THE UNIVERSITY OF MANITOBA

# MURINE TUMOR REACTIVE NATURAL ANTIBODIES

VERONICA MILLER

A Thesis

submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree of

Doctor of Philosphy

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BY

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A thesis submitted to the Faculty of Graduate Studies of
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DOCTOR OF PHILOSOPHY

To Harry G., Christopher S. B., and Janina K. Miller

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ABBREVIATIONS

| | |
|---|---|
| ADCC | antibody dependent cellular cytotoxicity |
| AK | anomalous killer cells |
| AT X BM | adult thymectomized, bone marrow reconstituted |
| α-AGM1 | anti-AGM1 antisera |
| bg | beige mutation |
| C. parvum | Corynebacterium parvum |
| CTL | cytotoxic T lymphocytes |
| DNP | 2,4-dinitrophenyl |
| E. coli | Escherichia coli |
| ELISA | Enzyme linked immunosorbent assay |
| FBS | fetal bovine serum |
| Gal | galactose |
| GalNac | N-acetyl-galactosamine |
| Ø-α-gal | phenyl-α-galactoside |
| Ø-β-gal | phenyl-β-galactoside |
| HBSS | Hank's balanced salt solution |
| HTLV | Human T cell leukemia virus |
| IdUR | iodo-deoxyuridine |
| IFN | interferon |
| IL-2 | interleukin 2 |
| $L1210_R$ | interferon resistant L1210 |
| $L1210_S$ | interferon sensitive L1210 |
| LAK | lymphokine activated killer cell |
| LGL | large granular lymphocyte |

| | |
|---|---|
| LPS | lipopolysaccharide |
| LY-F9 | L5178Y-F9 |
| mAb | monoclonal antibody |
| MHC | major histocompatibility complex |
| mNAb | monoclonal natural antibodies |
| MuLV | murine leukemia virus |
| NAb | natural antibody |
| NK | natural killer cell |
| $NK^R$ | natural killer resistant |
| $NK^S$ | natural killer sensitive |
| NP | 4-hydroxy-3-nitrophenyl |
| NR | natural resistance |
| NTA | natural thymocytotoxic antibody |
| NZB | New Zealand Black |
| PBS | phosphate buffered saline |
| PC | phosphorylcholine |
| PCM | PHA conditioned media |
| PEC | peritoneal exudate cells |
| PEG | polyethylene glycol |
| PNA | peanut agglutinin |
| poly I:C | polyinosinic polcytidiylic acid |
| RF | rheumatoid factor |
| RIA | radioimmunoassay |
| SLE | Systemic lupus erythematosus |
| S. mutans | Streptococcus mutants |
| S. pneumoniae | Streptococcus pneumoniae |

| | |
|---|---|
| TA | thymocyte antigen |
| xid | x-linked immunodeficiency mutation |

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ABSTRACT

Interferon (IFN) treatment of a natural killer cell resistant ($NK^R$) lymphoma enhanced its *in vitro* reactivity with natural antibodies (NAb), as measured in complement assays, absorption assays, and radio-immunoassays. The hypothesis that NAb contribute to natural resistance (NR) was tested by comparing the enhanced *in vitro* NAb reactivity of an IFN treated natural killer cell resistant lymphoma with enhanced elimination in an *in vivo* assay of NR.

In order to be able to examine the specificity of NAb in more detail, several murine hybridoma lines secreting tumor reactive monoclonal NAb were developed. Most of these monoclonal NAb were thymocyte reactive, but we were able to obtain tumor reactive mNAb that did not bind to thymocytes, confirming previous serum NAb studies. The tumor and thymocyte reactivity of a monoclonal antibody (49H.8 mAb) was examined in more detail because the specificity and binding characteristics are similar to serum NAb. We established that it detects a thymocyte differentiation antigen, which is different from the Thy 1 and AGM1 antigens. Although the 49H.8 mAb has a binding specificity similar to peanut agglutinin, it was concluded that the epitope recognized by the 49H.8 mAb is different, on the basis of ontogeny and blocking studies. The 49H.8 mAb also binds to a subpopulation of spleen cells. These cells were characterized using complement depletion of effector function and Flow Cytometric analysis. In this work, it was shown that the 49H.8 reactive antigen(s) is a marker for NK cells in the spleen.

# HISTORICAL REVIEW

## 1. NATURAL ANTIBODIES

The definition most commonly used for natural antibodies (NAb) is that they are antibodies in the serum of animals which have not been intentionally immunized. Several theories to account for their origin have been presented, including environmental stimulation, endogenous bacterial and viral stimulation, and stimulation by autologous antigen, as well as the theory that they represent original germ line sequences, before alteration of specificities due to somatic mutation has taken place. These theories will be discussed in Section 3. Although the concept of "immunization" cannot be excluded in some of these theories, the definition stated above will be used, stressing the fact that the animals used for the study of NAb have not been intentionally immunized. Two other points should be stressed in a discussion of the definition of NAb: 1) Although they are true antibodies, their binding affinity to their respective antigens may be lower than that of induced antibodies (1), and 2) The genetics of production of NAb to a defined antigen is not necessarily the same as that of induced antibodies to the same antigen. This latter point will be discussed in more detail in Section 5.

NAb's have been detected in the serum of many species, including mammalians, such as mice, rats, rabbits, guinea pigs, dogs, cows and humans (1-6), avian species such as chicken and ducks (2,5,7), and reptiles such as alligators (5). The most common example of NAb's in

humans are the anti-blood group antibodies (8,9). In mice, NAb's have been detected in most of the strains examined (10-13). The variation in the levels of NAb's was found to be considerable within the different strains (12-15), and genetic studies have revealed that the production of NAb is under genetic control, although differences exist in the various systems used for these studies (See Section 5).

A common method used for the detection of NAb is the complement toxicity assay (12,16-20). In addition, agglutination assays (21), enzyme-linked immunoassays (ELISA) (1,22), radioimmunoassays (RIA) (23,24), and immunofluorescence (25), have also been used. Most NAb are of the IgM class (12,26-29), and therefore easily detected by complement dependent assays, if cellular target antigens are being examined. Other classes of NAb that have been detected using non-complement dependent assays include IgG (various subclasses) (1,21,23-25,27,28,30,31), and IgA (1,24).

