three - four weeks the litters were weaned and separated according to sex.

Highly inbred strains of mice are frequently difficult to breed, therefore some special techniques were employed when mating CBA/N mice together. To synchronize the estrus cycle of the females, those to be bred were placed in a large cage within which was a wire cage containing one male mouse. After 24 hours, the male was removed and the females were bred as usual the following day. This method causes many of the females to be on the same estrus cycle and this is useful if large numbers of age-matched litters are required (150).

Pregnant CBA/N mice were treated with special care to maximize successful pregnancies and parturition. The females were placed one per cage or with a female known to be a dependable mother. They were left undisturbed, except when given fresh food and water, and their cages were placed away from cages containing male mice.

Sera

a) Bleeding

Mice were bled between the ages of 7 and 19 weeks, unless otherwise stated. Mice were first anesthetized with ether, an incision was made to expose the axillary artery which was cut through. The blood was quickly collected with a Pasteur pipette, transferred to 5 ml Falcon tubes (Fisher Scientific Ltd) and placed on ice in a styrofoam bucket. Immediately following the bleeding of each mouse, cervical dislocation was performed.

Testing sera on an individual mouse basis was achieved by restraining the animals in a metal cone and an incision was made in a tail vein. A maximum of 300 µl of blood was collected per mouse and immediately transferred to ice. This method of bleeding allowed the mice to be used in further experiments 1-2 weeks later.

b) Separation of sera and storage

Within 30 min. after bleeding the mice, the blood was allowed to clot at 4°C, centrifuged at 2500 rpm, and the serum was carefully removed and transferred to clean 5 ml Falcon tubes for storing. The serum was filtered through a 0.22 m Millex-GV filter unit (Millipore Products Division, Bedford, Mass) if it appeared cloudy. The tubes were carefully labelled and stored at -20 C until needed.

Tumor Cells

a) Origin

The RI-28 cell line was obtained through two cloning procedures (see d) in sloppy agar from the radiation induced T cell leukemia cell line (RĪ) syngeneic to the H-2^k CBA/H strain of mice (151). The RI-28 leukemia cell culture was subcloned again to generate RI-28/6, RI-28/7 and RI-28/8 subclones. The RI-28 cell line was the only clone utilized *in vivo*.

Three allogeneic cell lines were also used namely the YAC-NIH tumor which is a lymphoma induced by Moloney virus in A/Sn mice (82), the SL2-5 lymphoma syngeneic to DBA/2 strain which is a subclone of SL2, a methylcholanthrene-induced lymphoma (80) and L5178Y-F9, a T cell lymphoma also syngeneic to DBA/2 mouse strain (80).

b) Tumor cell maintenance

The RI-28 leukemia cells as well as the subclones RI-28/6, RI-28/7 and RI-28/8 were grown as suspension cultures in sterily filtered Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with a 10% fetal bovine serum (FBS) penicillin (10,000 U/ml), streptomycin (10,000 mcg/ml) solution for each litre of medium (Gibco Laboratories, Grand Island, NY). The FBS was heat inactivated

prior to use in a 56°C waterbath for 45 min. The concentration of the cell culture was adjusted by aseptically transferring an aliquot of 7.5×10^5 - 1.25×10^6 cells to 25 mls of 10% RPMI every two days to maintain the cells in exponential growth between $3 - 5 \times 10^4$ to 1×10^6 per ml. The cells were cultured in sterile 100 ml glass bottles and incubated at 37°C in a 3% CO_2, humidified atmosphere incubator (Napco Controlled Environment Incubator, Portland, Oregon).

The YAC-NIH and SL2-5 lymphomas were maintained in sterily filtered Fisher's medium (FM) supplemented with 10% FBS and penicillin: streptomycin solution as for RPMI. Cells were transferred every two days as indicated for the RI-28 leukemia cell line.

c) Long term cell storage

i) Freezing. Aliquots containing $4-7 \times 10^6$ cells were centrifuged and resuspended in 1 ml of 10% RPMI containing 10% dimethyl sulfoxide. To prevent cellular damage cells were frozen slowly in an insulated container at -70°C in a Ultra Low Freezer (Revco Inc., West Columbia, S.C.). Cells were transferred to uninsulated cardboard boxes after 24 hours and stored at -70°C.

ii) Thawing. Every three months cells were removed from the frozen stock of original cell lines and recultured in suspension. Frozen cells were thawed quickly at

37°C and washed once with 10% RPMI to remove excess dimethyl sulfoxide. A concentration of 1 x 10^6 cells/ml was used to initiate growth in tissue culture.

d) Cloning

A modification of the Chu and Fisher technique (151) was made by Chow and Greenberg (11) and utilized to generate tumor cell clones. Agar Noble (Difco Laboratories, Detroit, Mich. USA) was mixed with double distilled and deionized water (ddd H_2O) by adding 100-200 mg to 5 ml respectively. This was autoclaved and diluted while still dissolved to 50 ml with 20% RPMI which had been heated to 44°C and concentrated agar was kept warm until ready to use. The tumor cells were serially diluted to 25 cells/ml and 5 cells/ml. All cells were aseptically transferred in a 2 ml volume such that 5 sterile tubes received 50 cells each and 10 tubes received 10 cells each. A volume of 3 ml of the agar solution was added to each tube producing a final agar concentration of 1-2 mg/ml. After gentle mixing the tubes were placed in a 4°C fridge for 7 minutes to allow the agar to solidify slightly. The tubes were then transferred to a 37°C incubator for 7-14 days until colonies were visibly embedded in the agar. Ten each of small, medium and large sized colonies were removed with sterile Pasteur pipettes and transferred to 10 ml glass culture tubes. Only the cloning procedures displaying a

cloning efficiency of 90% or more were used as sources for subcloned cells. The cells were set to a concentration of 1 x 10^6 cells/ml with 10% RPMI and monitored until they could be maintained as suspension cultures in 100 ml glass bottle.

e) Cell centrifugation and washing

All cell washes were done in RPMI media or Hank's Balanced Salt Solution (HBSS) containing 0% fetal bovine serum followed by centrifugation at 210 g for 10 min unless indicated otherwise.

Tumor Frequency

a) CBA/N and CBA/J

A titration of 10^1, 10^2, 10^3, 10^4, 10^5 RI-28 cells was carried out in CBA/J mice first to determine an inoculum which would result in a tumor frequency of approximately 25%. An aliquot of 100 l of RI-28 cells suspended in HBSS was injected into the middle of a shaved area in the lower back of each mouse. This threshold RI-28 inoculum was injected into age and sex matched CBA/N and CBA/J mice which were monitored for a minimum period of 21 days and the tumors and deaths were recorded for each experiment. At the termination of each experiment, all mice were killed quickly with chloroform gas.

b) (CBA/N x CBA/J) F1 male and female

A titration of RI-28 cells to determine the threshold inoculum resulting in a tumor frequency of 25% was also carried out in F1 mice as described in a. Age matched male and female F1 mice were inoculated with the RI-28 cell line and monitored as in a. Prior to injection of RI-28, each F1 mouse was ear-marked and individually tail bled as outlined previously under Sera a) so that the serum could be tested for NAb activity.

FACS Detected NAb

Aliquots of 3-5 x 10^5 tumor cells were resuspended in 200 l of 50% RPMI or an equivalent amount of whole or diluted serum obtained from age and sex matched CBA/J and CBA/N mice or from age matched male and female F1 mice. The cells and sera were incubated at 4°C for 1 hour or for 2 hours at 37°C or twice for 45 min, at 4°C in 3% CO_2. Cells were then washed once, and resuspended in 100 µl of a 1/10 dilution of fluorescein isothyocyante (FITC) conjugated goat IgG (7S) anti-mouse IgG (Meloy, Springfield, Va) and a 1/10 dilution of fluorescein conjugated FITC goat $F(ab')_2$ anti-mouse IgM (Tago, Burlingame, Ca).

The fluorescein conjugated second antibodies were diluted with 10% RPMI containing 10-15 ml of a 1 M solu-

tion of Hepes buffer (Gibco Laboratories, Grand Island, NY) per 500 ml of media. Cells and second antibody were incubated for 30 min at RT, washed once and fixed with 100 µl of cold FBS containing 1% formaldehyde for 5 min at RT and resuspended in 500 µl RPMI. Linear fluorescence of the cells was analyzed at 4 C using a Coulter Epics V Multiparameter Sensor system. If whole serum decreased the cell viability, then serial dilutions were made and the dilution displaying good fluorescence without the loss of viability was used for that pool of serum. The background fluorescence following incubation in 10% RPMI followed by the second, FITC labelled antibodies was subtracted. While Nab binding assays performed for comparison were carried out at the same high voltage and gain, these parameters were adjusted for each NAb binding experiment so that the mean channel total linear fluorescence of a positive sample was in the center of the scale (channel 0-255).

Cytolysis Assays

a) Natural antibody and complement

i) Assay. A two step assay was used which has been previously described by Wolosin and Greenberg (68). The RI-28 cells and subclones were pelleted by centrifugation and resuspended in ^{51}Cr (as Na_2CrO_4), approximately 100 $\mu Ci/10^7$ cells in 100 µl. This cell suspension was incubated in a 37 C waterbath for 45 minutes. After labelling, excess ^{51}Cr was removed by washing twice and aliquots

of 5 x 10^5 cells were mixed in 5 ml Falcon tubes with 200 µl of test serum as a source of natural antibody or an equivalent amount of 50% RPMI for control cells. After incubation at 37°C in 3% CO_2 for 1 to 2 hours, the cells were washed once and resuspended in 320 µl of 10% RPMI. Using a 96 well V bottom microtitre plate an aliquot of 20 µl of the cell suspension was added to 20 µl of a 1/5 dilution of RI-28-absorbed rabbit serum (Buxted Rabbit Co., Buxted, Eng) as a source of complement. The complement had been thawed quickly at RT and diluted 1/5 with 10% RPMI immediately before being added to the test serum treated cells. The tests were carried out in quadruplicate in V-bottom microtiter plates and incubated at 37°C for one hour. HBSS was then added to raise the total volume to 200 µl per well, plates were centrifuged, and aliquots of 100 µl were removed from the supernatant for quantitation of ^{51}Cr release in an LKB Wallac 1282 Compugamma Universal Gamma Counter. If whole serum caused a decrease in cell viability the serum to be used was serially diluted with RPMI and the dilution displaying the best complement mediated NAb cytolysis was used in all further assays with that particular pool.

ii) **Specific absorption of complement.** In order to grow large numbers of RI-28 cells for the specific absorption of rabbit serum, CBA/J mice were irradiated with 450 rads and then injected with 3 x 10^6 cells ip. Five to

seven days later, the animals were sacrificed and ip cells were harvested by injecting 5 mls of HBSS into the peritoneal cavity and removing as much volume as possible. This was repeated 4-5 times per mouse. The resulting cells were centrifuged and treated with 5 mls of a 0.83% ammonium chloride (NH_4Cl) solution at RT for 4 min to lyse any red blood cells present. The cells were washed twice and an aliquot of 3×10^8 cells was pelleted and incubated at 4°C for 1 hour with 1 ml of the reconstituted, lyophilized rabbit serum (Buxted, etc). During the incubation the cell suspension was gently vortexed at 20 minute intervals. The cells were then centrifuged at 210 g for 8 min followed by 500 g for 2 min. The supernatant was removed and aliquots of 125 μl were stored at -70°C for use as a source of complement.

b) Natural killer cell cytolysis

In vitro natural killer cell cytolysis was tested by using a method described by Greenberg et al (91). The ^{51}Cr labelling of the RI-28, RI-28 subclones, YAC-NIH and SL2-5 cells was carried out as for the complement mediated cytolysis assay. After washing the concentration of each cell line was adjusted to 1×10^5 cells/ml with 10% RPMI.

Spleens from age and sex matched CBA/J and CBA/N mice or age matched male and female F1 mice were removed and the cells obtained from 2 to 4 animals were pooled in a

single cell suspension. The spleen cells were washed once and red blood cells were lysed with NH_4Cl as described in the specific complement absorption. After washing, the spleen cells were adjusted to a concentration of 1.5×10^7 cells/ml. Appropriate aliquots of spleen effector cells were added to 1×10^4 target tumor cells (100 µl of the 1×10^5 cell/ml tumor cell suspension in V-bottom wells of a 96 well microtitre plate), so that effector lymphocytes to tumor cell ratios (E:T) equalled 150:1, 75:1 and 37.5:1 unless otherwise specified. The final volume per microtiter well was adjusted to 200 µl with 10% RPMI, and the plates were centrifuged at 35 g for 4 minutes to bring the effector lymphocytes into contact with the target tumor cells. The incubation was carried out at 37°C in 3% CO_2 for 18 hours, the plates were centrifuged at 180 g for 10 min and 100 µl of supernatant from each well was tested for ^{51}Cr in the gamma counter.

c) Activated macrophages

i) In vivo activated peritoneal macrophages. C. parvum (Wellcome Research Laboratories, Beckenham, Eng), at a concentration of 2.5 mg/mouse was injected ip, in order to activate their peritoneal macrophages (153). After 5 days the animals were sacrificed, peritoneal cells were removed, and red blood cells were lysed with NH_4Cl as out

lined previously. After washing, the cells were resuspended in 10% RPMI at a concentration of 2.5 x 10^6 cells/ml and appropriate aliquots were added to flat bottom, 96 well, microtiter plates (Gibco Canada Inc, Burlington, Ont). Macrophages were allowed to adhere by incubating the plates at 37°C in 6% CO_2 for 75 min after which the cells were washed by adding aliquots of 100 μl RPMI to each well and suctioning off the supernatant. Finally, 100 μl of 10% RPMI was added to each well including control wells which contained no macrophage cells. Tumor targets were labelled with ^{51}Cr, washed and resuspended to a concentration of 1 x 10^5 cells/ml with 10% RPMI. Aliquots of 1 x 10^4 tumor targets were added to each well and the plates were incubated for 18 hrs to 36 hrs at 37 C in 6% CO_2. After the incubation, plates were centrifuged at 180 g for 10 min and 100 μl of supernatant from each well was analyzed for ^{51}Cr release as outlined previously. Effector to target ratios ranged from 90:1 to 10:1.

ii) In vitro activated bone marrow macrophages. Procedures for Con-A supernates of spleen cells containing lymphokine, bone marrow culture for activating macrophages and macrophage cytolytic assays were modifications of the techniques described by Roussel and Greenberg (154). Bone marrow from individual age-matched F1 male and female mice was removed asceptically from their femures, washed with HBSS and red blood cells were lysed with NH_4Cl. After

washing, the cells were resuspended in RPMI medium containing 10% FBS, 10% horse serum and 10% L cell conditioned medium. The concentration of bone marrow cells was adjusted to 3.2×10^4 cells/ml. Aliquots of 25 ml were transferred to petri dishes and incubated for 5 days at 37°C in 10% CO_2. After 5 days the media was poured off and the adherent bone marrow cells were harvested from the petri dishes by treatment with 3 ml of PBS containing 0.02% EDTA at pH 7.2 for 10 min at RT. The adherent cells were washed twice and adjusted to a concentration of 1.5×10^6 cells/ml in 10% RPMI. Aliquots of 0.1 ml were added to flat bottom, 96 well, microtiter plates and the macrophage were allowed to adhere by incubating the plates at 37°C in 10% CO_2 for 2 hrs. The media was then removed and 0.1 ml of Con A supernatant was added to each well and incubated for 12 hrs at 37°C in 6% CO_2. The Con A supernatant was removed and 0.1 ml of 10% RPMI containing 2 μg/ml LPS was added to each well and incubated for 1 hr at 37°C in 10% CO_2. The supernatant was removed, RI-28 cells labelled with [^{131}I]dUrd were added to each well at an E:T of 15:1 and incubated for 48 hrs at 37°C in 6% CO_2. The RI-28 cells were labelled by incubating 10^6 cells in 1 μCi of [^{131}I]dUrd for 4 hours at 37°C in 10% RPMI, washed and resuspended to an appropriate concentration. Finally, the plates were centrifuged and 0.1 ml of supernatant was analyzed as per the ^{51}Cr release assays.

The Con A supernatants were prepared by first removing spleens from 3-4 DBA/2 mice and these were pooled in a single cell suspension. The red blood cells were lysed with NH_4Cl as previously described, and the remaining spleen cells were washed in HBSS, resuspended to 5 x 10^6 cells/ml in 10% RPMI containing 2-5 μg/ml Con A and incubated for 72 hrs at 37°C. The cells were then removed by centrifugation and the supernatant was collected. The Con A was removed from the supernatant by adding G-25 Sephadex at a concentration of 1 g/100 ml and the Con A was allowed to absorb for 1 hr at 4°C. The G-25 Sephadex was removed by centrifugation and the remaining supernatant was passed through a filter and frozen in appropriate aliquots. The supernatant was diluted 10% with media for use in the macrophage cytolysis assay.

d) Calculations of cytolysis

Activated macrophage cytotoxicity and natural killer cell cytotoxicity was determined by the amount of ^{51}Cr or [^{131}I]dUrd released from lysed tumor target cells and calculated as follows:

$$\frac{\%^{51}Cr \text{ release (experimental)} - \%^{51}Cr \text{ release (spontaneous)}}{100 - \%^{51}Cr \text{ release (spontaneous)}} \times 100$$

The spontaneous release of ^{51}Cr from tumor target cells incubated with 10% RPMI ranged from 5 to 25%.