## 2. SPECIFICITY OF NAb

Some of the antigens recognized by NAb have been identified and characterized in detail; others such as some cell surface membrane antigens have not been characterized at all. A general statement that can be made regarding NAb specificities is that they are to a large extent carbohydrate reactive (6,23,32-25). Although the sugars found in mammalian cell glycoprotein and glycolipid structures are relatively restricted, they have the potential of forming a wide array of antigenic determinants, based on the degree of complexity of the sugar components as well as the amount of branching (36).

Carbohydrate reactive NAb can sometimes differentiate between structural differences of carbohydrate sequences, such as between $\alpha(1\rightarrow 3)$ and $\alpha(1\rightarrow 4)$-linked galactose residues (33) and between $\alpha$- and $\beta$- linked galactosides (37). Other specifities include common conformational structures, such as $\alpha$- helices (38) and phosphodiester groups (39). It has also been found that NAb are highly crossreactive (1,38-41), although in some cases it has been possible to define particular antigens and differentiate these from other closely related antigens using NAb (42).

In the following sections, some of the more prominent NAb reactive antigens will be discussed. As the interest in the study of NAb's, their origin and specificities has increased in the recent years, the literature has become extensive. Therefore, only representative examples will be listed in each category.

## 2.1 Cell Surface Antigens

NAb to cell surface antigens are present in most species examined and include NAb to cryptic antigens, differentiation antigens, major histocompatibility antigens, thymocyte antigens, and tumor related antigens. These antigens have been detected using syngeneic (10,12,16,19), allogeneic (12,15,19) and xenogeneic (2,3,18,43) sera.

2.1.1. Blood group antigens: NAb to human blood group antigens include those to the ABO blood groups, as well as to precursor structures such as the T and Tn antigens (8,9,29). For example, anti-T antibodies are found in all humans, but not in mice (29,44).

These are predominantly of the IgM class (29). The levels of these antibodies remain constant throughout adult life, although changes have been observed in patients with certain carcinomas. The T antigen, βGal(1→3)αGalNac, is a precursor of the MN Blood Group antigens and can be detected easily on enzyme treated erythrocytes. It has also been found on several carcinomas (45), as well as fetal tissue (29). ABO blood group antigens have also been found on tumor cells originating from tissue not usually expressing these antigens, and can be detected by NAb (46).

2.1.2. Cryptic antigens: It has been shown by several laboratories that enzyme treatment of cells such as lymphocytes and erythrocytes reveals NAb reactive determinants which are not detectable on normal cells (3,10,11,34,47-49). The anti-T antibody system described above is one example of NAb recognition of cryptic antigens on erythrocytes. Although other specificities have not been examined in such detail, one can conclude that these antibodies have carbohydrate reactivities, based on the fact that their detection is dependent on neuraminidase treatment. In addition, Rogentine et.al. (34) have shown that NAb reactivity with neuraminidase treated lymphocytes can be inhibited by some carbohydrates. Another study revealing the carbohydrate specifities of NAb reactive with neuraminidase treated cells is that of Imai et.al. (37), in which they found that sugars such as phenyl-β-galactoside, lactose and melibiose could inhibit this interaction.

Human NAb reactivity with a non-carbohydrate cryptic antigen was described recently (31). This antibody was found to be of the IgG

class and reacted with cryptic regions of the band 3 protein of human erythrocytes.

2.1.3. Thymocyte antigens: The thymocyte reactivity of NAb has been studied mainly in the murine strains. It was found that all strains of mice examined had natural thymocytotoxic antibodies (NTA), although the levels showed considerable variation between strains (50-52). NTA do not display alloreactivity (50,53). In the study cited above (37), Imai et.al. showed that the NTA population of NZB mice was heterogenous, and that it could be divided into two major subpopulations: those reacting with normal thymocytes, and those reactive with neuraminidase treated thymocytes and lymphocytes. This latter population was inhibitable by several sugars, and was reactive with a selective subpopulation of peripheral T cells.

In examining the cell surface determinants recognized by NTA, Parker et.al. (53) found that the NTA reactive antigen co-capped with the Thy-1 glycoprotein, and concluded that NTA are reactive with this major glyocprotein on the thymocyte surface, or with a closely related molecule. The Thy-1 antigen is heavily glycosylated, and the carbohydrate structure changes during differentiation (54). Thus, it is possible that NTA are reactive with (a) carbohydrate determinant(s) on the Thy-1 glycoprotein which is (are) absent on peripheral T cells.

Other antigens present on murine thymocytes which are recognized by NAb are antigens related to endogenous murine leukemia viruses (MuLV) (42,55-57). In fact, NAb have been used to define some of these antigens (42). Mouse thymocytes express on their surface a

glycoprotein (gp70) related to the env gene product of MuLV. Four antigenic systems have been described : $G_{IX}$, $G_{RADA1}$, $G_{AKSL2}$, and $G_{ERLD}$, (the G standing for Ludwig Gross, the discoverer of the class of virus, followed by the designation of the prototype leukemia cell line used for defining the antigen). These antigens are expressed in varying amounts on thymocytes of different mouse strains (42), and are not always associated with the production of complete viral particles. Some strains of mice (eg AKR), express all four antigens, and other strains (eg BALB/c), express none of these, although they may appear on tumors of BALB/c origin (42). These antigens are accessible on the thymocyte surface, rendering the thymocytes susceptible to NAb and complement lysis in contrast to the group specific antigen (gs) also present on the gp70 molecule which is detectable only after membrane disruption (58).

2.1.4. Major Histocompatibility (MHC) antigens: NAb to MHC or MHC-linked antigens have been described by three laboratories. In previous studies from our own laboratory (15), using congenic mouse strains we had shown that NAb from H-2b mice reactive with an H-2d tumor could be absorbed with splenocytes from H-2d mice, but not other congenic strains. Longenecker et.al. have shown that sera from a variety of different species contain NAb which are reactive against chicken MHC antigens (5). This was confirmed further by the production of murine monoclonal NAb, which revealed that the determinants recognized by the chicken red blood cell reactive NAb were predominantly of the polymorphic type, including MHC antigens rather than species specific antigens (43). The third group that has

identified anti-MHC NAb is that of Ivanyi et.al. (59,60). They have detected anti-MHC antibodies in the serum of aged, normal mice, and have also been able to confirm their findings by producing monoclonal NAb with MHC specificity from spleens of non-immunized mice (61). These antibodies recognized public Class I determinants, and one was reactive with determinants on syngeneic K and D antigens (62). The presence of anti-self MHC reactive antibody producing B cell clones was also established earlier in a series of experiments carried out by Risser and Grunwald (63). They immunized H-$2^{b/k}$ hybrid mice with a parental Abelson virus-induced lymphoma (H-$2^{b/b}$) and found that some of the mice produced antibodies reactive with private specificities on the H-$2D^{b}$ and H-$2K^{b}$ glycoproteins. Whether any of these MHC reactive antibodies have carbohydrate specificities has not been reported, but since MHC antigens are glycosylated (64), and immunization may lead to α-MHC antibodies which are carbohydrate reactive (64), it is possible that the anti-MHC NAb are also carbohydrate reactive.

2.1.5. Viral antigens: Some of the antigens related to murine oncoviruses have already been described (Section 2.1.3) since these can also be considered to be thymocyte antigens, in some cases being expressed as differentiation antigens (42). In addition to the gp70 glycoprotein, normal murine serum also contain NAb to the gp45 and p15 viral determinants (66,67). In a survey of murine monoclonal NAb, Colnaghi et.al. also demonstrated that some of these antibodies had antiviral activity, reacting with murine ecotropic virus related structures (28).

Human NAb to gp70 glycoprotein have also been described (32,68). One study has demonstrated the carbohydrate nature of these antigenic determinants (32), which has not yet been established in the murine system. Barbacid et.al. (68) have presented evidence that the antigens recognized by the anti viral gp70 NAb in human sera are actually cell surface antigens of the cell line used to grow the viruses, but this has not been substantiated by others. Human antibodies to viral antigens have also been found when endemic populations were screened for the presence of Human T-Cell Leukemia Virus (HTLV) (69). These anti-HTLV antibodies were found in patients with Adult T-Cell Leukemia, as well as in 10-37% of non-tumor bearing residents of endemic areas in Japan. The antibodies reacted with the p24 and p19 proteins, both of which are specific to this virus (70).

2.1.6. Tumor cell associated antigens: Many laboratories have reported the presence of tumor reactive NAb in normal serum (12,13, 16,19,28,71). From the above discussion of cell surface antigens recognized by NAb, it is evident that many of these are also present on tumor cells as is the case for the MuLV and HTLV related antigens in the murine and human system respectively, and the T/Tn antigens in the human system. Other normal cellular antigens such as the MHC and blood group antigens may also be present on tumor cells. For example, in an analysis of tumor reactive NAb, our laboratory, using congenic strains of mice has shown that a population of tumor reactive NAb can be absorbed by spleen cells of certain haplotypes but not others (15), as discussed in Section 2.1.4. It is also

possible, as proposed by Rogentine et.al. (48) that some of the NAb reactive antigens that are normally cryptic and require neuraminidase treatment for their detection on normal cells, or are exposed on aged cells due to desialization, are expressed in unsubstituted form on tumor cells due to alterations in the glycosylation of cell surface glycoproteins and glycolipids, or due to increased enzymatic cleavage of the terminal sialic acid molecules.

In a recent study, Grönberg et.al. (35) have shown the binding of NAb to the YAC lymphoma can be inhibited by purified C-type virus particles and gp70 molecules, as well as by bacterial sonicates. They also found that NAb bound less to tunicamycin treated tumors, a treatment which prevents N-linked glycosylation of cell surface proteins.

Another general group of tumor associated antigens recognized by NAb are differentiation antigens, such as the Mel 1 and AH antigens on melanomas detected by human IgG and IgM NAb respectivelly (27). The Mel 1 antigen is expressed by fetal, but not adult fibroblasts, in addition to several epithelial cancer cell lines. It is absent on glioma and B-cell lines, and melanomas can be classified into Mel $1^+$ and Mel $1^-$ subsets. In contrast, the AH antigen is found on a large proportion of melanomas, all astrocytomas, and some sarcomas, but is absent on epithelial cancers. This antigen has not yet been detected on normal cultured cells, and Houghton et.al. (27) have classified it as a Class 2 antigen, that is, antigens expressed on various tumors of similar origin, but not on normal tissue. Their absence on normal tissue would be difficult to confirm, however.

In conclusion, it is unlikely that tumor reactive NAb recognize antigens that are truly tumor specific, but rather, that they react with antigens present on some normal tissue, such as thymocytes or aged erythrocytes. Their detection on tumor cells could be due to the fact that they are expressed to a higher degree on tumor cells.

## 2.2 Bacterial and parasitic antigens

NAb to bacterial antigens have been frequently described (7,24, 26,39,72). For example, a common antibody in the serum of BALB/c mice is the T15 idiotype bearing antibody reactive with phosphoryl-choline (PC), a component of Streptococcus pneumoniae (72). Other determinants are those of certain strains of Escherichia coli (E. coli), which are crossreactive with the carbohydrate sequences responsible for the human ABO blood group determinants (8,73,74). The anti-T antibodies in human serum have also been shown to react with E. coli, strain $O_{86}$ (7). In addition, it has been shown that anti-DNA antibodies derived in monoclonal form from MRL-lpr/lpr mice bind to bacteria common to the murine intestinal flora, such as Streptococcus faecalis, Staphylococcus aureus, and E. coli (39).