The complement mediated NAb cytotoxicity was calculated as above except that lysis of tumor cells by complement alone was also incorporated into the calculation:

$$\frac{\%^{51}Cr \text{ release (NAb+C)} - \%^{51}Cr \text{ release (NAb)} - \%^{51}Cr \text{ release (C)}}{100 - \%^{51}Cr \text{ release (NAb)} - \%^{51}Cr \text{ release (C)}} \times 100$$

The spontaneous release of ^{51}Cr by tumor cells incubated with only NAb was comparable to control cells that had been treated with 10% RPMI and ranged from 5 to 25%. The toxicity of specifically absorbed rabbit complement was less than 3%.

Statistics

a) Student's t test

All cytotoxicity assays were analyzed by the t-dependent or t-independent Student's t-test to determine the statistical significance for differences between the percent cytolysis of the RI-28 leukemia cell line, subclones of RI-28, YAC-NIH lymphoma, L5178Y-F9 lymphoma or SL2-5 lymphoma exposed to CBA/N vs CBA/J mediators or to (CBA/N x CBA/J) F1 male vs female mediators.

The presence of NAbs in the serum of the inbred and F1 mice detected by FACS were also statistically analyzed with the t-dependent Student's t-test through a comparison of mean fluorescence channels.

The cumulative tumor frequencies of CBA/J vs xid-bearing CBA/N mice or (CBA/N x CBA/J) F1 females vs xid-bearing F1 males were analyzed using the Student's t-independent test.

b) Mantel-Haenszel Chi-squared test

This test was used to analyze the maximum cumulative tumor frequency in F1 mice which exhibited fluorescence-detected NAb binding above 60 channels with the tumor frequency in F1 mice with NAb binding below 60 channels (155).

c) Correlation coefficient analysis

This test was used to compare the NAb binding, NK cytolytic activity and macrophage cytolytic activity in individual (CBA/N x CBA/J)F1 male and female mice where each mouse provided serum, spleen and bone marrow. Correlation coefficients were determined for NAb binding vs NK cytolysis, NAb binding vs macrophage cytolysis and NK cytolysis vs macrophage cytolysis.

RESULTS

Differences in tumor susceptibility in CBA/N and CBA/J mice

Mice expressing the recessive xid defect are known to have low levels of circulating IgG_3 and IgM antibodies compared to normal or heterozygous animals and the immunoglobulin classes IgG and IgM have been representative of natural antibodies in normal unstimulated serum (80,-142,149). Antitumor activity of NAb has been shown for all tumors tested but the accumulating in vitro and in vivo evidence for NAb as an important component of NR has not so far been supported by in vivo genetic models (25,72,75,80,157). Therefore, by taking advantage of the Ig deficiency in CBA/N mice, the fate of small tumor foci of syngeneic RI-28 leukemia cells was examined in both normal and xid bearing mice and serum NAb levels were determined initially for two separate strains. Menard, Colnaghi and Porta showed evidence for an inverse relationship between NAb levels and tumor frequency when small numbers of tumor cells were used (156).

There is evidence to suggest that tumors develop through the growth and progression of a single or a few transformed cells and it seems likely that immune surveillance would be optimal during the very early development of the tumor mass (10,158).

An experimental protocol developed by Greenberg and Greene (1976) involves injecting the lowest number of cells that is capable of permitting the growth of a subcutaneous tumor (81). Since the CBA/N mouse strain is immunodeficient with respect to the levels of immunoglobulin it was worthwhile to determine if this would correlate with their ability to handle threshold s.c. tumor inocula. In a series of four experiments CBA/J and CBA/N mice between 7 and 14 weeks of age were administered the s.c. RI-28 inoculum and the tumor frequency, latency and deaths were monitored over periods of 3 weeks. It was found that the CBA/N mice always displayed a higher tumor incidence than the CBA/J mice (Table I). Since both CBA/N sexes exhibited this phenomenon, the data was compiled and revealed a two-fold increase in s.c. tumor occurrence for the immune defective CBA/N animals which was statistically significant (Fig. 1). The tumor frequency, age, sex and numbers of animals used in each experiment is given in Table I. For each experiment, the appearance of RI-28 s.c. tumors was recorded and the average latency for CBA/J and CBA/N mice was 15 days and 10.5 days, respectively (Table II).

NAb Analysis for CBA/J and CBA/N Mice

Once a significant difference was established between

CBA/J and CBA/N mice with respect to the fate of a small RI-28 s.c. tumor inocula, the normal serum of each strain of mouse was analyzed for natural anti-RI-28 antibody. The presence of tumor reactive NAb can be demonstrated in a complement-mediated cytotoxicity assay or the tumor bound NAb can be labelled with fluoresceinated anti-mouse immunoglobulin and detected by FACS (72,75,149,157).

i) NAb and complement mediated cytotoxicity

In a two step assay, normal sera taken from mature mice were incubated with the RI-28 leukemia cell line or subclones of RI-28 and lysed with rabbit serum as a source of complement. The CBA/N immunoglobulin deficient serum displayed almost undetectable cytolysis of the tumor targets whereas the activity of CBA/J serum was significantly higher (Table III). The RI-28 and each of its subclones varied slightly with respect to lysis by normal CBA/J serum.

ii) NAb bound to RI-28 detected by FACS

Since the lytic procedure is designed to detect complement activity and tumor cell binding NAb, it was necessary to analyze IgG and IgM anti-RI-28 binding by FACS. Whole or diluted, age and sex matched normal sera from CBA/J or CBA/N mice was incubated with RI-28 cells and the relative levels of NAb bound to the tumor target were detected by fluoresceinated anti-mouse-IgG and anti-mouse-IgM. The

fluorescence displayed by RI-28 cells treated with pooled CBA/J serum was 5 times greater than RI-28 cells treated with immune deficient CBA/N sera (example of a typical profile in Figure 2). That is, CBA/J NAb derived from mice of a variety of ages (6 weeks to 8 mos) showed a greater ability to bind RI-28 than the NAb derived from CBA/N mice (Table IV). Male and female littermates were also examined and the same result was found (Expt. 2a, 2b, Table IV). The ability of CBA/J NAb to bind to the RI-28 target cells suggested that the presence of such NAb was somehow inversely related to the tumor incidence exhibited by CBA/J animals *in vivo*.

Natural Killer Cell Activity of CBA/J and CBA/N Mice

Since effectors such as NK cells, and macrophages in addition to NAb, have been implicated in tumor surveillance and progression, comparisons of the effectiveness of these cells derived from CBA/J and CBA/N mice were tested *in vitro*. The cytolytic activity of splenic NK cells from CBA/N and CBA/J strain mice was examined *in vitro* against both syngeneic and allogeneic tumor cell lines. Initial experiments indicated that the syngeneic RI-28 tumor target was NK resistant (NK^r), therefore, subclones RI-28/6 and RI-28/8 were examined and found to be NK sensitive (NK^s). The allogeneic cell lines tested included YAC-NIH and SL2-5 lymphomas and these were also NK^s.

The CBA/N and CBA/J splenic NK cells showed no significant difference in their ability to lyse either the syngeneic or the allogeneic tumor targets with the possible exception of subclone RI-28/8 which was more susceptible to the xid-bearing CBA/N effectors (Expt. 3, Table V).

The YAC-NIH cell line proved to be the most sensitive to both CBA/N and CBA/J NK cells with maximum lysis of 58.1% and 51.5% respectively, at an effector to target ratio 150:1 (Table V). Thus, the NK activity in CBA/J mice was never greater than that of the xid-bearing CBA/N mice.

The data presented here supports the hypothesis that NK cells were not the important contributing NR effector responsible for the differences seen in the tumor frequency of RI-28 exhibited by CBA/J and CBA/N mice. Since the RI-28 tumor target appears to be NK^r this lends further evidence to suggest that the NAb differences in these two strains of mice may be responsible for the tumor frequency differences.

Activated Macrophage Activity of CBA/J and CBA/N Mice

Many reports have indicated that macrophages may play a

role in tumor cytotoxicity against tumors (159-163). It has also been shown that soluble factors released by T cells and NK cells that have been exposed to tumor targets can activate macrophages (164-167). Therefore, it was important to determine if the cytolytic activity of activated macrophages from the immune deficient CBA/N mice was less than the cytolytic activity of activated macrophages from CBA/J mice.

The syngeneic RI-28 cell line was found to be fairly resistant to cytolysis by activated peritoneal macrophages from both mouse strains, therefore macrophage-sensitive targets subclone RI-28/8 and the allogeneic L5178Y-F9 cell lines were also assayed. No statistically significant difference was found in the cytolytic activity of macrophages from the immune deficient CBA/N mice and the normal CBA/J mice when the L5178Y-F9 tumor target was used. The cytolytic activity of activated peritoneal macrophages from the immune deficient CBA/N mice was consistently higher than the cytolytic activity of CBA/J macrophages when tested against the syngeneic RI-28 tumor target and the subclone RI-28/8 however, these differences were not significant (Table VI). Since the xid-bearing CBA/N mice displayed slightly increased macrophage activity this could not account for their increased tumor frequency.

Tumor Frequency in F1 Male and Female Mice

It was important to establish if the difference seen in CBA/J and CBA/N mice tumor frequency was actually due to the xid defect. Therefore, the two strains of mice were bred so that the resulting male progeny were xid positive and the female progeny were xid negative. Since the xid defect is recessive and carried on the X chromosome, the CBA/N females were cross-bred with CBA/J males to obtain heterozygous, phenotypically normal F1 females and hemizygous, deficient F1 males.

The results of four individual experiments indicated that the tumor frequency of threshold RI-28 sc inocula in (CBA/N x CBA/J) F1 male mice was twice as high as the frequency in female F1 mice and the difference was significant (Figure 3). The mice tested were all between the ages of 10 to 11 weeks and in each experiment the tumor frequency was higher for the (CBA/N x CBA/J) F1 male mice (Table VII). The latency of tumor occurence was slightly but not significantly lower in the F1 male mice at 12 days compared to 14 days for the F1 female mice (Table VIII).

The differences found in tumor frequencies for the F1 male and female mice coincided with the data obtained for the CBA/N and CBA/J mice. That is, animals which exhibit the xid defect display a higher tumor frequency as well as

a slightly lower tumor latency compared to their normal counterparts.

NAb analysis for F1 mice

NAb binding as well as NAb plus complement cytotoxic activity against the RI-28 tumor target was examined in vitro in an attempt to add further support for the data obtained in the CBA/N and CBA/J studies.

i) NAb and complement mediated cytotoxicity

The cytolysis of tumor targets RI-28 and subclone RI-28/8 by serum NAb from (CBA/N x CBA/J) F1 female and F1 male mice was compared. The F1 female sera displayed cytolytic activity that was 12 times greater than the cytolysis of RI-28 by sera from hemizygous immune deficient F1 male mice. The same result was found when the subclone RI-28/8 was tested except that cytolysis by F1 female sera was 4 times higher than serum NAb obtained from F1 male mice (see Table IX). This data is comparable to the results obtained for CBA/J and CBA/N mice. That is, sera from the xid-bearing CBA/N mice and F1 males displayed a lower ability to lyse the tumor targets than sera from normal CBA/J and F1 female mice respectively.

ii) NAb Binding to RI-28

Since the NAb plus rabbit complement assay only detects

antibodies which are reactive with complement it was necessary to detect the anti-RI-28 NAbs by labelling with fluoresceinated anti-mouse immunoglobulin and measure the fluorescence by FACS. An example of a typical FACS profile is given in Figure 4. Sera from (CBA/N x CBA/J) F1 female mice contained IgG and IgM antibodies which bound 2 times better to the RI-28 tumor than sera from F1 male mice (Table X). Individual mice were also tested for anti-RI-28 NAbs and the same result was found. The twenty-eight F1 female mice that were examined showed an average of 2 times more fluorescence than the twenty-six F1 male mice.

Surprisingly, the B-cell deficient males displayed a heterogeneity which overlapped with the expected heterogeneity of the normal females and this heterogeneity was more evident in the (CBA/N x CBA/J) F1 mice than the CBA/J mice (Figure 5). Previous researchers have reported this heterogeneity in NAb levels between members of the same normal inbred mouse strain including CBA/J mice (68,75). The F1 male and female mice were tail bled separately at weekly intervals beginning 1, 2 or 3 weeks prior to tumor inoculation. Although the weekly bleeds from each set of male and female mice were tested on different days, correlations were shown for 3 of the 4 possible comparisons of sera from the same mice (Figure 5). This data suggests

that the level of variability in the analysis of repeated weekly bleeds is not inherent in the assay and probably reflects the kinetics of the serum NAb variability.

This data supports the evidence obtained from the NAb plus rabbit complement experiments. That is, sera from F1 female mice contains antibodies which are more reactive against the RI-28 tumor target than the antibodies found in the sera of the B cell deficient F1 male mice.

Natural Killer Cell Activity by F1 Male and Female Mice

As mentioned previously, it was necessary to examine other NR effector cells to ascertain if differences in tumor cytotoxicity by these cells could account for the differences found in tumor frequency seen in the (CBA/N x CBA/J) F1 male and female mice.

Splenic natural killer cells from (CBA/N x CBA/J) F1 male and female mice were tested and effector to target ratios of 150:1 and 75:1 are given in Table XI. There was a tendency for higher NK activity in F1 male mice when NKs targets were tested and the difference between males and females reached significance in one experiment carried out against the NK^S YAC-NIH target (Expt. 6, Table XI). In this case, spleens from 6 individual mice were removed and each mouse was tested for its NK cytolytic activity against RI-28 and YAC-NIH tumor targets. At an effector

to target ratio of 150:1 the immune deficient (CBA/N x CBA/J) F1 male mice displayed NK cytolytic activity against YAC-NIH that was 1.5 times higher than the NK cytolysis by normal F1 female mice.

This data supports the evidence compiled for the CBA/J and CBA/N splenic NK cytolytic activity. Since there was a tendency for NK activity to be slightly higher in the NAb deficient (CBA/N x CBA/J) F1 male mice it is unlikely that NK activity is responsible for the differences seen in the tumor frequencies of the F1 mice.

Activated Macrophage Cytolysis by F1 Male and Female Mice

The cytolytic activity of peritoneal macrophages from (CBA/N x CBA/J) F1 male and female mice was tested in the same manner as the experiments performed on CBA/N and CBA/J mice. Tumor targets included the syngeneic cell lines RI-28 and subclone RI-28/8 as well as the allogeneic L5178Y-F9 cell line. After activation of intraperitoneal macrophages by C. Parvum injection the adherent cells were harvested and incubated with the various tumor targets. No statistically significant difference could be detected between F1 male and female activated macrophages (Table XII). Since the macrophage activity of (CBA/N x CBA/J) F1 male and female mice was equivalent in vitro this suggested that these effectors are not the principal effector

cells responsible for the increased tumor frequency exhibited by F1 male mice.

Anti-RI-28-NAb and Tumor Incidence in Individual F1 Mice

In three of the four (CBA/N x CBA/J) F1 tumor frequency experiments the animals were individually tail bled one week prior to the sc RI-28 inocula and the sera were tested for anti-RI-28 NAb by FACS. The mice were ear marked and the tumor incidence of each mouse was recorded at the end of each tumor frequency experiment. The mean percent tumor incidence in the immune deficient males was twice that of the females as stated previously (see Figure 3). There was little variability in ranking from one bleed to the next (Figure 5) therefore the last bleed before tumor inoculation was plotted against the fate of threshold tumor inoculum for each mouse (Figure 6, panels A, B and C).

The individual sera revealed extensive and overlapping heterogeneity for anti-RI-28 NAb binding activity for F1 males and females, however, the females averaged a mean channel fluorescence that was almost double that of the males. A correlation was found between tumor susceptibility from small foci and low fluorescence-detected NAb

binding activities for male and female mice. That is, tumors developed in 77.3% of the animals with low NAb levels (mean binding below 60 channels). Tumors also appeared in animals with mean binding above 60 channels but in only 26% of them.

The data suggests that there was an inverse relationship between tumor incidence and anti-RI-28 NAbs and this was found to be statistically significant by Mantel-Haenszel Chi-squared analysis. Mice exhibiting low levels of NAb displayed a higher tumor incidence than those mice which had high levels of anti-RI-28 NAbs.