Another series of NAb of interest in the human system are those reactive with Streptococcus mutans (S. mutans) an organisms which is thought to play an important role in the development of dental caries (75). NAb to S. mutans have been detected in serum and in saliva (76-78). Recently, Challacombe et.al. (24) have reported their analysis of the determinants being recognized by serum NAb reactive

with S. mutans. They found that the binding of antibodies to whole bacterial cells could be inhibited by a purified protein antigen (SA I/II), with glucosyltransferase, with C polysaccharide and with lipoteichoic acid. Thus, in this system, the antigens being recognized by human NAb include those that are specific to this bacteria, as well as some which are shared with other Gram-positive bacterial species.

NAb to pathogenic Gram negative bacteria are also present in human serum, as exemplified by the natural bactericidal activity of normal human serum for Neisseria gonorrhoeae (79). This bactericidal activity has been ascribed to IgM antibodies, but the nature of the bacterial determinants recognized has not been determined (26).

Human NAb reactive with Leishmania donovani have also been described, and is present in individuals from endemic areas as well as in donors with no history of exposure to leishmania. The antigens recognized by these antibodies have also not been characterized, but they are present on promastigotes and not on amastigotes (30).

## 2.3 Intracellular and serum protein antigens

The fact that much of the antibody activity found in normal serum is directed at autoantigens has already been discussed in the Section 2.1 in relation to cell surface antigens. Another group of autoantibodies frequently found in normal serum is reactive against sub-cellular structures and serum proteins. These include the anti-DNA antibodies which are prominent in patients with Systemic Lupus Erythematosus (SLE) (80,81). To this category also belong the

anti-immunoglobulin or Rheumatoid Factors (RF) (82). These types of antibody, although prominent in auto-immune diseases are not necessarily always associated with it (83-85). Anti-DNA as well as RF NAb have also been frequently described in mice, also not in strict association with autoimmune disease (38,86).

In a systematic study of normal human sera, Guilbert et.al. (1) have examined human NAb reactivity against nine common serum antigens. They have found that NAb to tubulin, actin, thyroglobulin, myoglobin, fetuin, transferrin, albumin, cytochrome c, and collagen are commonly present. Although binding of these antibodies to their respective antigens could be inhibited most efficiently by the same antigen, some crossreactivity was observed, which differentiated the NAb from induced antibodies. In addition, they confirmed the autoreactivity of these antibodies by staining cultured human hepatocytes.

In a further study, this group was able to produce monoclonal NAb from normal, non-immunized BALB/c splenocytes, which reacted with thyroglobulin, myosin, actin, tubulin, spectrin and dsDNA (38). Some crossreactivity was observed in this study as well. Analysing one of their monoclonal NAb in more detail, they found that it reacted predominantly with the light meromyosin subfragment of myosin, which is rich in $\alpha$-helices. Thus, they postulated that NAb recognize common structural determinants present on several molecules found in nature. Evidence for this concept has also been presented in other reports, in which it was demonstrated that phosphodiester groups were responsible for the crossreactivity of human anti-DNA antibodies with phospholipids (87). In this context, it is worth pointing out again

the study by Carroll et.al. (39), in which they demonstrated that murine monoclonal anti-DNA antibodies bind to endogenous bacteria, that contain phosphate esters such as teichoic and lipoteichoic acids, lipopolysaccaharides, and phospholipids. The antibodies bound to bacterial preparations that had been pretreated with DNase, thus ruling out the possibility that the binding was due to the presence of DNA on the cell surface. In addition, these antibodies were able to bind to phosholipid extracted from the bacterial cell walls.

The analysis of NAb reactive with normal immunoglobulins (RF) has also been carried out at the level of serum antibodies as well as monoclonal antibodies (88,89). It was found that these RF antibodies could be separated into groups defined by their isotypic and allotypic specificities, and that both allotypic and isotypic determinants recognized by mouse RF are located in the $C_H3$ domain of $IgG_1$, and $IgG_{2a}$ and $IgG_{2b}$ immunoglobulins (89).

Natural anti-idiotypic antibodies have also been descibed. These have been detected after immunization with antigen (90-92), in autoimmune diseases (22), as well as obtained in monoclonal form from neonatal mice (93).

## 2.4 Synthetic Haptens

Another interesting group of NAb are those reactive with synthetic haptens, such as oxazalone, 2,4-dinitrophenyl (DNP), and 4-hydroxy-3-nitrophenyl (NP) (94-96). These have also been found in a wide variety of species. They are important in that they provide a system in which studies of the origin, genetics, and regulation of

NAb can be carried out without the complicating factors of non-intenional exposure to environmental antigens.

## 2.5 Heterogeneity and crossreactivity of NAb

A survey of the antigenic determinants recognized by NAb, as described in this section, would indicate that NAb's are a rather heterogenous population, their specificities ranging from natural determinants found on bacteria and autologous cells, to synthetic haptens which would normally not have been encountered by the individual under study. In spite of the large number of antigens that have been identified as being NAb reactive, there are some striking examples of rather selective recognition. An example of this are the polymorphic versus species specific antigens on chicken red blood cells that are recognized by murine NAb (43).

When discussing the heterogeneity of NAb, it is important to point out that each laboratory may have used different systems for their identification, and in this way, some crossreactivities may not have been detected. The crossreactivity between DNA and phospholipids has already been stressed (39,87). More recently, anti-DNA antibodies have also been shown to react with proteoglycans, such as hyaluronic acid and chondroitin sulfates (40). Another striking example of crossreactivity is the fact that Klebsiella pneumonia polysaccaride K30 specific monoclonal antibodies bind to DNA, and that this binding can be inhibited by the K30 polysaccaride (97). These authors also established that the anti-Klebsiella and anti-DNA antibodies share idiotypic determinants.

In a study of monoclonal autoantibodies, Haspel et.al. (98) detected multiple organ reactive antibodies, which appeared frequently after immunization, as well as in normal animals (98, 99). These antibodies reacted with organ sites such as the anterior (but not posterior) pituitary, pancreatic islets (but not pancreatic acinar tissue), small intestine, and stomach.

It has also been reported that RF antibodies cross-react with nuclear protein antigens (100-102).

It is possible, that some of the NAb populations differentiated on the basis of antigenic specificity, may in fact be overlapping. A systematic study of NAb in monoclonal form, many specificities of which are now becoming available, would be useful in resolving this question.

## 3. ORIGIN OF NAb

The theories explaining the origin of NAb's put forward by different individuals often reflect the particular system they were using for their studies. As is evident from the discussion in Section 2, it may be difficult to classify all NAb's as one group in terms of origin and regulation. Some of the proposed models will be discussed in this section.

### 3.1 Environmental Stimulation

This has been the most common model proposed to account for the prevalence of NAb in normal serum. If one assumes that NAb arise

from "natural immunization", then the difference between NAb and induced antibody lies only in the method of immunization. Whether some of the differences in affinity and genetic control seen in the production of NAb versus immune antibodies can be ascribed to "natural" versus "intentional" immunization is not clear at present, and would be difficult to test in the cases where the antigens involved are a part of the individual's endogenous and external environment. In this context it should be pointed out that there are well characterized differences between the mucosal and gut associated lymphoid systems and the systemic immune system (103). For example, it has been reported that there are differences in B cell repertoire directed against phosphoryl choline (PC) (104), and that the B cell subpopulation missing in spleens of CBA/N mice, which are unable to respond to TI-2 antigens (see Section 4.3.1), is not lacking in CBA/N mucosal tissues (105). Cooperation between the two systems, which occurs when antigen is introduced via the two routes, may affect the antiidiotypic regulatory circuits (106). The immune response to antigen may be quite different, depending on the route of immunization (107,108). These considerations are important in view of the fact that the environmental antigens reponsible for the production of NAb according to this model, would be introduced to the host via the mucosal system. Yet, comparisons between NAb and induced antibody have generally used the systemic route of induction (1,96).

A considerable amount of evidence has been accumulated in support of the environmental stimulation model. This includes studies such as the one by Galili et.al. (33) mentioned above, where it was found that the structure recognized by their NAb ($\alpha(1\rightarrow3)$Gal) was also

present on bacteria present in the environment of the individual. Numerous other examples of this type exist, such as the ABO blood group determinants (8,73,74), the T antigen (7), and the study by Carroll et.al. (39) showing that anti-DNA NAb bind to endogenous bacteria. In a more direct approach to this question, Springer and Tegtmeyer (7) established that the production of anti-T antibodies in normal serum was dependent on the presence of certain strains of E. coli in the gut of the individual under study. Others have shown that certain NAb specifities are absent in the sera of animals raised under germ-free conditions, as for example, RF NAb (86) and anti-PC NAb (72). In the latter case, it was shown that this specificity of NAb was produced if the mice were exposed to normal environment (72). A more recent study indicated that although anti-PC antibodies were lower in germ-free mice, they were nevertheless present in detectable levels (104).

Genetic studies of the anti-MuLV NAb production in mice have indicated that the presence of these antibodies in backcrosses correlated with the presence of viral particles (66). In this system however, there are also exceptions where mice do produce anti-MuLV NAb in the absence of viral particles. In humans, anti-HTLV NAb have been found in individuals who do not have signs of leukemia, but these were residing in the environment were this virus is prevalent, therefore exposure cannot be ruled out (70).

Although the evidence listed above strongly argues in favor that at least some NAb specificities are the result of environmental stimulation, this model cannot account for all NAb populations. In addition to the exceptions cited in the anti-MuLV system, results

from other studies indicate that additional mechanisms must be operative. For example, it has been shown that mice raised under germ free conditions do produce serum NAb reactive with human lymphocytes (18). Spleen cells and bone marrow cells from germ-free mice produce as many background IgM plagues, and their antigen-specificity repertoire (measured against a variety of haptens) were similar (109). Spleen cells from germ free mice have been used to produce monoclonal NAb (25). Three additional studies, using monoclonal antibodies, have shown that neonatal mice do produce NAb reactive with self-antigens (25,93,110). These studies would argue that factors other than environmental stimulation are responsible for the production of some NAb specificities.

## 3.2 Autologous antigen stimulation

As has already been stressed, many of the NAb are autoreactive, and these specificities are present in mice and humans who have autoimmune diseases, as well as normals (38,83-85). Therefore, it is possible that some autoantigens are responsible for the stimulation of B cells producing NAb. This would be the case for natural auto-anti-idiotypic antibodies (22,90-93). Other self antigens that possibly fulfill this role are those on aged erythrocytes and lymphocytes, which can also be detected after enzymatic treatment of these cell types (3,10,11,34,47-49). For example, Cunliffe and Cox (111), showed that NAb reactive with isologous murine immunoglobulins cross-reacted with bromelain-treated murine erythrocytes. The high frequency of murine sponataneous plaques reactive with sheep-erythro-

cytes could be explained on the basis that sheep-erythrocyte antigens cross-reacted with bromelain-treated murine erythrocytes (112), whereas horse erythrocytes, against which few spontaneous plaques were observed, did not display this cross-reactivity. Other laboratories have shown that tumor reactive NAb could be absorbed by normal plasma cells (16) and brain tissue (113). These studies do not, of course, confirm that NAb are produced as a result of autoantigenic stimulation, since the possibility that these NAb are not reacting with environmental antigens is difficult to rule out.

## 3.3 Polyclonal B-cell Activation

B-cells can be polyclonally activated by a variety of agents such as bacterial endotoxins and peptidoglycans (114,115). Lipopolysaccharide (LPS) from *E. coli* and other gram negative organisms is frequently used for this purpose. Therefore, the role of endogenous bacteria in NAb production could be expanded to include this mechanism in addition to direct antigenic stimulation. Several studies have shown that *in vivo* and *in vitro* LPS stimulation increases serum NAb levels as well as the frequency of spontaneous plaque forming cells in splenic cell populations (20,71,114-119). Glycoprotein bacterial extracts can also be polyclonal activators (120). Coulie and Van Snick have shown that RF producing B cells are activated during secondary immune responses to various antigens, such as hen lyzozyme, human transferrin in mice (121,122) and tetanus toxoid in humans (123). Therefore, it is conceivable that some of the NAb's detected in the serum of normal individuals are a result of poly-

clonally activated B-cells. This model of NAb production does not explain the presence of NAb in mice raised under germ-free conditions and neonatal mice (18,25,93,109,110). It also does not answer the question of why, in some instances, NAb of restricted specifities are produced.