Conversely, individual CBA/N mice displayed sera NAb activity which appeared to be relatively low and homogeneous. The sera from CBA/J littermates showed NAb activity which was high and relatively heterogeneous similar to reports of other normal inbred strains exhibiting NAb heterogeneity. The mean binding of the CBA/J NAb was approximately ten times that of the CBA/N (Figure 6, panel D).

NAb and NK levels in individual F1 mice

Since the (CBA/N x CBA/J) F1 mice displayed an increased heterogeneity in their levels of NAb, tests were carried out to determine if their NK cytolytic activity behaved in the same manner. If a correlation exists

between the NAb levels and NK activity, such a relationship might explain the differential tumor susceptibility in F1 mice.

In three separate experiments in which individual F1 male and females provided serum, spleen and bone marrow, a comparison was made between serum anti-RI-28 levels and the percentage NK cytolysis (Figure 7). As before, the NAb binding by F1 males was heterogeneous and overlapping with the females in 2 of the 3 experiments. Among the F1 males, 23.5% (12/51) of them were apparently NAb-positive and 15.7% (8/51) exhibited high activity. Yet, there was no direct relationship between the NK cytolytic activity and NAb levels. As before, the xid-bearing F1 males displayed NK activity that was actually higher than the normal F1 females and the difference was significant ($p < 0.0001 - 0.05$).

NAb and activated macrophage activity in individual F1 mice

A comparison was also made between the serum anti-RI-28 levels and the cytolytic activity of Con-A stimulated bone marrow macrophages (Figure 8). In only one of the three experiments was there shown a direct correlation between NAb and macrophage activity. However, this relationship was based on a large range of NAb binding (6.3 - 251.7

mean channel fluorescence) compared to a small range of macrophage activity (52.8 - 78.5% cytolysis). The other two experiments display a tendency toward an inverse relationship between the NAb binding and macrophage activity. Considering these experiments with the lack of significant difference in macrophage cytolysis by male and female F1's, the data does not support a direct correspondence between NAb and macrophage cytolysis.

NK cell and activated macrophage activity in individual F1 mice

Lastly, a comparison was made between the NK activity and activated macrophage cytolysis of individual male and female F1 mice (Figure 9). None of the three experiments displayed a correlation between these cellular effectors of natural immunity. The data seems to suggest that the regulation of NK cells and macrophage cells occurs independently since there was a difference in lysis by male and female splenic NK cells yet no difference in macrophage cytolysis.

TABLE I

Tumor Frequency in CBA/J and CBA/N Mice[a]

Expt.	Sex	% Tumor Frequency	
		CBA/J +/+♀ or +/-♂	CBA/N xid/xid♀ or xid/-♂
1	♀	50 (5/10)	87.5 (7/8)
2	♀	33.3 (2/6)	80 (4/5)
3	♂	12.5 (1/8)	57.1 (4/7)
4	♂	28.6 (2/7)	100 (3/3)
	♀	N.D.	25 (1/4)

a A threshold s.c. inocula of 10^4 RI-28 cells was administered to each mouse. The mice were age matched in each experiment and the ages ranged from 6 to 14 weeks old. The tumor frequencies were recorded at 21 days after sc inocula. The ratio of tumor-bearing animals to total mice per group is given in parentheses.

TABLE II

Latency of Tumor Occurence in CBA/J and CBA/N Mice[a]

Expt.	Sex	Days after s.c. inoculum	
		CBA/J +/+♀ or +/-♂	CBA/N xid/xid♀ or xid/-♂
1	♀	7,7,14,14,21	7,7,7,7,7,7,14
2	♀	14,17	14,14,10,10
3	♂	17	10,10,17,14
4	♂	18,18	11,11,12
	♀	N.D.	11
Mean latency ± S.E.		15 ± 1.5 day	10.5 ± 0.7 days

a The data represents the total number of days between the administration of the s.c. inoculum of RI-28 and the initial appearance of palpable s.c. tumors. The results were analyzed by Student's t-independent test and the mean latency differences were statistically significant ($p < 0.01$).

TABLE III

C-Dependent Cytolysis of Serum NAb from CBA/J and CBA/N Mice[a]

Expt.	Tumor Target	% Cytolysis ± S.E.		P
		CBA/J +/+♀ or +/-♂	CBA/N xid/xid♀ or xid/-♂	
1	RI-28 (7)	11.1 ± 3.0	0.3 ± 0.1	< 0.02
2	RI-28/6 (3)	16.2 ± 4.1	2.4 ± 2.4	< 0.03
3	RI-28/8 (7)	21.1 ± 4.4	0.9 ± 0.9	< 0.01
4	RI-28/7 (1)	34.3	0.0	N.S.

a Target cells were incubated with sera from age and sex matched inbred mice 12 wk to 8 mo of age. The numbers of independent tests are given in parentheses. The results were analyzed with the Student's t-dependent tests and significant differences between xid-bearing and normal mice were demonstrated.

TABLE IV

Binding of Serum NAb from CBA/J and CBA/N Mice[a]

Expt.	Tumor Target	FITC Antibody	Mean Channel Fluorescence ± S.E.		P
			CBA/J +/+♀ or +/-♂	CBA/N xid/xid♀ or xid/-♂	
1	RI-28 (7)	αIgG + αIgM	44.0 ± 13.5	7.9 ± 1.8	< 0.03
2 a.	RI-28	αIgG + αIgM	197.9 ± 34.9 (2)	17.5 ± 1.2 (3)	N.S.
2 b.	RI-28	αIgG + αIgM	171.2 ± 22.6 (2)	20.7 ± 0.8 (2)	N.S.
2 c.	RI-28	αIgG + αIgM	184.6 ± 21.5 (4)	18.8 ± 1.2 (5)	< 0.003

[a] Pooled sera was used for experiment 1 and the number of independent tests is shown in parentheses. The binding of sera from male and female littermates was examined separately in experiments 2a and 2b respectively and the number of mice tested are given in parentheses. Data from male 2a and female 2b mice were combined in 2c for statistical purposes and significance was determined by the Student's t-independent analysis.

TABLE V

Splenic NK Cytolysis by CBA/J and CBA/N Mice[a]

Expt.	Tumor Target	% Cytolysis ± S.E.			
		CBA/J +/+♀ or +/-♂		CBA/N xid/xid♀ or xid/-♂	
		150:1	75:1	150:1	75:1
1	RI-28 (3)	0.3 ± 0.1	0.9 ± 0.8	2.2 ± 0.9	3.5 ± 1.7
2	RI-28/6 (5)	49.2 ± 8.5	38.8 ± 7.2	57.7 ± 7.1	46.7 ± 6.2
3	RI-28/8 (3)	19.7 ± 7.3	21.8 ± 8.8	29.6 ± 7.9	23.0 ± 5.1
4	YAC-NIH (5)	51.5 ± 8.9	47.3 ± 8.8	58.1 ± 4.2	50.7 ± 5.9
5	SL2-5 (4)	40.6 ± 10.0	29.3 ± 8.7	37.2 ± 5.5	27.6 ± 3.9

[a] Spleen cells were pooled from 2-3 mice in each experiment and the mice were sex and age matched. The animals tested were between the ages of 7 and 13 weeks. No statistically significant differences were detected except for experiment 3 at an E:T ratio of 150:1 in which the xid-bearing CBA/N mice exhibited a greater activity than the normal CBA/J mice according to the Student's t-independent analysis ($p < 0.01$). The number of independent assays are given in parentheses.

TABLE VI

Activated Macrophage Cytolysis by CBA/J and CBA/N Mice[a]

Expt.	Tumor Target	% Cytolysis ± S.E.		P
		CBA/J +/+♀ or +/-♂	CBA/N xid/xid♀ or xid/-♂	
1	RI-28 (3)	5.6 ± 3.0	15.4 ± 5.5	N.S.
2	L5178Y-F9 (3)	27.1 ± 13.2	26.3 ± 13.2	N.S.
3	RI-28/8 (1)	8.7	29.7	N.S.

a Data is given at an E/T ratio of 50/1 for the RI-28 and the subclone RI-28/8. The L5178Y-F9 is more sensitive to macrophage and the data is given at an E/T ratio of 10/1. No significant differences were observed using the t-independent or t-dependent Student's test. The number of independent assays are given in parentheses.

TABLE VII

Tumor Frequency in (CBA/N x CBA/J) F1 Male and Female Mice[a]

Expt.	% Tumor Frequency	
	(CBA/N x CBA/J) F1 ♀ xid/+	(CBA/N x CBA/J) F1 ♂ xid/-
1	14.3 (1/7)	42.9 (3/7)
2	75 (6/8)	83.3 (5/6)
3	33.3 (2/6)	71.4 (5/7)
4	25 (1/4)	66.7 (4/6)

a A threshold s.c. inocula of 2 x 10^4 RI-28 cells was administered to each mouse. The mice were 11 weeks of age in each experiment. The tumor frequencies were recorded at 21 days after sc inocula. The ratio of tumor-bearing animals to total mice per group is given in parentheses.

TABLE VIII

Latency of Tumor Occurence in (CBA/N x CBA/J) F1 Male and Female Mice[a]

Expt.	Days after s.c. inoculum	
	(CBA/N x CBA/J) F1 xid/+ ♀	(CBA/N x CBA/J) F1 xid/- ♂
1	10	11,11,16
2	10,10,11,11,13,18	10,10,10,11,13
3	13,20	9,9,9,9,13
4	20	12,12,14,20
Mean Latency ± S.E.	14 ± 1.4 days	12 ± 0.7 days

[a] The data represents the total number of days between the administration of the s.c. inoculum of RI-28 and the initial appearance of palpable s.c. tumors. Significant differences could not be demonstrated using Student's t-independent or t-dependent analysis.

TABLE IX

C-Dependent Cytolysis of Serum NAb from (CBA/N x CBA/J) F1 Male and Female Mice[a]

Expt.	Tumor Target	% Cytolysis ± S.E.		P
		(CBA/N x CBA/J) F1 ♀ xid/+	(CBA/N x CBA/J) F1 ♂ xid/-	
1	RI-28 (5)	27.6 ± 7.4	2.2 ± 1.0	< 0.04
2	RI-28/8 (3)	16.0 ± 6.6	3.6 ± 2.5	< 0.04

a The F1 male and female mice were age matched in each experiment and the ages of mice tested ranged 9 - 13 weeks. The RI-28 cells were incubated with whole or ½ dilutions of sera and the RI-28/8 cells were incubated with ¼ dilutions of sera. Statistically significant differences between male and female mice were shown by analysis with the Student's t-dependent test. The number of independent assays are given in parentheses.

TABLE X

Binding of Serum NAb from (CBA/N x CBA/J) F1
Male and Female Mice[a]

Expt.	Tumor Target	FITC Antibody	Mean Channel Fluorescence (CBA/N x CBA/J) F1 ♀ xid/+	Mean Channel Fluorescence (CBA/N x CBA/J) F1 ♂ xid/-	P
1	RI-28 (8)	αIgG + αIgM	64.7 ± 11.2	25.4 ± 7.7	< 0.02
2	RI-28	αIgG + αIgM	95.0 ± 10.5 (28)	56.3 ± 7.1 (26)	< 0.01
3 a	RI-28 (4)	αIgG	66.3 ± 10.8	13.9 ± 7.9	< 0.01
3 b	RI-28 (4)	αIgM	42.7 ± 25.7	2.2 ± 0.9	N.S.

[a] Pooled sera was used except for experiment 2 in which sera from individual mice was tested. The number of mice examined in experiment 2 is shown in parentheses. The male and female mice were age matched and ages ranged from 7 to 19 weeks. Statistical significance is shown using the Student's t-independent analysis. The number of independent assays is given in parentheses.

TABLE XI

Splenic NK Cytolysis of (CBA/N x CBA/J) F1 Male and Female Mice[a]

Expt.	Tumor Target	% Cytolysis ± S.E.			
		(CBA/N x CBA/J) F1 ♀ xid/+		CBA/N x CBA/J) F1 ♂ xid/-	
		150:1	75:1	150:1	75:1
1	RI-28 (6)	5.7 ± 6.0	1.0 ± 0.5	1.6 ± 1.2	1.1 ± 0.3
2	RI-28/8 (1)	7.5	7.0	24.9	25.3
3	SL2-5 (2)	14.4 ± 7.2	15.9 ± 9.5	23.2 ± 3.3	21.5 ± 4.5
4	YAC-NIH (3)	33.5 ± 10.3	32.4 ± 11.2	43.2 ± 5.2	38.2 ± 6.1
5	RI-28	5.4 ± 0.6 (6)	4.3 ± 0.3 (6)	1.7 ± 0.7 (6)	2.1 ± 0.8 (6)
6	YAC-NIH	27.0 ± 1.2 (6)	22.8 ± 0.8 (6)	39.1 ± 2.6 (6)	30.7 ± 3.4 (6)

[a] Spleen cells were pooled from 2-3 mice except in experiments 5 and 6 where individual mice were tested. The number of individual mice is given in parentheses for experiments 5 and 6. No statistically significant differences were detected except for experiment 6 at an E:T ratio of 150:1 in which the xid-bearing males exhibited a greater activity than the normal females according to the Student's t-independent analysis ($p < 0.01$). The numbers of independent tests are given in parentheses.

TABLE XII

Activated Macrophage Cytolysis by (CBA/N x CBA/J) F1 Male and Female Mice[a]

Expt.	Tumor Target	% Cytolysis ± S.E. (CBA/N x CBA/J) F1 ♀ xid/+	% Cytolysis ± S.E. (CBA/N x CBA/J) F1 ♂ xid/-	P
1	RI-28 (7)	7.8 ± 2.4	10.5 ± 2.5	N.S.
2	RI-28/8 (1)	47.0	49.4	N.S.
3	L5178Y-F9 (4)	30.2 ± 3.2	34.0 ± 4.2	N.S.

a The data are given for RI-28 and subclone RI-28/8 at an E/T ratio of 50/1 and for the L5178Y-F9 at 10/1. Mice were age matched and between the ages of 10 to 19 weeks. No significant differences were observed by analysis with the Student's t-independent or t-dependent test. The number of independent assays are given in parentheses.

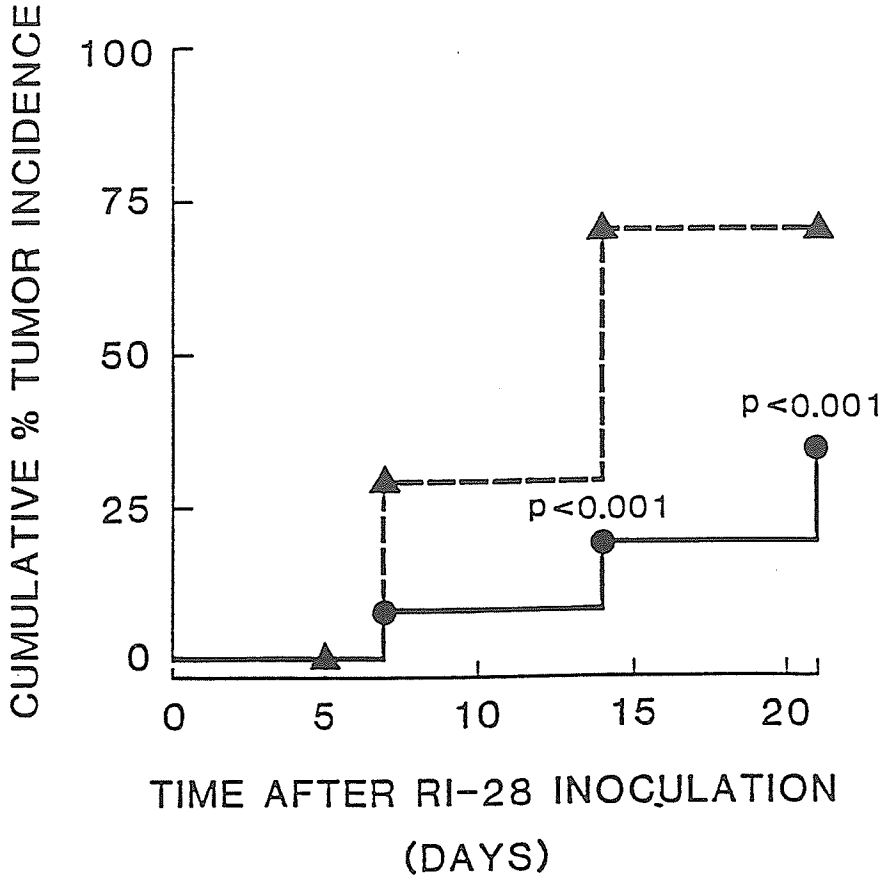

Figure 1: Cumulative percentage tumor incidence in CBA/J and CBA/N mice. The CBA/J mice (●) and xid-bearing CBA/N mice (▲) were injected sc with 10^4 RI-28 cells in a 0.1 ml volume. The animals were tested in four sex and age matched groups between 6 and 14 weeks of age. In total, 31 CBA/J mice and 27 CBA/N mice were tested. The cumulative percentage tumor frequencies were significantly different as indicated using the Student's t-independent test.