Although agents such as LPS have been termed polyclonal B cell activators, implying a lack of selectivity in this process (124-126), this is probably not a correct interpretation. For example, Bretscher (127) has proposed that LPS activates not all B cells, but only those that are undergoing antigen dependent activation in the host. According to this view polyclonal activators such as LPS increase the levels of NAb specificities already present in the normal serum, but it does not really account for the origin of the "original" NAb.

Other findings have been that LPS stimulates predominantly autoantibody production, directed against immunoglobulin, ssDNA, and bromelain treated erythrocytes (114,116,117).

In conclusion, polyclonal B cell activation may account for the enhancement of the detectable levels of NAb but it is not a good model to account for their origin.

## 3.4 NAb and Germline V region Sequences

The fact the NAb can be detected in animals raised under germ-free conditions, and can be produced in monoclonal form from neonatal spleens has led some authors to propose that these are representative of germline V region sequences representative of

autoreactive specificities, that are expressed throughout the ontogeny of immune development (38,110,128). That the NAb are frequently antibodies of low affinity, which react with a variety of self-antigens would support this concept, in that it had been established earlier that the antibody repertoire of fetal and neonatal mice is more restricted then that of the adult and that the affinity of these early antibodies is lower (129). The finding that certain NAb specificities seem to be represented at a higher frequency in the neonatal spleen cell population compared to adult has also been interpreted to support this model (110). According to this model, somatic mutation taking place as these B cells are activated would result in additional specificities being generated, with the consequent dilution of the neonatally expressed germ line V region sequences (110). The same authors have also detected frequent cross-reactive idiotypes among NAb populations (128). The anti-idiotypic antibodies were raised against two monoclonal NAb, and reacted with natural serum antibodies, as well as with a large number of subsequently raised monoclonal NAb. Examination of human anti-DNA antibodies has also revealed that some idiotypes are expressed in the serum of different individuals (130). These findings, together with those of Homberg et.al. (93) that anti-idiotypic antibodies are present among neonatal NAb would suggest that an internal idiotypic network may be functioning at this level.

Corley (131) has proposed that cooperation between natural anti-idiotypic antibody and autoreactive T cells is a possible mechanism for continous activation of the B cells expressing the appropriate germline encoded idiotypes. This activation would then

allow somatic mutation and antigen dependent selection for other specificities.

Dominant idiotypes representative of germline sequences have been frequently observed in response to bacterial immunization, and these idiotypes are also present in normal serum (132). Bottomly (132) has pointed out that the most frequently analyzed idiotypic systems are those in which the first antibody is directed against common environmental pathogens, such as the T15-anti-T15 system in the response to PC containing pneumococcus. She proposed that these V region sequences have been selected throughout the evolutionary history of the host as a protective mechanism against environmental pathogens. In the PC specific antibody system, three predominant idiotypes have been identified: T15, M603 and M167 (133). The $T15^+$ antibodies show less variation due to somatic mutation than the other two idiotypes, both in their heavy and light chains (134,135). Exact translation of the T15 $V_H$ gene results in heavy chains characteristic of the $T15^+$ antibody, whereas the M603 and M167 idiotypes, although utilizing the same $V_H$ gene, differ on the average by five amino acids from the germ line gene. Since the $T15^+$ antibodies have been shown to be optimally protective against murine S. pneumoniae infections (133), these authors have also suggested that evolutionary pressures resulted in the production of these germline encoded NAb as a protective mechanism against environmental pathogens.

Klinman and Stone (136) have shown, using the in vivo splenic fragment assay, that the T15 idiotype is expressed in a dominant manner already at the pre-B cell level, although $T15^-$ PC binding antibodies also exist. Thus they concluded that the T15 idiotype

dominance is not due to environmental selection. A more recent *in vitro* culture system has confirmed that antibody specificities are expressed in a well defined sequence, again in the absence of environmental selection (137). This would appear to contradict earlier studies showing that the T15 idiotype is not represented in the serum of animals raised under germ-free conditions (72). One explanation would be that the natural production of this antibody in germ-free mice is low, and environmental stimulation is necessary to activate the $T15^+$ B cells sufficiently to bring the serum levels of $T15^+$ antibodies to detectable levels. The finding of Gerhard and Cebra (104) that detectable levels of $T15^+$ anti-PC antibodies do exist in germ free animals, but that their levels are enhanced by exposure to a conventional environment would support this interpretation.

In conclusion, it is likely that all of the models discussed in this section play a role in the origin of NAb, as defined in Section 1. Nab, or at least a subpopulation of these, may be representative of germ line sequences, and their production enhanced by either autologous or environmental stimulation. Since anti-self reactive antibodies have been shown to crossreact with bacteria (7,33,39,40, 97) the repertoires stimulated by either of these two mechanisms may be largely overlapping. Furthermore, it cannot be excluded that, as B cells expressing germ line sequences are activated and undergo somatic mutation, some of the resulting specificites may also be NAb-like. This is supported by the finding that a somatic mutant of an anti-PC antibody, with a one amino acid substitution, lost

its PC binding specificity and binds to ds-DNA, protamine and cardiolipin (138).

## 3.5 Nab Producing B Cells

Recently, a unique B cell population has been identified which is characterized by the Ly 1 marker. This marker was initially thought to be present only on T cells and therefore designated Lyt 1, but several laboratories have confirmed its presence on about 2% of splenic IgM$^+$ cells of normal mice (139,140). This Ly 1$^+$ B cell is characterized by having high surface IgM and low surface IgD densities (139). In spleens of NZB mice, which produce high levels of autoantibodies, the frequency of this population is significantly higher (up to 10% of splenic IgM$^+$ cells) (139).

There is a striking difference between Ly 1$^+$ and Ly 1$^-$ B cells in terms of autoantibody and immune antibody production: Ly 1$^+$ B cells are reponsible for virtually all spontaneous IgM secretion in NZB as well as LPS stimulated BALB/c spleen cell populations, whereas immune antibodies are produced predominantly by the Ly 1- population (139). Specificity studies of these autoantibodies have shown that they include thymocytotoxic and anti-DNA antibodies (141).

It is also interesting to note that these cells are more frequent in neonatal spleens. As other B cell subpopulations increase in frequency, the Ly 1 B cells are diluted out to a final frequency of about 2% in adult spleens of non-NZB mice (139). This provides an additional explanation for the higher incidence of NAb

directed at autoantigens in neonatal mice as discussed above (Section 4.3).

Although the Ly 1 B cell is rather infrequent in normal spleens, in the peritoneal cavity it represents approximately one half of all the B cells (142). Peritoneal cells have been known for a long time to be a particularly good source of autoantibody producing B cells (11,112,117).

Another cell associated with NAb production is the Lyb $5^+$ B cell, which is lacking in mice bearing the X-linked immunodeficiency syndrome mutation (xid). These mice have lower levels of NAb and do not respond to immunization with TI-2 antigens (Section 4.1) In terms of total splenic B cells, xid mice lack the surface $IgM^{low}$, $IgD^{high}$ population normally considered to be the mature B cell population (143), but their levels of LY $1^+$ B cells is similar to that of other mice. The relationship between these B cell populations is not clear at present. One study has shown that approximately 70% of Ly $1^+$ B cells are Lyb $5^+$ (as determined by Lyb 5 antisera and complement depletion) and that the level of contribution of Lyb $5^+$ B cells to different autoantibody specificities varies, from 96% for anti-DNA antibodies to 67% for anti-bromelain treated red blood cell antibodies (140).

It appears therefore, that IgM autoantibodies are produced by Ly $1^+$, Lyb $5^+$ B cells. How these relate the studies using monoclonal NAb has not been examined yet. However, it is clear that not all NAb are produced by this cell population. Included in the exceptions would be the non-IgM Nab.

# 4. REGULATION OF NAb PRODUCTION

From the discussion in Section 2 it is evident that NAb are a heterogenous population in terms of their specificities. This can also be extended to their origin, as discussed in Section 3. It is therefore not surprising, that different regulatory mechanisms be operative in the maintenance of NAb levels. These include genetic regulation and T-cell dependent and independent regulatory mechanisms.

## 4.1 T Cell Regulation of NAb

Several laboratories have shown that the production of NAb is T cell independent. NAb's are found in normal quantities in the serum of nude mice, as well as in adult thymectomized, irradiated, and bone marrow reconstituted mice (AT x BM) (50,70,96,144). The antigens tested include induced and spontaneous tumors, as well as haptens. Although the production of NAb may be T independent, some authors have indicated that their levels are regulated by T suppressor cells (19,28,145,146). This has not been a universal finding, however (15,146). Mènard and colleagues (19) found that levels of NAb in BALB/c mice to a syngeneic fibrosarcoma were higher in older mice than in young mice, and that T-deprivation of young mice resulted in levels as high as that of the 40 week old mice. These same authors found, however, that levels of anti-thymocyte NAb were not influenced by T cell deprivation (146). In fact, transfer of young T cells into older mice resulted in lower tumor (fibrosarcoma) reactive NAb

levels and higher thymocytotoxic NAb levels. T cell suppression of NAb levels has also been documented for NAb reactive with the EL4 lymphoma (28) and anti-erythrocyte antibodies (145). The analysis of DBA/2 NAb to syngenic T cell lymphomas also did not reveal T cell regulation (15).

## 4.2 Genetic Regulation of NAb

4.2.1. Studies with mutant mice: The use of mice with immunological deficiencies due to a genetic mutation has proven to be useful in the study of the role of various immunological systems. In relation to NAb, the models that have been most often used are the nude (nu) mutation and the X-linked immunodeficiency (xid) mutation in the CBA/N mouse. Studies with the athymic nude mice have been reviewed in Section 4.1.

CBA/N mice, bearing the xid mutation, were found to be incapable of producing antibodies to certain T-independent antigens, termed TI-2 antigens, including pneumococcal polysaccharides, and polyriboinosinic:polyribocytidilic acid (poly I·C). Other thymus-independent antigens which do elicit a response in CBA/N mice ahave been designated TI-1 antigens (147,148). Mice affected by the xid mutation have lower serum level antibodies to tumors (149), and they are also unresponsive to LPS stimulation for a variety of auto-antibodies (150). The xid gene, when congenic in the autoimmune strain NZB, resulted in a much lower level of autoantibody production (151) Although this mutation results in some clear NAb deficiencies, the lack of NAb production and LPS responsiveness is not

universal. For example, spontaneous plaque forming cells to bromelain-treated mouse red blood cells could be stimulated with LPS, although not to the same extent as in normal mice (152). Studies from our laboratory have also shown that xid/- F1 males do respond to LPS in antibody production to a murine lymphoma (153), and, like the results of Rosenberg (152), the increases were not as pronounced as in F1 females, or normal CBA/J mice. As discussed in Section 3.5, CBA/N mice are deficient in a subpopulation of cells characterized by the LYb 5 marker. Although initially it was assumed that this deficiency was absolute, more recent findings have shown that a small number of Lyb $5^+$ B cells are present in xid bearing mice and that there are qualitative differences between the $Lyb5^-$ cells of xid and normal mice (140,154,155). This provides an explanation for the fact that their unresponsiveness is not absolute. It has also been possible to examine the effect of the nu mutation in a model using double mutant mice which bear both the nude and the xid mutation (156,157). The fact that these mice had very low serum immunoglobulin levels, as well as low LPS responsiveness suggests that a sub-population of NAb producing B cells may be controlled by the nude locus. Hardy et.al. (158) have developed one model, using monoclonal antibodies against B cell lineage associate antigens (BLA 1 and BLA 2), and were able to distinguish deficiencies in CBA/N mice (lacking BLA $1\text{-}2^-$ and BLA $1^+2^-$ cells) and CBA/nu mice (lacking BLA $1\text{-}2^+$ cells, but having more BLA $1\text{-}2^-$ cells).