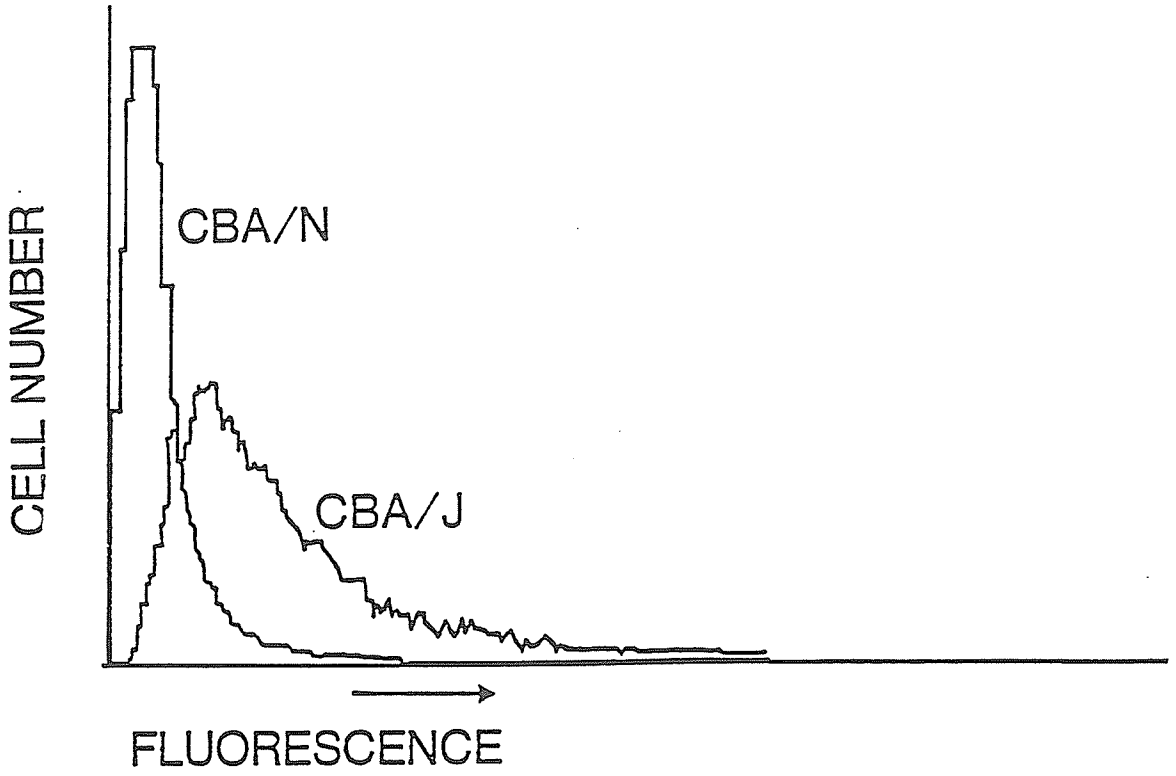

Figure 2: Linear fluorescence profile of CBA/J and CBA/N normal serum NAb.
This is an example of a typical linear fluorescence profile comparing CBA/J and CBA/N normal serum for the presence of anti-RI-28 IgG and IgM antibodies. Background fluorescence has been subtracted.

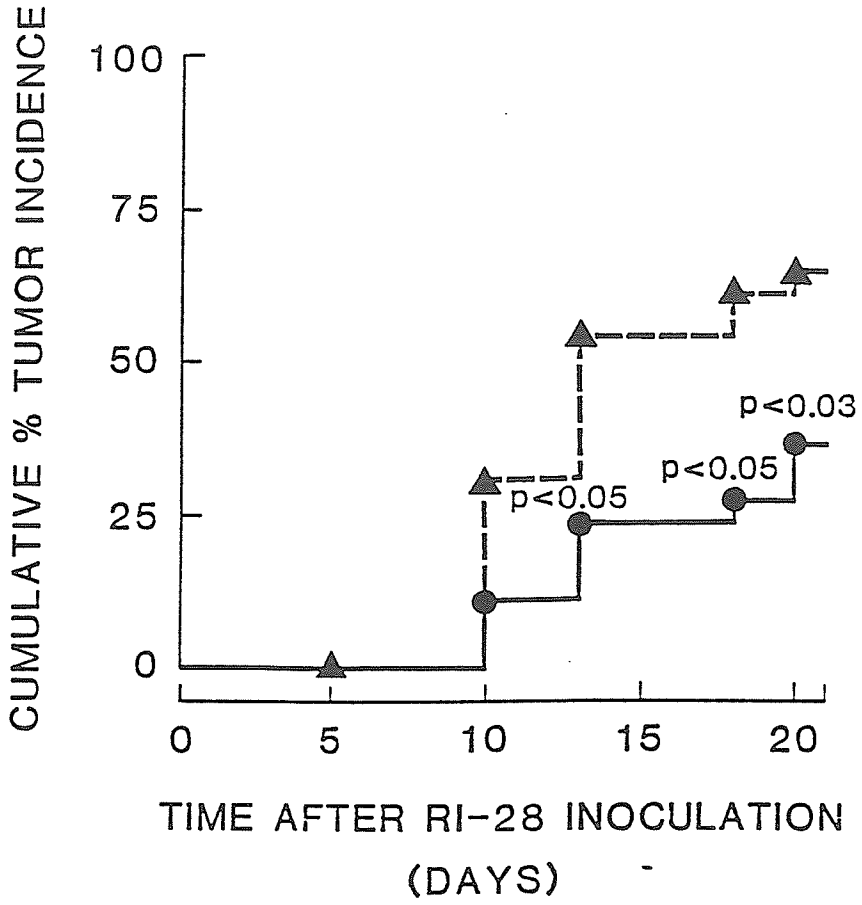

Figure 3: Cumulative percentage tumor incidence in (CBA/N x CBA/J) F1 male and female mice. The F1 female mice (●) and xid-bearing F1 males (▲) were injected sc with 2 x 10^4 RI-28 cells in a 0.1 ml volume. Four experiments were performed with age matched 11 week old male and female littermates. In total, 25 F1 females and 26 F1 males were tested. The cumulative percentage tumor frequencies were significantly different as indicated using the Student's t-independent test.

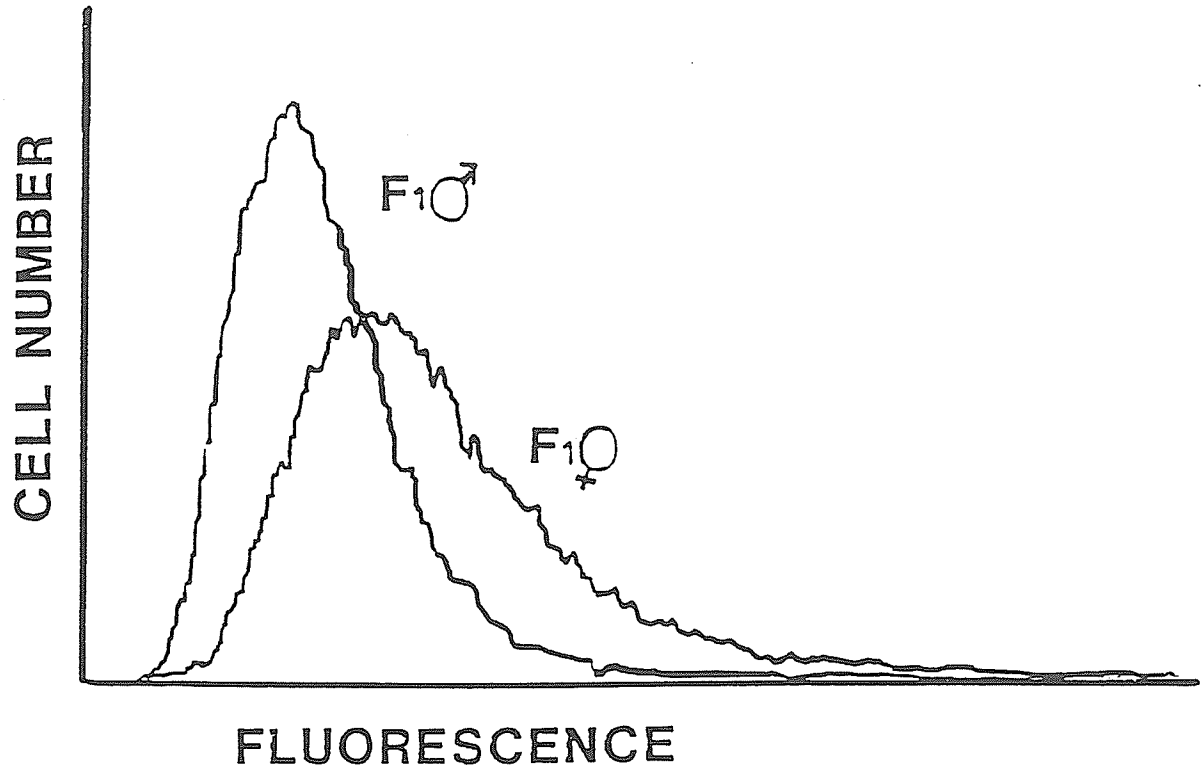

Figure 4: Linear fluorescence profile of (CBA/N x CBA/J) F1 normal serum NAb binding.
This is an example of a typical linear fluorescence profile comparing F1 male and female normal serum for the presence of anti-RI-28 IgG and IgM antibodies. Background fluorescence has been subtracted.

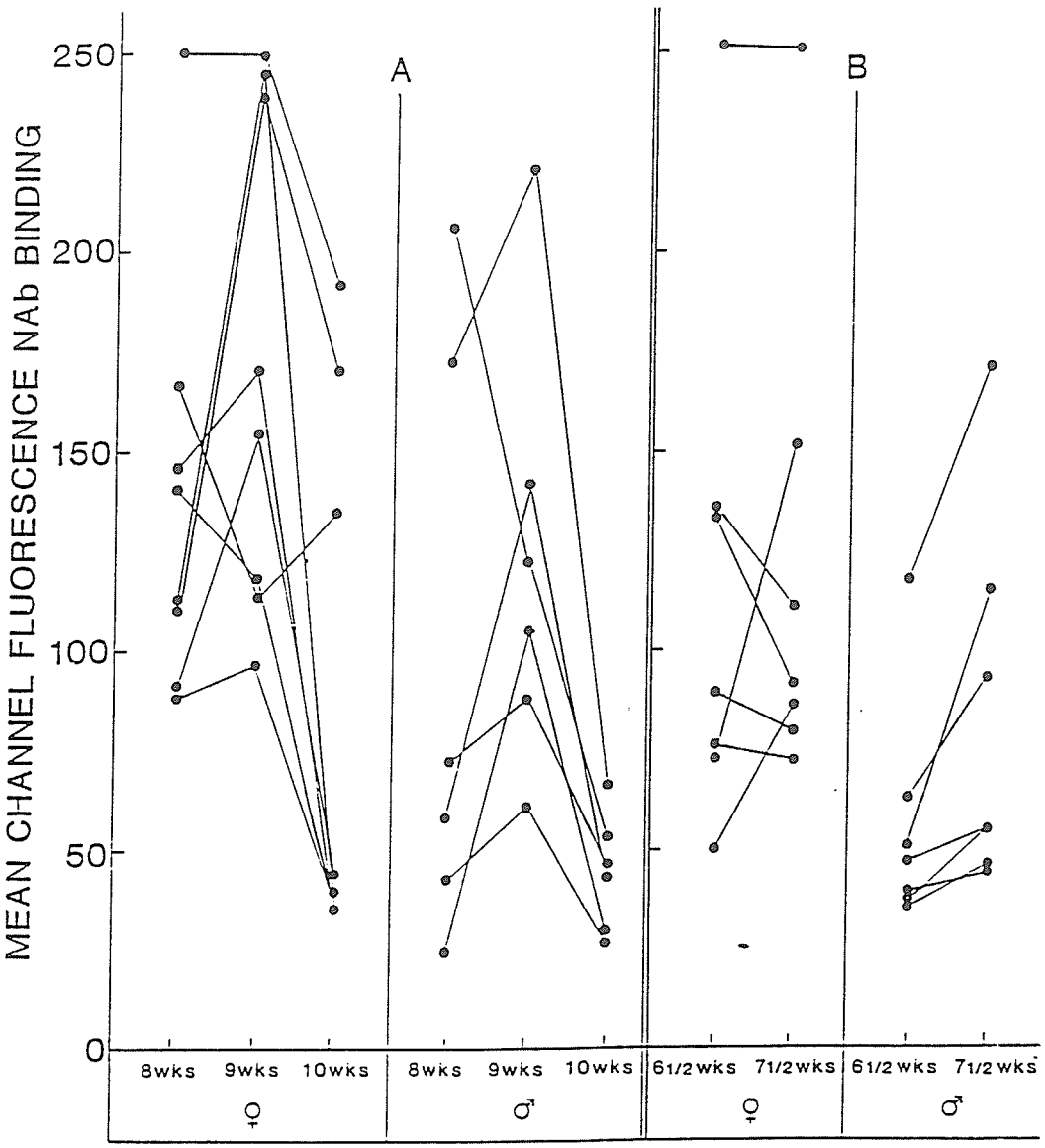

Figure 5: NAb binding from individual (CBA/N x CBA/J) F1 mice. Age matched male and female F1's were tail bled at A) 8, 9 and 10 and B) 6½ and 7½ weeks. Male and female sera from each bleed were tested simultaneously. In experiment A significant direct correlations were seen for NAb binding for bleeds 1 and 3 ($p < 0.03$). Bleeds 1 and 2 were not significant ($p < 0.07$). In experiment B significant direct correlation was achieved ($p < 0.001$).

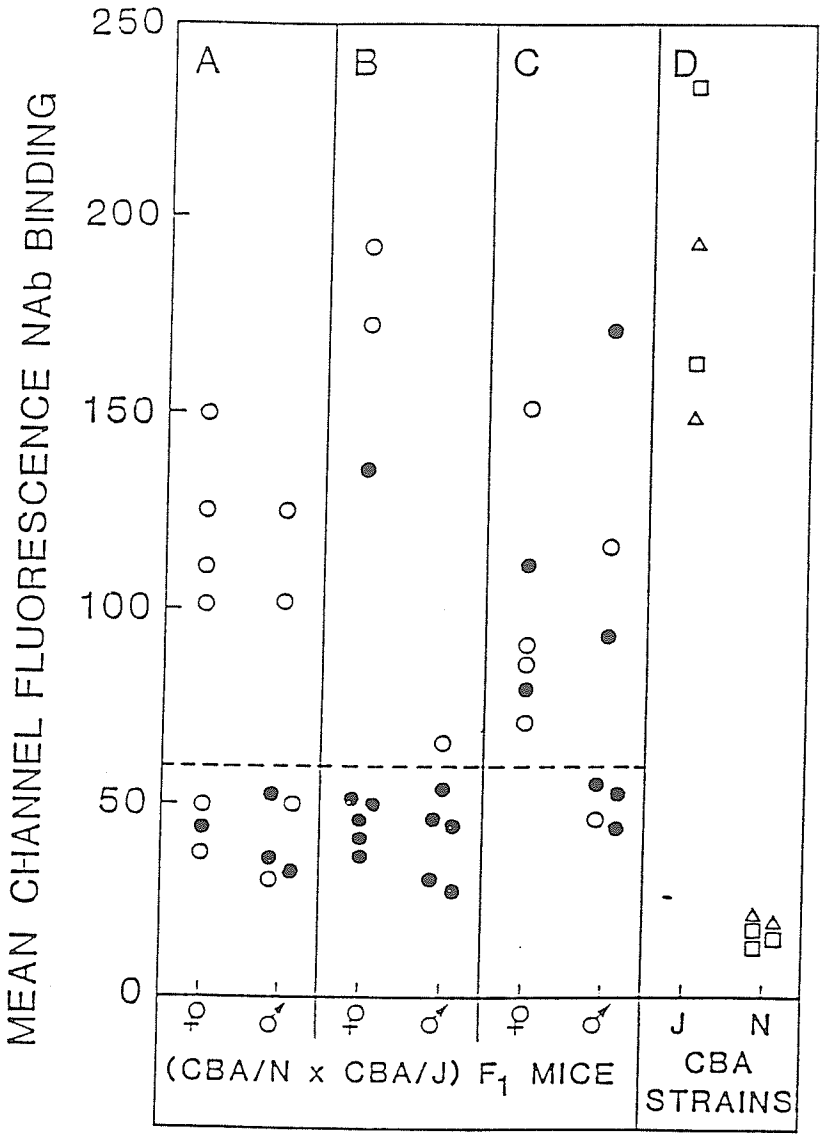

Figure 6: NAb binding activity and tumor susceptibility of individual (CBA/N x CBA/J) F1 mice. Age matched F1 mice were tail bled at A) 11, B) 10 and C) 7½ weeks of age and 1 week later they were challenged with a sc inoculum of 2×10^4 RI-28 cells. Closed circles (●) represent animals that developed a tumor and open circles (○) those the remained tumor free. The maximum cumulative tumor frequency for F1 mice which exhibited NAb binding above 60 channels (---) vs. the tumor frequency in mice with NAb binding below 60 channels was significantly different ($p<0.003$) using the Mantel-Haenszel Chi-squared test. Panel D represents individual bleeds from 6 week old CBA/N and CBA/J mice (male, □ and female, △ littermates) which were not subsequently exposed to a tumor inoculum. Mouse sera was diluted 1/5 in 10% RPMI.

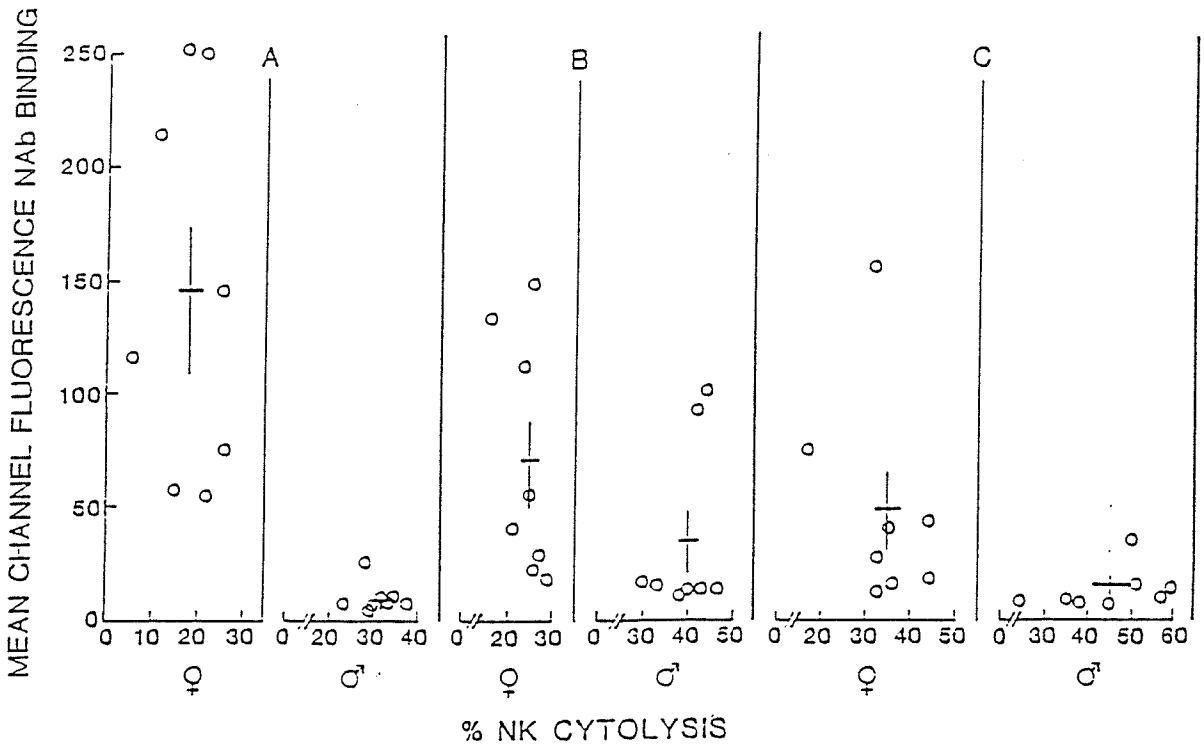

Figure 7: NAb binding and NK cytolysis of individual (CBA/N x CBA/J) F1 mice. Serum NAb, spleens and bone marrow were removed from groups of male and female mice ages A) 9, B) 9 and C) 8-9 weeks. Serum NAb binding against RI-28 and NK cell cytolysis of SL2-5 cells were examined with male and female mice of each series being tested simultaneously. Means $\pm$ S.E. are indicated (+).

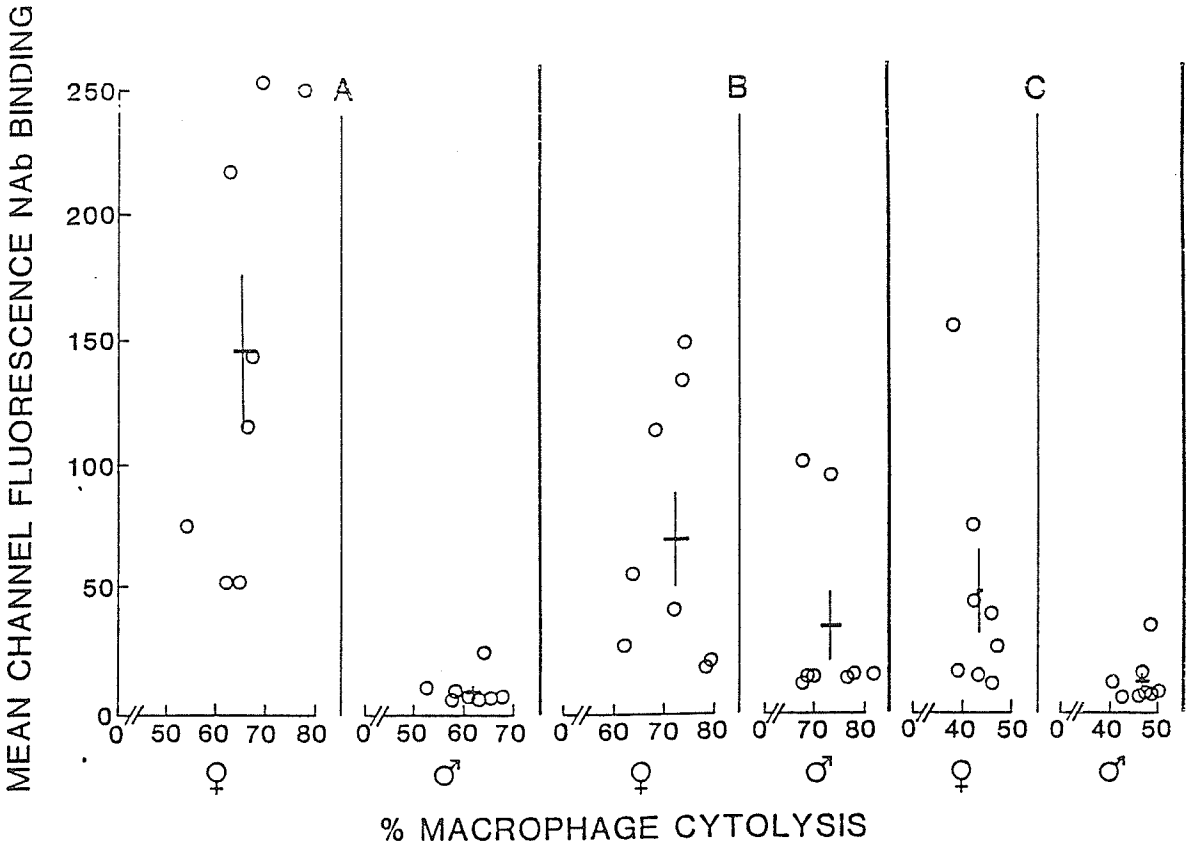

Figure 8: NAb binding and in vitro activated macrophage activity of individual (CBA/N x CBA/J) F1 mice. Serum NAb and in vitro Con A stimulated macrophage activity of the same individual F1 mice described in Figure 7 were both assessed against RI-28 cells. Male and female mice of each series were tested simultaneously. Means ± S.E. are indicated (+).

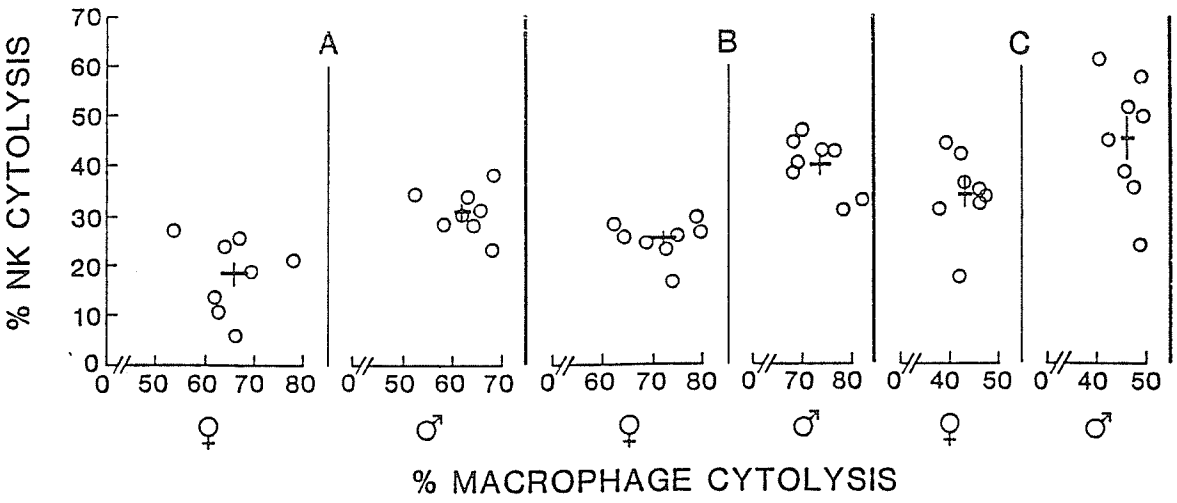

Figure 9: NK cytolysis and in vitro activated macrophage activity of individual (CBA/$\overline{N}$ x CBA/J) F1 mice. NK cytolysis and in vitro Con-A stimulated macrophage activity of the same individual F1 mice described in Figure 7 were assessed against SL2-5 and RI-28 cells respectively. Male and female mice of each series were tested simultaneously. Means $\pm$ S.E. are indicated (+).

DISCUSSION

In this study mice expressing the xid defect, namely, CBA/N mice and (CBA/N x CBA/J)F1 male mice, were examined for their ability to handle threshold sc tumor inocula compared to their normal counterparts, CBA/J and F1 female mice respectively. The xid-bearing mice are known to have low levels of IgG3 and IgM antibodies in their serum (142), therefore these mice were used as an *in vivo* genetic model for examining NAb participation in tumor surveillance. Since NK cells, macrophages and NAb have been implicated in the host first line of defense against small tumor inocula, we also analyzed the function of these effectors *in vitro.*

The tumorigenicity experiments revealed that the B-cell deficient mice had higher tumor frequencies than normal mice following the sc inoculation of a threshold dose of the RI-28 lymphoma. That is, the CBA/N mice and the (CBA/N x CBA/J) F1 male mice exhibited tumor frequencies which were twice as high as the CBA/J or F1 female mice. The average latency of tumor development was generally later in normal mice, but this difference was more evident in comparisons of the CBA/N and CBA/J inbred mice than the F1 male and female hybrids.

This inverse relationship between NAb and tumor incidence has been alluded to by previous investigators. For example, Menard etal, found that BALB/c mice which exhibited

elevated levels of anti-tumor antibodies in their serum were more resistant to tumor challenge (156). Chow et al found that the administration of adjuvants to DBA/2 mice increased the NAb activity in addition to decreasing the tumor frequencies of L5178Y or P815 lymphoma threshold sc inocula. They also demonstrated that tumors which were coated with NAb were less tumorigenic than uncoated tumors (25). Similarly, Tough and Chow were able to isolate by cell sorting high NAb binding SL2-5 and L5178Y-F9 cells following 12-0-tetradecanoylphorbol-13-acetate treatment as well as high and low NAb binding L5178Y-F9 cells from a heterogenous cell culture. The high NAb binding variants all exhibited reduced tumorigenicity of threshold sc inocula in syngeneic DBA/2 mice (169). Finally, Agassy-Cahalon et al prepared hybridomas which secreted IgM NAb that were reactive with L5178Y lymphoma cells. They found that one hybridoma secreted monoclonal NAb that was cytotoxic with only mouse lymphoma cell lines. Mice were first treated with urethane to induce carcinogenesis, then they were given the IgM NAb's two times a week for 5 months, after which time the tumor foci in the lungs were examined. Those mice that had been treated with the cytotoxic monoclonal NAb displayed a reduced number of foci (170). The above research, along with the data herein of an inverse relationship between tumorigenicity and NAb levels in xid-bearing mice, suggests that

NAb may play a key role in the defense against small tumor foci.

The splenic NK cell activity of normal and xid-bearing mice was compared by in vitro cytolysis against syngeneic and allogeneic tumor cell lines. There was no statistically significant difference in the NK activity displayed by the mice and in general the B-cell deficient CBA/N and F1 male mice displayed slightly higher cytolysis of the tumor cell lines tested. Martin and Martin reported that even though CBA/HN mice have reduced levels of anti-tumor reactive NAb, these mice do not exhibit a higher incidence of spontaneous tumors (149). The slightly increased NK activity displayed by the CBA/N and xid-bearing F1 male mice in this study may provide a rationale for their observation if, in fact, the NK activity we observed is associated with xid expression.

Chow et al have shown that an NKr SL2 lymphoma subclone displayed increased tumorigenicity in mice (25). Since the RI-28 leukemia was also found to be NKr, one might predict that the tumor frequencies in both normal and xid-bearing mice would also be high. However, the normal CBA/J and F1 female mice exhibited reduced tumorigenicity compared to their xid-bearing counterparts; therefore, some other effector mechanism must be contributing to the difference. It is proposed that the serum NAb levels of the normal mice is

likely responsible.

Similar results were obtained when C.parvum activated peritoneal macrophages were tested for their ability to lyse the syngeneic RI-28 leukemia cell and the allogeneic L5178Y-F9 lymphoma. The normal CBA/J and xid/+ F1 females exhibited activated macrophage activity which was essentially equivalent to the activity displayed by the CBA/N and xid/- F1 males respectively. The RI-28 was also found to be fairly resistant to activated peritoneal macrophages. Again, this data suggests that the differences in tumor frequency must be due to anti-tumor responses other than the macrophage activity.

The serum NAb levels of xid-bearing mice and normal mice were analyzed in vitro in complement-mediated cytolysis assays as well as in assays which measured bound IgG and IgM by FACS. As was predicted, the serum anti-RI-28 natural antibodies from CBA/J and F1 female mice were far more capable of lysing the tumor target in the presence of complement whereas the sera from xid-bearing CBA/N and F1 male mice exhibited very little, if any, cytolysis of the RI-28 leukemia cell. Since this data represents only complement-binding NAb, we measured the IgG and IgM which could bind to the RI-28 cell line. The sera from normal CBA/J mice clearly exhibited a greater amount of bound IgG and IgM anti-RI-

28 antibodies than sera from CBA/N mice. This difference was very apparent when age matched litters of CBA/N and CBA/J mice were examined individually (Figure 6, panel D).

When the (CBA/N x CBA/J) F1 male and female mice were examined for bound NAb, it was found that the differences were not as evident. That is, in two of the eight experiments performed using pooled sera, the amount of bound anti-RI-28 IgG and IgM antibodies from F1 males was equivalent to the F1 females; however, sera from heterozygous females still had a mean of the mean fluorescence channel which was 2.5 times higher than the hemizygous males. These findings prompted the assessment of the NAb levels of the F1 mice on an individual basis, and we found that the NAb binding activity of sera from male and female mice was overlapping and very heterogeneous.

Due to this observation of heterogeneity in NAb levels, mice were individually tail bled and tested for NAb binding activity one week prior to the RI-28 threshold sc tumor inoculum in three of the four F1 tumorigenicity experiments. This would enable us to see if there was a relationship between tumor susceptibility in individual mice and their anti-RI-28 NAb levels. It was found that F1 mice, either male or female, which had low serum NAb exhibited a tumor incidence that was approximately three times that of the F1

mice displaying high serum NAb. Thus, this data based on individual mice, supported the hypothesis that NAb participates in the host defense against small tumor foci *in vivo*.

The heterogeneity demonstrated in F1 sera NAb activity might also be accompanied by similar variable macrophage and NK activity which could contribute to the increased tumorigenicity displayed by the F1 males. This possibility was tested in three separate experiments in which individual F1 male and female mice provided bone marrow (as a source of macrophage), spleen cells and serum.

The *in vitro* activated bone marrow macrophages displayed no consistent direct correlation with the NAb levels. However, since the RI-28 cell was found to be sensitive to cytolysis by bone marrow macrophages, it is possible that macrophages may have contributed to the resistance demonstrated by tumor-free mice. It has been shown that the antitumor cytotoxicity exhibited by macrophages can be augmented by the addition of antibodies from tumor bearing mice (90). Furthermore, the suppression of tumor growth by a hyperimmune antitumor serum could be enhanced by stimulating intraperitoneal macrophages (89) and NAb may also act as an opsonin for macrophages (168).

In two of the three experiments in which NAb activity was compared to NK activity, no direct correlation could be

demonstrated. In fact, one of the experiments exhibited an inverse relationship between the NAb and NK levels of individual mice. This finding along with the NKr nature of the RI-28 cell suggests that NK cell activity did not contribute to the tumor resistance exhibited by those mice with high serum NAb levels.

The heterogeneity and variation in NAb levels exhibited by individual F1 mice has been previously documented by Pierotti and Colnaghi (157). They found that natural anti-EL4 lymphona antibodies could be detected in the serum of C3Hf mice but not in C57BL mice. The F1 hybrids of these mice also had anti-tumor NAb and the variability demonstrated by individuals was evident whether or not they were caged together or if they were derived from the same or different litters. Martin and Martin also found that individual (CBA/HN x DBA/2) F1 mice had variable levels of NAb detected by cytotoxicity activity against an NB1 neuroblastoma cell line (149).

In analyzing the NAb levels in individual B-cell deficient F1 males, it was surprising to find that some of these mice had very high binding anti-RI-28 NAb. It is possible that these mice represent male responder mice which have been examined by Kenny and Guelde (171). As was mentioned

previously, xid-bearing F1 males normally do not express antiphosphocholine antibodies of the dominant T15+ idiotype (122, 123), but rather they produce decreased levels of T15- idiotype antibody. Kenny found that approximately 65% of (CBA/N x BALB/c) F1 male mice express the T15- idiotype anti-PC antibody, 25% produce very low levels of the T15+ idiotype and 10% respond as well as females to phospocholine and the antibodies produced were of T15+ idiotype. These F1 male responder mice were found to have normal levels of serum IgM and they could respond to TNP-Ficoll. They suggested that these mice had developed an Lyb5+ B cell population normally absent in xid-bearing mice by a reversion of the xid defect to the normal phenotype or that the X-linked gene was "leaky" (171). In addition to this, Dighiero et al have shown that hybridomas derived from (CBA/N x BALB/c) F1 male mice secrete natural autoantibodies. Therefore xid-bearing mice have the genetic information needed to produce these antibodies yet they do not secrete them normally (174). If a reversion of the xid phenotype had occurred in our F1 males it is possible that their high binding NAb activity may be due to natural autoantibody production. Finally, Kenny et al demonstrated that the xid gene may act

synergistically with other genes since C3H/He.xid congenic mice exhibited immune defects which were more extreme than in the CBA/N mice (172). An interaction between genes has been shown in NZB.xid congenic mice whereby polyclonal B cell activators can augment the production of autoantibodies which normally are not produced in these congenic mice (173). Therefore, it is possible that the CBA/J mice used in this study possess a gene which can partially counteract the xid defect in F1 males.

The heterogeneous NAb binding exhibited by individual F1 females may possibly be due to the inactivation of one of the X chromosomes in the heterozygous females. Nahm et al have found that splenic B cells from F1 females exhibited an unbalanced mosaicism in favor of the normal non-xid chromosome, and conversely, other splenocytes and tissue cells display balanced mosaicism. They concluded that if the xid gene affects the B cells directly it would eliminate or select against the xid+ B cells. Therefore, they suggested that the xid gene(s) only affects B cells which produce IgG3 since nearly all of the IgG3-secreting hybridomas expressed the normal X chromosome, whereas many of the IgG1-secreting hybridomas could express the mutant xid X chromosome (175). Forrester et al confirmed these results and in addition they demonstrated that pre-B cells revealed normal mosaicism and

that non-random X chromosome inactivation increases with age (176). This unbalanced mosaicism has also been demonstrated in the B cells of humans suffering from X-chromosome-linked severe combined immunodificiency (177). The variable NAb levels of the (CBA/N x CBA/J) F1 females in this study may be the result of a range of balance in the X chromosome inactivation seen in B cells.

Several investigators have found that NAb are able to cross-react with a wide spectrum of determinants (69, 178-180). For example, Colnaghi et al produced a panel of hybridoma cells from normal murine spleen cells and a mouse myeloma cell line in which they selected for high anti-EL4 lymphoma responses. The resulting monoclonal NAb's were found to be cross-reactive with antigenic determinants found on various tumor cell lines and normal tissue cells (69). This raises the possibility that the natural anti-RI-28 antibodies detected in the sera of CBA/J mice and (CBA/N x CBA/J) F1 mice included autoantibodies. Interestingly, Hayakawa et al have demonstrated that splenic Ly-1+ B cells from normal BALB/C mice and autoimmune NZB mice are largely responsible for the production of IgM autoantibodies (187). In addition to this, Smith et al found that CBA/N or NZB.xid congenic mice have autoantibody producing Ly1+ B cells which are predominantly Lyb5+ B cells. Although this population

of cells represents only a small percentage of the entire B cell repetoire of xid-bearing animals, it may account for some of the RI-28 tumor resistance demonstrated in the CBA/J and responder (CBA/N x CBA/J) F1 mice.

NAb can exert its antitimor effects by a number of possible mechanisms. Several investigators have provided evidence which suggests that antitumor antiserum can interfere with tumor cells through antibody-dependent cellular cytotoxicity reactions (84, 91). The in vitro cytolysis by NAb suggest that they might act on tumor cells directly by complement-mediated cytolysis (188) or NAb may act as an opsonin to enhance phagocytosis of tumor cells (168). NAb may bind to growth factors which are required for tumor cell proliferation (189) or may block cell surface structures to inhibit the colonization, adherence or motility of tumor cells (190-192).

The physiological importance of these circulating antibodies remains uncertain, yet there is evidence which suggests some of the possible functions of NAb. For example, in 1975 Kay found that aged human red blood cells could be phagocytozed when they were incubated in human IgG NAb (193). Similarly, Khansari and Fudenberg demonstrated that the phagocytosis of old platelets required the presence of intact membrane bound IgG which had been eluted from

senescent red blood cells (194). In studying bone marrow graft rejection, Warner and Dennert found that NK cells appeared to be involved. However, the rejection of allografts is H-2 specific and it was known that NK cells do not act against H-2 determinants. They found that mice have H-2 specific NAb and they suggested that the rejection may be mediated by antibody-dependent cellular cytotoxicity (195). Holmberg et al found that IgM NAb derived from hybridomas from 6-day-old BALB/c mice displayed a high degree of cross-reactivity with each other and they suggested that NAb may be involved in an idiotypic network in the developing immune system (196). Finally, NAb has been shown to be polyspecific and demonstrates an extensive cross-reactivity with cytoskeletal constituents (197). In conclusion, NAb may contribute to the removal of senescent or damaged cells, or NAb may be involved in some aspects of immune regulation through an idiotypic network. Since tumor cells are host cells which exhibit some sort of abnormal behaviour the antitumor responses of NAb are not surprising.

The data presented herein supports the hypothesis that Nab are important mediators in the host defense against small tumor foci. The xid-bearing CBA/N mice and (CBA/N x CBA/J) F1 male mice were more susceptible to threshold sc inocula of syngeneic RI-28 than the normal CBA/J and F1

female mice. The tumor incidence in individual F1 hybrids correlated inversely with their levels of anti-RI-28 antibodies prior to tumor challenge. The NK cells and activated macrophages derived from the xid/- and xid/xid mice exhibited normal levels of cytolysis *in vitro* but these mice displayed reduced complement-mediated Nab lysis and reduced levels of fluorescence detected anti-RI-28 antibodies. Therefore, the differences in susceptibility to threshold tumor inocula would appear to be due to Nab levels rather than the NK and macrophage activity. This data represents the first genetic evidence that NAb contribute to tumor resistance *in vivo.*

REFERENCES

1. **Rous, P. and J.W. Beard.** 1935. The progression to carcinoma of virus-induced papillomas (Shope). J. Exp. Med. 62:523.

2. **Rous, P. and J.G. Kidd.** 1939. A comparison of virus-induced rabbit tumors with the tumors of unknown cause elicited by tarring. J. Exp. Med. 69:399.

3. **Foulds, L.** 1954. Tumor progression: a review. Cancer Res. 14:327.

4. **Friedewald, W.F. and P. Rous.** 1944. The initiating and promoting elements in tumor production. An analysis of the effects of tar, benzpyrene and methyl-cholanthrene on rabbit skin. J. Exp. Med. 80:101.

5. **Roy, J.** 1950. The structure and progression of vesical tumors induced in mice by 2-acetyl aminofluorene. Microscop. Soc. 70:173.

6. **Klein, G.** 1951. Comparative studies of mouse tumors with respect to their capacity for growth as "ascites tumors" and their average nucleic acid content per cell. Exper. Cell Res. 2:518.

7. **Klein, G. and L. Revesz.** 1953. Quantitative studies on the multiplication of neoplastic cells in vivo. I. Growth curves of the Ehrlich and MClM ascites tumors. J. Nat. Cancer Inst. 14:229.

8. **Foulds, L.** 1969. Neoplastic development, Vol. 1, Academic Press. London. pp 1.

9. **Foulds, L.** 1975. Neoplastic development, Vol. 2, Academic Press, London.

10. **Nowell, P.C.** 1976. The clonal evolution of tumor cell populations. Science. 194:23.

11. **Chow, D.A. and A.H. Greenberg.** 1980. The generation of tumor heterogeneity in vivo. Int. J. Cancer. 25:261.

12. **Chow, D.A., M. Ray, and A.H. Greenberg.** 1983. In vivo generation and selection of variants with altered sensitivity to natural resistance (NR): A model of tumor progression. Int. J. Cancer. 31:99.

13. **Ehrlich, P.** 1951. The collected papers of Paul Ehrlich. 2:550. London: Pergamon.

14. **Thomas, L.** 1959. Cellular and humoral aspects of the hypersensitivity state, ed. H. S. Lawrence. New York: Hoeber. pp. 529.

15. **Foley, E.J.** 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. Cancer Res. 13:835.

16. **Lotzova, E.** 1985. Effector immune mechanisms in cancer. Nat. Immun. Cell Growth Regul. 4:293.

17. **Stutman, O.** 1975. Immunodepression and malignancy. Adv. Cancer Res. 22:261.

18. **Rygaard, J. and C.O. Povlsen.** 1976. The nude mouse vs. the hypothesis of immunological surveillance. Transplant Rev. 28:43.

19. **Simpson, E. and S.L. Nehlsen.** 1971. Prolonged administration of antithymocyte serum in mice. II. Histopathological investigations. Clin. Exp. Immunol. 9:79.

20. **Karre, K., G.O. Klein, R. Kiessling, G. Klein and J.C. Roder.** 1980. In vitro NK-activity and in vivo resistance to leukemia: Studies of beige, beige/nude and wild type hosts on C57BL background. Int. J. Cancer 26:789.

21. **Haller, O., M. Hansson, R. Kiessling and H. Wigzell.** 1977. Role of non-conventional natural killer cells in resistance against syngeneic tumour cells in vivo. Nature. 270:609.

22. **Kasai, M., J.C. Leclerc, L. McVay-Boudreau, F.W. Shin and H. Cantor.** 1979. Direct evidence that natural killer cells in nonimmune spleen cell populations prevent tumor growth in vivo. J. Exp. Med. 149:1260.

23. **Chow, D.A., M.I. Greene and A.H. Greenberg.** 1979. Macrophage-dependent, NK-cell-independent "natural" surveillance of tumors in syngeneic mice. Int. J. Cancer 23:788.

24. **Keller, R.** 1980. Regulatory capacities of mononuclear phagocytes with particular reference to natural immunity against tumors. In: Herberman, R.B. ed. Natural cell-mediated immunity against tumors. New York. Academic Press. 1980: 1219.

25. **Chow, D.A., L.B. Wolosin and A.H. Greenberg.** 1981. Murine natural antitumor antibodies. II. The contribution of natural antibodies to tumor surveillance. Int. J. Cancer. 27:459.

26. **Urban, J.L., R.C. Burton, J.M. Holland, M.L. Kripke and H. Schreiber.** 1982. Mechanisms of syngeneic tumor rejection. J. Exp. Med. 155:557.

27. **Riccardi, C., A. Santoni, T. Barlozzari, P. Pucetti and R.B. Herberman.** 1980. In vivo natural reactivity of mice against tumor cells. Int. J. Cancer. 25:475.

28. **Flanagan, S.P.** 1966. "Nude", a new hairless gene with pleiotropic effects in the mouse. Genet. Res. 8:295.

29. **Wortis, H.H.** 1974. Immunological studies of nude mice. In: M.D. Cooper and N.L. Warner. ed. Contemporary topics in immunobiology, Plenum Press, New York and London. Vol. 3, pp. 243.

30. **Stutman, O.** 1974. Tumour development after 3 methyl-cholanthrene in immunologically deficient athymic-nude mice. Science. 183:534.

31. **Outzen, H.D., R.P. Custer, G.J. Eaton and R.T. Prehn.** 1975. Spontaneous and induced tumor incidence in germ-free nude mice. J. Reticuloendoth. Soc. 17:1.

32. **Gillette, R.W. and A. Fox.** 1975. The effect of T lymphocyte deficiency on tumour induction and growth. Cell. Immunol. 19:328.

33. **Herberman, R.B., M.E. Nunn, H.T. Holden, D.H. Lavrin.** 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int. J. Cancer. 16:230.

34. **Miller, S.C.** 1982. Production and renewal of murine natural killer cells in the spleen and bone marrow. J. Immunol. 129:2282.

35. **Herberman, R.B., M.E. Nunn and D.H. Lavrin.** 1975. Natural cytotoxicity reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. Int. J. Cancer 16:216.

36. **Shellam, G.R.** 1977. Gross-virus-induced lymphoma in the rat. V. Natural cytotoxic cells are non-T cells. Int. J. Cancer. 19:225.

37. **Kiessling, R., E. Klein, H. Pross and H. Wigzell.** 1975. "Natural" killer cell in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristic of the killer cell. Eur. J. Immunol. 5:117.

38. **Petranyi, G., R. Kiessling and G. Klein.** 1975. Genetic control of "natural" killer lymphocytes in the mouse. Immunogenetics. 2:53.

39. **Timonen, T., J.R. Ortaldo and R.B. Herberman.** 1981. Characteristics of human large granular lymphocytes and relationships to natural killer and K cells. J. Exp. Med. 153: 569.

40. **Herberman, R.B., J. Djeu, H.D. Kay, J.R. Ortaldo, C. Riccardi, G.D. Bonnard, H.T. Holden, R. Fagnani, A. Santoni and P. Puccetti.** 1979. Natural killer cells: Characteristics and regulation. Immunol. Rev. 44:43.

41. **Kiessling, R. and H. Wigzell.** 1979. An analysis of the murine NK cell as to structure, function and biological relevance. Immunol. Rev. 44:165.

42. **Warner, N.L. and A.T. Li.** 1979. Approaches to the identification of the natural killer cell and its target cell specificity. In: Immunobiology and immunotherapy of cancer. W. Terry and Y. Yamamura eds. Elsevier, North Holland. pp. 119.

43. **Cudkowicz, G. and P.S. Hochman.** 1979. Do natural killer cells engage in regulated reactions against self to ensure homeostasis? Immunol. Rev. 44:13.

44. **Herberman, R.B. and H.T. Holden.** 1978. Natural cell-mediated immunity. Adv. Cancer Res. 27:305.

45. **Kiessling, R., G. Petranyi, G. Klein and H. Wigzell.** 1975. Genetic variation of *in vitro* cytolytic activity and *in vivo* rejection potential of non-immunized semi-syngeneic mice against a mouse lymphoma line. Int. J. Cancer. 15:933.

46. **Talmadge, J.E., K.M. Meyers, D.J. Prieur and J.R. Starkey.** 1980. Role of NK cells in tumor growth and metastasis in beige mice. Nature. 284:622.

47. **Warner, N.L., M.F.A. Woodruff and R.C. Burton.** 1977. Inhibition of the growth of lymphoid tumours in syngeneic athymic (nude) mice. Int. J. Cancer. 20:146.

48. **Gorer, P.A.** 1956. Some recent work on tumor immunity. Adv. Cancer Res. 4:149.

49. **Hibbs, J.B., L.H. Lambert and J.S. Remington.** 1972. Possible role of macrophage mediated nonspecific cytotoxicity tumour resistance. Nature New Biol. 235:48.

50. **Evans, R., H. Cox and P. Alexander.** 1973. Immunologically specific activation of macrophages armed with the specific macrophage arming factor (SMAF). Proc. Soc. Exp. Biol. Med. 143:256.

51. **Pels, E., R.A. Weger and W. Den Otter.** 1984. Lymphocyte-induced macrophage cytotoxicity. Production of Specific Macrophage Arming Factor by sensitized Lyt 1+ 2+ T-lumphocytes. Int. Arch. Allergy Appl. Immunol. 74:140.

52. **Fidler, I.J., J.H. Darnell and M.B. Budmen.** 1976. In vitro activation of mouse macrophages by rat lymphocyte mediators. J. Immunol. 117:666.

53. **Piessens, W.F., W. Churchill and S.D. Sharma.** 1981. On the killing of syngeneic tumor cells by guinea pig macrophages activated in vitro with lymphocyte mediators. Lymphokines 3:293.

54. **Alexander, P. and R. Evans.** 1971. Endotoxin and double-stranded RNA render macrophages cytotoxic. Nature New Biol. 232:76.

55. **Keller, R.** 1973. Cytostatic elimination of syngeneic rat tumor cells in vitro by nonspecific activated macrophages. J. Exp. Med 138:625.

56. **Scott, M.T.** 1974. Corynebacterium parvum as an immunotherapeutic anti-cancer agent. Semin. Oncol. 1:367.

57. **Evans, R.** 1972. Macrophages in syngeneic animal tumours. Transplantation. 14:468.

58. **Alexander, P.** 1976. The functions of the macrophage in malignant disease. Ann. Rev. Med. 27:207.

59. **Levy, M.H. and E.F. Wheelock.** 1975. Effects of intravenous silica on immune and non-immune functions of the murine host. J. Immunol. 115:41.

60. **Keller, R.** 1976. Promotion of tumour growth in vivo by antimacrophage agents. J. Natl. Cancer Inst. 57:1355.

61. **Germain, R., R. Williams and B. Benacerraf.** 1975. Specific and nonspecific antitumor immunity. II. Macrophage-mediated nonspecific effector activity induced by BCG and similar agents. J. Natl. Cancer Inst. 54:709.

62. **Zbar, B., I.D. Bernstein, G.L. Bartlett, M.J. Hanna Jr. and H.J. Rapp.** 1975. Immunotherapy of cancer: Regression of intradermal tumors and prevention of growth of lymph node metastases after intralesional injection of living Mycobacterium bovis. J. Natl. Cancer Inst. 49:119.

63. **Alexander, P.A., S.A. Eccles and C.L.L. Gauci.** 1976. The significance of macrophages in human and experimental tumors. Ann. N.Y. Acad. Sci. 276:124.

64. **Nowinski, R.C. and G.L. Koehler.** 1974. Antibody to leukemia virus: widespread occurrence in inbred mice. Science. 185:869.

65. **Robert-Guroff, M., Y. Nakao, K. Notake, Y. Ito, A. Sliski and R.C. Gallo.** 1982. Natural antibodies to human retrovirus HTLV in a cluster of Japanese patients with adult T cell leukemia. Science. 215:975.

66. **Henle, G., G. Henle and C.H. Horowitz.** 1974. Antibodies to Epstein-Barr virus associated nuclear antigen in infectious mononucleosis. Cancer. 34:1368.

67. **Wolosin, L.B. and A.H. Greenberg.** 1981. Genetics of natural antitumor antibody production. Antibodies to MHC-linked determinants detected in the serum of unstimulated mice. J. Immunol. 126:1456.

68. **Martin, S.E. and W.J. Martin.** 1975. Interspecies brain antigen detected by naturally occurring mouse anti-brain autoantibody. Proc. Nat. Acad. Sci. 72:1036.

69. **Colnaghi, M.I., S. Menard, E. Tagliabue and G. Della Torre.** 1982. Heterogeneity of the natural antitumor immune response in mice shown by monoclonal antibodies. J. Immunol. 128:2759.

70. **Winchester, R.J., Shu Man Fu, J.B. Winfield and H.G. Kunkel.** 1975. Immunofluorescent studies on antibodies directed to a membrane structure present in lymphocytes and erythrocytes. J. Immunol. 114:410.

71. **Herberman, R.B. and T. Aoki.** 1972. Immune and natural antibodies to syngeneic murine plasma cell tumors. J. Exp. Med. 136:94.

72. **Aoki, T., E.A. Boyse and L.J. Old.** 1966. Occurrence of natural antibody to the G (Gross) leukemia antigen in mice. Cancer Res. 26:1415.

73. **Sato, H., E.A. Boyse, T. Aoki, C. Iritani and L.J. Old.** 1973. Leukemia associated transplantation antigens related to murine leukemia virus. The X-1 system: Immune response controlled by a locus linked to H-2. J. Exp. Med. 138: 593.

74. **Martin, S.E. and W.J. Martin.** 1975. Anti-tumor antibodies in normal mouse sera. Int. J. Cancer. 15:658.

75. **Guilbert, B., G. Dighiero and S. Aurameus.** 1982. Naturally occurring antibodies against nine common antigens in human sera. I. Detection, isolation, and characterization. J. Immunol. 128:2779.

76. **Mittal, K.K., S. Ferrone, M.R. Mickey, M.A. Pellegrino, R.A. Reisfeld and P.I. Terasaki.** 1973. Serological characterization of natural antihuman lymphocytotoxic antibodies in mammalian sera. Transplantation. 16:287.

77. **Leiberman, R., M. Potter, E.B. Mushinski, W. Humphrey Jr. and S. Rudikoff.** 1974. Genetics of the new IgVH (T15) idiotype marker in the mouse regulating natural antibody to phosphoryl choline. J. Exp. Med. 139:983.

78. **Borocchi, M. and M.I. Colnaghi.** 1983. Natural antibodies to oncogenic viruses, tumour cells and other antigens. Clin. Immunol. Allergy. 3:365.

79. **Martin, S.E. and W.J. Martin.** 1975. Naturally occurring cytotoxic tumour reactive antibodies directed against type C viral envelope antigens. Nature. 256:498.

80. **Wolosin, L.B. and A.H. Greenberg.** 1979. Murine natural antitumor antibodies. I. Rapid in vivo binding of natural antibody by tumor cells in syngeneic mice. Int. J. Cancer. 230:519.

81. **Greenberg, A.H. and M. Greene.** 1976. Non-adaptive rejection of small tumor inocula as a model of immune surveillance. Nature (Lond.) 264:356.

82. **Chow, D.A., L.B. Wolosin and A.H. Greenberg.** 1981. Genetics, regulation, and specificity of murine natural antitumor antibodies and natural killer cells. J. Natl. Cancer Inst. 67:445.

83. **Miller, V.E., B. Pohajdak and A.H. Greenberg.** 1983. Murine natural antitumor antibodies. III. Interferon treatment of a natural killer-resistant lymphoma: Augmentation of natural antibody reactivity and susceptibility to in vivo natural resistance. J. Natl. Cancer Inst. 71:377.

84. **Perlmann, P.** 1976. Cellular immunity-antibody dependent cytotoxicity. Clin. Immunobiol. 3:107.

85. **Hellström, I. and K.E. Hellström.** 1969. Studies on cellular immunity and its serum mediated inhibition in Moloney-virus-induced mouse sarcomas. Int. J. Cancer. 4:587.

86. **Hellström, I. and K.E. Hellström.** 1970. Colony inhibition studies on blocking and non-blocking serum effects on cellular immunity to Moloney sarcomas. Int. J. Cancer. 5:195.

87. **Tamerius, J., J. Nepom, I. Hellström and K.E. Hellström.** 1976. Tumor-associated blocking factors: isolation from sera of tumor-bearing mice. J. Immunol. 116:724.

88. **Thomson, D.M.P., K. Steele, and P. Alexander.** 1973. The presence of tumour-specific membrane antigen in the serum of rats with chemically induced sarcomata. Br. J. Cancer. 27:27.

89. **Matthews, T.D., K.J. Weinhold, A.J. Langlois and D.P. Bolognesi.** 1985. Immunologic Control of a retrovirus-associated murine adenocarcinoma. VI. Augmentation of antibody-dependent killing following quantitative and qualitative changes in host peritoneal cells. J. Natl. Cancer Inst. 75:703.

90. **Haskill, J.S., M. Key, L.A. Radov, E. Parthenais, J.H. Korn, J.W. Fett, Y. Yamamura, F. DeLustro, J. Vesley and G. Gant.** 1979. The importance of antibody and macrophages in spontaneous and drug-induced regression of the T1699 mammary adenocarcinoma. J. Reticulo. Soc. 26:417.

91. **Greenberg, A.H., L. Shen and G. Medley.** 1975. Characteristics of the effector cells mediating cytotoxicity against antibody-coated target cells. I. Phagocytic and non-phagocytic effector cell activity against erythrocyte and tumour target cells in a 51Cr release cytotoxicity assay and [125I]IUdR growth inhibition assay. Immunol. 29:719.

92. **Pohajdak, B., J.L. Gomez, J.A. Wilkins and A.H. Greenberg.** 1984. Tumor-activated NK cells trigger monocyte oxidative metabolism. J. Immunol. 133:2430.

93. **Gomez, J., B. Pohajdak, S. O'Neill, J. Wilkins and A.H. Greenberg.** 1985. Activation of rat and human alvoeolar macrophage intracellular microbicidal activity by a preformed LGL cytokine. J. Immunol. 135:1194.

94. **Urban, J.L. and H. Schreiber.** 1983. Selection of macrophage-resistant progressor tumor variants by the normal host. J. Exp. Med. 157:642.

95. **Amsbaugh, D.F., C.T. Hansen, B. Prescott, P.W. Stashak, D.R. Barthold and P.J. Baker.** 1972. Genetic control of the antibody response to Type III pneumococcal polysaccharide in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness. J. Exp. Med. 136:931.

96. **Scher, I., M.D. Frantz and A.D. Steinberg.** 1973. The genetics of the immune response to a synthetic double-stranded RNA in a mutant CBA mouse strain. J. Immunol. 110:1396.

97. **Lyon, M.F., E.V. Hulse and C.E. Rowe.** 1965. Foam-cell reticulosis of mice: An inherited condition resembling Gaucher's and Niermann-Pick disease. J. Med. Genetics 2:99.

98. **Mosier, D.E.** 1978. Derivation and characteristics of the CBA/N subline. In: H.C. Morse ed. Origins of Inbred Mice. Academic Press. p.471.

99. **Shultz, L.D. and C.L. Sidman.** 1987. Genetically determined murine models of immunodeficiency. Ann. Rev. Immunol. 5:367.

100. **Feldmann, M. and A. Basten.** 1971. The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. J. Exp. Med. 134:103.

101. **Felton, L.D.** 1949. The significance of antigen in animal tissues. J. Immunol. 61:107.

102. **Sela, M., E. Mozes and G.M. Shearer.** 1972. Thymus-independence of slowly metabolizing immunogens. Proc. Natl. Acad. Sci. 69:2696.

103. **Mosier, D.E., J.J. Mond and E.A. Goldings.** 1977. The ontogeny of thymic independent antibody responses in vitro in normal mice and mice with an X-linked B cell defect. J. Immunol. 119:1874.

104. **Mosier, D.E., I.M. Zitron, J.J. Mond, A. Ahmed, I. Scher and W.E. Paul.** 1977. Surface immunoglobulin D as a functional receptor for a subclass of B lymphocytes. Immunol. Rev. 37:89.

105. **Cohen, P.L., I. Scher and D.E. Mosier.** 1976. In vitro studies of the genetically determined unresponsiveness to thymus-independent antigens in CBA/N mice. J. Immunol. 116:301.

106. **Mosier, D.E., I. Scher and W.E. Paul.** 1976. In vitro responses of CBA/N mice: Spleen cells of mice with an X-linked defect that precludes immune responses to several thymus-independent antigens can respond to TNP-lipopolysaccharide. J. Immunol. 117:1363.

107. **Mond, J.J., I. Scher, D.E. Mosier, M. Blaese and W.E. Paul.** 1978. T-independent responses in B cell defective CBA/N mice to Brucella abortus and to TNP-conjugates of Brucella abortus. Eur. J. Immunol. 8:459.

108. **Boswell, H.S., A. Ahmed, I. Scher and A. Singer.** 1980. Role of accessory cells in B cell activation. III. Cellular analysis of primary immune response defects in CBA/N mice: Presence of an accessory all-B cell interaction defect. J. Immunol. 125:1340.

109. **Mond, J.J., P.K.A. Mongini, D. Sieckmann and W.E. Paul.** 1980. Role of T lymphocytes in the response to TNP-AECM-Ficoll. J. Immunol. 125:1066.

110. **Slack, J., G.P. Der-Balian, M. Nahm and J.M. Davie.** 1980. Subclass restriction of murine antibodies. II. The IgG plaque-forming cell response to thymus-independent type 1 and type 2 antigens in normal mice and mice expressing an X-linked immunodeficiency. J. Exp. Med. 151:853.

111. **Morrissey, P.J., H.S. Boswell, I. Scher and A. Sanger.** 1981. Role of accessory cells in B cell activation. IV. Ia + accessory cells are required for the in vitro generation of thymic independent type 2 antibody responses to polysaccharide antigens. J. Immunol. 127:1345.

112. **Scher, I., A.D. Berning and R. Asofsky.** 1979. X-linked B lymphocyte defect in CBA/N mice. IV. Cellular and Environmental influences on the thymus dependent IgG anti-sheep red blood cell response. J. Immunol. 123:477.

113. **Stein, K.E., C.A. Brennan, J.J. Mond, O. Makela and W.E. Paul.** 1980. Antibody affinity in mice with the CBA/N defect. J. Immunol. 124:1798.

114. **Greenstein, J.L., E. Lord, J.W. Kappler and P.C. Marrack.** 1981. Analysis of the response of B cells from CBA/N defective mice to nonspecific T cell help. J. Exp. Med. 154:1608.

115. **Boswell, H.S., M.I. Nerenberg, I. Scher and A. Singer.** 1980. Role of accessory cells in B cell activation. III. Cellular analysis of primary immune response deficits in CBA/N mice. Presence of an accessory cell-B cell interaction defect. J. Exp. Med. 152:1194.

116. **O'Brien, A.D., I. Scher, G.H. Campbell, R.P. MacDermott and S.B. Formal.** 1979. Susceptibility of CBA/N mice to infection with Salmonella typhimurium: Influence of the X-linked gene controlling B lymphocyte function. J. Immunol. 123:720.

117. **O'Brien, A.D., I. Scher and E.S. Metcalf.** 1981. Genetically conferred defect in anto-salmonella antibody formation renders CBA/N mice innately susceptible to Salmonella typhimurium infection. J. Immunol. 126:1368.

118. **Hunter, K.W. Jr., F.D. Finkelman, G.T. Strickland, P.C. Sayles and I. Scher.** 1979. Defective resistance to Plasmodium yoeli in CBA/N mice. J. Immunol. 123:133.

119. **Brown, A.R., C.A. Crandall and R.B. Crandall.** 1977. The immune response and acquired resistance to Ascaris suum infection in mice with an X-linked B lymphocyte defect. J. Parasitol. 63:950.

120. **Quintáns, J.** 1977. The "patchy" immunodeficiency of CBA/N mice. Eur. J. Immunol. 7:749.

121. **Perlmutter, R.M., M. Nahm, K.E. Stein, J. Slack, I. Zitron, W.E.Paul and J.M. Davie.** 1970. Immunoglobulin subclass-specific immunodeficiency in mice with an X-linked B-lymphocyte defect. J. Exp. Med. 149:993.

122. **Kenny, J., G. Guelde, J. Claflin and I. Scher.** 1981. Altered idiotype response to phosphocholine in mice bearing an X-linked immune defect. J. Immunol. 127:-1629.

123. **Köhler, H., S. Smyk and J. Fung.** 1981. Immune response to phosphorylcholine. VIII. The response of CBA/N mice to PC-LPS. J. Immunol. 126:1790.

124. **Kenny, J.J., L.S. Wicker, G. Guelde and I. Scher.** 1982. Regulation of T15 idiotype dominance: I. Mice expressing the xid immune defect provide normal help to T15+ B cell precursors. J. Immunol. 129:1534.

125. **Amsbaugh, D.F., C.T. Hansen, B. Prescott, P.W. Stashak, R. Asofsky and P.J. Baker.** 1974. Genetic control of the antibody response to type 3 pneumococcal polysaccharide in mice. II. Relationship between IgM immunoglobulin levels and the ability to give an IgM antibody response. J. Exp. Med. 139:1499.

126. **Scher, I., A. Ahmed, D.M. Strong, A.D. Steinberg and W.E. Paul.** 1975. X-linked B-lymphocyte immune defect in CBA/HN mice. I. Studies of the function and composition of spleen cells. J. Exp. Med. 141:788.

127. **Rosenstreich, D.L., S.N. Vogel, A. Jacques, L.M. Wahl, I. Scher and S.E. Mergenhagen.** 1978. Differential endotoxin sensitivity of lymphocytes and macrophages from mice with an X-linked defect in B cell maturation. J. Immunol. 121:685.

128. **Boswell, H.S., S.O. Sharrow and A. Singer.** 1980. Role of accessory cells in B cell activation. II. The interaction of B-cells with accessory cells results in the exclusive activation of an Lyb 5+ B cell subpopulation. J. Immunol. 125:1340.

129. **Nunn, M.E., and R.B. Herberman.** 1979. Natural cytotoxicity of mouse, rat, and human lymphocytes against heterologous target cells. J. Natl. Cancer Inst. 62:-765.

130. **Gerson, J.M., H.T. Holden and R.B. Herberman.** 1980. Systematic and in situ natural killer activity in C57BL/6N and CBA/N mice bearing murine sarcoma virus-induced regressor tumors. J. Natl. Cancer Inst. 65:905.

131. **Sieckmann, D.G., R. Asofsky, D.E. Mosier, I.M. Zitron and W.E. Paul.** 1978. Activiation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response. J. Exp. Med. 147:814.

132. **Sieckmann, D.G., I. Scher, R. Asofsky, D.E. Mosier and W.E. Paul.** 1978. Activation of mouse lymphocytes by anti-immunoglobulin. II. A thymus-independent response by a mature subset of B lymphocytes. J. Exp. Med. 148:1628.

133. **Sieckmann, D.G., F. Finkelman and I. Scher.** 1980. Induction of DNA synthesis by heterologous anti-d in mouse spleen cell cultures. Fed. Proc. Fed. Am. Soc. Exp. Biol. 39:806.

134. **Sieckmann, D.G.** 1980. The use of anti-immunoglobulins to induce a signal for cell division in B lymphocytes via their membrane IgM and IgD. Immunol. Rev. 52:181.

135. **Metcalf, E.S. and N.R. Klinman.** 1977. In vitro tolerance induction of bone marrow cells: a marker for B cell maturation. J. Immunol. 118:2111.

136. **Metcalf, E.S., I. Scher and N.R. Klinman.** 1980. Susceptibility to in vitro tolerance induction of adult B cells from mice with an X-linked B-cell defect. J. Exp. Med. 151:486.

137. **Kincade, P.W.** 1977. Defective colony formation by B lymphocytes from CBA/N and C3H/HeJ mice. J. Exp. Med. 145:249.

138. **Scher, I., A.D. Berning, S. Kessler and F.D. Finkelman.** 1980. Development of B lymphocytes in the mouse; studies of the frequency and distribution of surface IgM and IgD in normal and immune-defective CBA/N F1 mice. J. Immunol. 125:1686.

139. **Huber, B.T., P.P. Jones and D.A. Thorley-Lawson.** 1981. Structural analysis of a new B-cell-differentiation antigen associated with products of the I-A subregion of the H-2 complex. Proc. Natl. Acad. Sci. U.S.A. 78:4525.

140. **Ahmed, A. and I. Scher.** 1979. In: B lymphocytes in the immune response. M. Cooper, D. Mosier, I Scher, and E. Vitetta, eds. Elsevier, Amsterdam. p.115.

141. **Glasebrook, A.D., J. Quintans, L. Eisenberg and F.W. Fitch.** 1981. Alloreactive cloned T cell lines. II. Polyclonal stimulation of B cells by a cloned helper T cell line. J. Immunol. 126:240.

142. **Scher, I.** 1982. The CBA/N mouse strain: An experimental model illustrating the influence of the X-chromosome on immunity. Adv. Immunol. 33:1.

143. **Huber, B., R.K. Gershon and H. Cantor.** 1977. Identification of a B-cell surface structure involved in antigen-dependent triggering: absence of this structure on B-cells from CBA/N mutant mice. J. Exp. Med. 145:10.

144. **Ahmed, A., I. Scher, S.O. Shjarrow, A.H. Smith, W.E. Paul, D.H. Sachs and K.W. Sell.** 1977. B-lymphocyte heterogeneity: development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation alloantigens. J. Exp. Med. 145:101.

145. **Subbarao, B., A. Ahmed, W.E. Paul, I. Scher, R. Lieberman and D.E. Mosier.** 1979. Lyb-7, a new B cell alloantigen controlled by genes linked to the IgCH locus. J. Immunol. 122:2279.

146. **Smith, H.R., L.J. Yaffe, T.M. Chused, E.S. Raveche, D.M. Klinman and A.D. Steinberg.** 1985. Analysis of B-cell subpopulations. I. Relationships among splenic xid Lyl+, and Lyb5+ B cells. Cell. Immunol. 92:190.

147. **Yaffe, L. and I. Scher.** 1981. Analysis of receptiveness of B cells to the T-cell factors TRF and AEF in CBA/N and DBA/2Ha immune defective mice. Fed. Proc. Fed. Am. Soc. Exp. Biol. 40:1112.

148. **Greenberg, A.H., D.A. Chow and L.B. Wolosin.** 1983. Natural antibodies: origin, genetics, specificity and role in host resistance to tumors. Clin. Immunol. Allergy. 3:389.

149. **Martin, S.E. and W.J. Martin.** 1975. X-Chromosome-linked defect of CBA/HN mice in production of tumor-reactive naturally occurring IgM antibodies. J. Immunol. 115:5-02.

150. **The Calas Training Manual.** 1974. Animal Resources Division of the Health Protection Branch, Health and Welfare Canada and Canadian Association for Laboratory Animal Science and the Canadian Council on Animal Care. p.49.

151. **Longenecker, B.M., A.F.R. Rahman, J.B. Leight, R.A. Purser, A.H. Greenberg, D.J. Williams, O. Keller, P.K. Petrik, T.Y. Thay, M.R. Suresh and A.A. Noujaim.** 1984. Monoclonal antibody against a cryptic carbohydrate antigen of murine and human lymphocytes. I. Antigen expression in non-cryptic or unsubstituted form on certain murine lymphomas, on a spontaneous murine mammary carcinoma, and on several human adenocarcinomas. Int. J. Cancer. 33:123.

152. **Chu, M-Y. and G.A. Fisher.** 1968. Effects of cytosine arabinoside on the cell viability and uptake of deoxypyrimidine nucleosides in L5178Y cells. Biochem. Pharmacol. 17:741.

153. **Ghaffar, A., R.T. Cullen, N. Dunbar and M.F.A. Woodruff.** 1974. Anti-tumour effect in vitro of lymphocytes and macrophages from mice treated with Corynebacterium parvum. Br. J. Cancer. 29:199.

154. **Roussel, E. and A.H. Greenberg.** 1989. Identification of a macrophage-activating factor in granules of the RNK large granular lymphocyte leukemia. J. Immunol. 142:543.

155. **Mantel, N. and W. Haenszel.** 1959. Statistical aspects of the analysis of data from retrospective studies of disease. J. Natl. Cancer Inst. 22:719.

156. **Menard, S., M.I. Colnaghi and G.D. Porta.** 1977. Natural antitumor serum reactivity in BALB/c mice. I. Characterization and interference with tumor growth. Int. J. Cancer. 19:267.

157. **Pierotti, M.A. and M.I. Colnaghi.** 1976. Natural antibodies directed against murine lymphosarcoma cells: Variability of level in individual mice. Int. J. Cancer. 18:223.

158. **Möller, G. and E. Möller.** 1976. The concept of immunological surveillance against neoplasia. Transplant. Rev. 28:3.

159. **Wiltrout, R.H., M.J. Brunda and H.T. Holden.** 1982. Variation in selectivity of tumor cell cytolysis by murine macrophages, macrophage-like cell lines and NK cells. Int. J. Cancer 30:335.

160. **Roder, J.C. et al.** 1979. A functional comparison of tumor cell killing by activated macrophages and natural killer cells. Eur. J. Immunol. 9:283.

161. **LaPosta, V.J. and I. Kotlarski.** 1982. The interaction of activated macrophages with normal and neoplastic cells. Aust. J. Exp. Biol. Med. Sci. 60:513.

162. **Brooks, C.G. et al.** 1981. The specificity of rat natural killer cells and cytotoxic macrophages on solid tumor-derived target cells and selected variants. J. Immunol. 127:2477.

163. **Keller, R.** 1978. Macrophage-mediated natural cytotoxicity against various target cells in vitro. I. Macrophages from diverse anatomical sites and different strains of rats and mice. Br. J. Cancer. 37:732.

164. **Schreiber, R.D.** 1984. Identification of gamma-interferon as a murine macrophage-activating factor for tumor cytotoxicity. Contemp. Top. Immunobiol. 13:171.

165. **Piessens, W.F., W.H. Churchill and J.R. David.** 1975. Macrophages activated in vitro with lymphocyte mediators kill neoplastic but not normal cells. J. Immunol. 114:293.

166. **Meltzer, M.S., W.R. Benjamin and J.J. Farrar.** 1982. Macrophage activation for tumor cytotoxicity: induction of macrophage tumoricidal activity by lymphokines from EL-4, a continuous T cell line. J. Immunol. 129:2802.

167. **Gemsa, D. et al.** 1983. Macrophage-activating factors from different T cell clones induce distinct macrophage functions. J.Immunol. 131:833.

168. **Bennet, B., L.J. Old and E.A. Boyse.** 1963. Opsonization of cells by isoantibody in vitro. Nature (Lond). 198:10.

169. **Tough, D.F. and D.A. Chow.** 1988. Tumorigenicity of murine lymphomas selected through fluorescence-detected natural antibody binding. Cancer Res. 48:270.

170. **Agassy-Cahalon, L., M. Yaakubowicz, I.P. Witz and N. Smorodinsky.** 1988. The immune system during the

precancer period: naturally-occurring tumor reative monoclonal antibodies and urethane carcinogenesis. Immunol. Letters. 18:181.

171. **Kenny, J.J. and G. Guelde.** 1981. Normal immune responsiveness to phosphocholine and TNP-Ficoll in immune defective (CBA/N x BALB/c) F1 male mice expressing T15 idiotypic antibodies. In: B lymphocytes in the immune response: Functional, developmental, and interactive properties. Klinman, Mosier, Scher and Vitetta eds. Elsevier, North Holland. p.77.

172. **Kenny, J.J., G. Guelde, C. Hansen and J.J. Mond.** 1987. Synergistic effects of the xid gene in X chromosome congenic mice. I. Inability of C3.CBA/N mice to respond to thymus-dependent antigens in adoptive transfer assays. J. Immunol. 138:1363.

173. **Smathers, P.A., B.J. Steinberg, J.P. Reeves and A.D. Steinberg.** 1982. Effects of polyclonal immune stimulators upon NZB.xid congenic mice. J. Immunol. 128:-1414.

174. **Dighiero, G., P. Poncet, S. Rouyre and J.-C. Mazie.** 1986. Newborn xid mice carry the genetic information for the production of natural autoantibodies against DNA, cytoskeletal proteins, and TNP. J. Immunol. 136:4000.

175. **Nahm, M.H., J.W. Paslay and J.M. Davie.** 1983. Unbalanced X chromosome mosaicism in B cells of mice with X-linked immunodeficiency. 1983. J. Exp. Med. 158:-920.

176. **Forrester, L.M., J.D. Ansell and H.S. Micklem.** 1987. Development of B lymphocytes in mice heterozygous for the X-linked immunodeficiency (xid) mutation. J. Exp. Med. 165:949.

177. **Conley, M.E., A. Lavoie, C. Briggs, P. Brown, C. Guerra and J.M. Puck.** 1988. Nonrandom X chromosome inactivation in B cells from carriers of X chromosome-linked severe combined immunodeficiency. Proc. Natl. Acad. Sci. 85:3090.

178. **Klinman, D.M., S. Banks, A. Hartman and A.D. Steinberg.** 1988. Natural murine autoantibodies and conventional antibodies exhibit similar degrees of antigenic cross-reactivity. J. Clin. Invest. 82:652.

179. **Dighiero, G., P. Lymperi, D. Holmberg, I. Lundquist, A. Coutinho and S. Avrameas.** 1985. High frequency of natural autoantibodies in normal newborn mice. J. Immunol. 134:765.

180. **Prabhada, B.S., J.S. Junzo, T. Onodera and A.L. Notkins.** 1984. Lymphocytes capable of making monoclonal autoantibodies that react with multiple organs are a common feature of the normal B cell repetoire. J. Immunol. 133:2385.

181. **Hardy, R.R., K. Hayakawa, J. Haaijman and L.A. Herzenberg.** 1983. B cell subpopulations identifiable by two-color fluorescence analysis using a dual-laser FACS. Ann. N.Y. Acad. Sci.

182. **Manohar, V., E. Brown, W.M. Leiserson and T.M. Chused.** 1982. Expression of Lyt-1 by a subset of B lymphocytes. J. Immunol. 129:532.

183. **Hayakawa, K., R.R. Hardy, D.R. Parks and L.A. Herzenberg.** 1983. The "Ly-1 B" cell subpopulation in normal, immunodefective and autoimmune mice. J. Exp. Med. 157:202.

184. **Hayakawa, K., R.R. Hardy and L.A. Herzenberg.** 1986. Peritoneal Ly-1 B cells: genetic control, autoantibody production increased lambda light chain expression. Eur. J. Immunol. 16:450.

185. **Förster, I. and K. Rajewsky.** 1987. Expansion and functional activity of Ly-1+ B cells upon transfer of peritoneal cells into allotype-congenic newborn mice. Eur. J. Immunol. 17:521.

186. **Hardy, R.R., K. Hayakawa, M. Shimizu, K. Yamasaki and T. Kishimoto.** 1987. Rheumatoid factor secretion from human Leu-1+ B cells. Science. 236:81.

187. **Hayakawa, K., R.R. Hardy, M. Honda, L.A. Herzenberg, A.D. Steinberg and L.A. Herzenberg.** 1984. Ly-1 B cells : functionally distinct lymphocytes that secrete IgM autoantibodies. Proc. Natl. Acad. Sci. 81:2494.

188. **Ran, M., G. Klein and I.P. Witz.** 1976. Tumor-bound immunoglobulins. Evidence for the in vivo coating of tumor cells by potentially cytotoxic anti-tumor antibodies. Int. J. Cancer. 17:90.

189. **Cuttitta, F., D.N. Carney, J. Mulshine, T.W. Moody, J. Fedorko, A. Fischler and J.D. Minna.** 1985. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature. 316:823.

190. **Castronovo, V., J.M. Foidart, M. LiVecchi, J.B. Foidart, M. Bracke, M. Mareel and P. Mahieu.** 1987. Human anti-alpha-galactosyl IgG reduces the lung colonization by murine MO4 cells. Inv. Metas. 7:325.

191. **Guirguis, R., I. Margulies, G. Taraboletti, E. Schiffman and L. Liotta.** 1987. Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration. Nature. 329:261.

192. **Roos, E. and F.E. Roossien.** 1987. Involvement of leukocyte function-associated antigen-1 (LFA-1) in the invasion of hepatocyte cultures by lymphoma and T-cell hybridoma cells. J. Cell. Biol. 105:553.

193. **Kay, M.M.** 1975. Mechanism of removal of senescent cells by human macrophages in situ. Proc. Natl. Acad. Sci. 72:3521.

194. **Khansari, N. and H.H. Fudenberg.** 1983. Immune elimination of aging platelets by autologous monocytes: role of membrane-specific autoantibody. Eur. J. Immunol. 13:990.

195. **Warner, J.F. and G. Dennert.** 1985. Bone marrow graft rejection as a function of antibody-directed natural killer cells. J. Exp. Med. 161:563.

196. **Holmberg, D., S. Forsgren, F. Ivars and A. Coutinho.** 1984. Reactions among IgM antibodies derived from normal neonatal mice. Eur. J. Immunol. 14:435.

197. **Ternyck, T. and S. Avrameas.** 1986. Murine natural monoclonal autoantibodies: A study of their polyspecificities and their affinities. Immunol. Rev. 94:99.

198. **Grönberg, A., E. Ericksson, F. Sinangil, M. Ronnholm, T. Feizi, P. Minden and R. Kiessling.** 1985. Comparison between murine natural antibodies and natural killer cells: Recognition of separate target structures as revealed by differential in vitro expression and dependence on glycosylation. J. Natl. Cancer Inst. 74:67.

199. **Brown, G.W. and D.A. Chow.** 1985. Characterization of tumor progression from threshold tumor inocula: Evidence for natural resistance. Int. J. Cancer 35:385.

200. **Chow, D.A.** 1984. Tumor selection in vivo for reduced sensitivity to natural resistance and natural antibodies. J. Natl. Cancer Inst. 72:339.

201. **Brown, G.W., T.P. Lesiuk and D.A. Chow.** 1986. Phenotypic alterations in tumors that developed from threshold subcutaneous inocula. J. Immunol. 136:3116.