4.2.2. Other genetic models: The variation in the levels of NAb in different inbred strains of mice suggested that the production of

NAb may be under genetic control. Studies of the genetics of NAb production have included viral, hapten, and tumor antibodies. In a genetic analysis of anti-MuLV antibodies, Nowinski et.al. (66) found that in backcrosses (C57L x (AKR x C57L), the mice could be segregated into those containing infectious MuLV, and those that were MuLV non-producers. The producers were all NAb positive, while the non-producers inluded both antibody positive and antibody negative progeny. Further studies (159) revealed that the antibody producers could be divided into high and low producers. The level of antibody production was associated with the $SS^{h/l}$ and $SS^{l/l}$ phenotypes, respectivelly.

The genetics of the natural thymocytotoxic antibody (NTA) system has been studied extensively, since it was suggested that these antibodies may play a role in the pathogenesis of the autoimmune disease characteristic of strains such as the NZB which produce excessive amounts of these antibodies. Genetic analysis revealed however, that many mice had high levels of NTA, and that the amount of NTA found did not correlate with the presence or absence of autoimmune disease (52). In addition, studies of xid/NZB congenic mice showed that although these mice had lower amounts of NTA the T cell abnormalities seen in normal NZB controls were not abrogated (151). In genetic backcross studies between NZB (high NTA producers) and DBA/2 (low NTA producers), it was found that the frequency of NTA producers was intermediate, consistent with a co-dominant pattern of inheritance (160). No evidence for H-2 linkage was found. These studies also showed that the production of NTA, anti-DNA, and

anti-erythrocyte antibodies was regulated independently than that of NTA.

Genetic studies of anti-tumor NAb have been carried out in several laboratories, including our own. It was noted initially that the levels of NAb that were detected was dependent on the strain - tumor combination that was used (12,15). In an analysis of NAb reactive with a DBA/2 lymphoma, it was found that strains such as CBA/J ($H\text{-}2^k$) and C57Bl/6 ($H\text{-}2^b$) were high producers, and the DBA/2 ($H\text{-}2^d$) strain was a low producer (15). Analysis of genetic backcrosses revealed a recessive mode of inheritance of high NAb levels, and it was found that low levels of NAb were associated with the $H\text{-}2^d$ haplotype. These studies also included an analysis of serum NAb levels of congenic mice, and these confirmed the association of low NAb levels with the $H\text{-}2^d$ haplotype.

More recent studies from our laboratory have extended these findings further by combining the effect of the H-2 controlled gene with that of the xid mutation, in an analysis of serum NAb reactivity against the $H\text{-}2^d$ lymphoma L5178Y-F9 (153). It was found that while (CBA/N x CBA/J)F1 males were responsive to LPS induction of NAb, (CBA/N x DBA/2)F1 males were not. In backcross studies, the frequency of LPS responders was consistent with the expected frequencies made on the basis that the $H\text{-}2^d$ haplotype was associated with low responsivenes. The interpretation from these studies was that at least two populations of anti-L5178Y-F9 reactive NAb exist, one controlled by $H\text{-}2^d$, and one by the xid locus.

Results from other laboratories have led to some different interpretations of the inheritance pattern of tumor reactive NAb.

For example, Colnaghi et.al. found that high NAb levels were inherited in a dominant pattern in contrast to the recessive inheritance seen in our studies (15). These differences may be a reflection of the heterogeneity of the NAb populations that have been examined, as well as the heterogeneity of the tumors, and their subclones that were used for the studies.

The last system to be discussed in this section is that of the anti-hapten NAb. These studies, carried out primarily by Mäkelä and colleagues have provided some interesting results, comparing NAb to induced antibody production. It was found that the high antibody level found in natural serum to oxazalone was inherited recessively and was allotype linked in contrast to induced antibodies, high levels of which were inherited dominantly (94-96). Studies with anti-NP antibodies confirmed the difference in the genetics between induced and naturally occurring antibodies in that the distribution of high and low affinity anti-NP antibodies was distributed in a codominant fashion in F1 hybrids of high and low affinity NAb producing parental strains. Again, in contrast to the NAb pattern, induced antibodies are dominant for the high affinity strain.

In conclusion, these various genetic models confirm that different genetic mechanisms regulate NAb production, as is expected in view of the heterogenous nature of NAb.

## 5. ROLE OF NAb

The prevalence of NAb in all species examined raises the question of their function. An indication of what this may be can be

extracted from the antigens that have been identified as being NAb reactive, such as environmental bacteria and aged or damaged erythrocytes. Various physiological and defensive functions have been attributed to NAb, and these will be discussed separately in this section.

## 5.1 Physiological role of NAb

Depletion of circulating aged, damaged cells, as well as degradation products, is one of the major physiological functions that has been attributed to NAb (161-165). Studies by Khansari and Fudenberg (164-165) and Kay (162) have demonstrated that NAb coated erythrocytes are phagocytosed by macrophages. The antibodies involved in this interaction are predominantly of the IgG class. These studies have included separating young and old erythrocytes from the circulation, as well as artificially aging the erythrocytes, and it was shown that the NAb involved in this opsonization only bound to naturally aged or enzyme treated erythrocytes (162). Kay determined that the IgG from normal human serum responsible for the enhanced phagocytosis was bound *in situ* to senescent erythrocytes (162). The anti-α-galactose IgG identified by Galili *et.al*. (21,33) was found to be present in relatively high quantities in all normal human serum (1 % of total IgG, with a titer ranging from 1:800 to 1:1600). This antibody did not bind to normal human red blood cells, a finding not surprising in view of its high titer. It did bind however, to pronase treated human red blood cells, as well as to thalassemic red blood cells, which resemble prematurely aged erythro-

cytes (166). Although these authors did not carry out functional studies, such as phagocytosis by monocytes, it is likely that this antibody is involved in this function, and representative of the antibody populations described in the studies discussed above.

A role for NAb in the elimination of aging platelets has also been described (167). In these studies, removal of IgG bound to aging platelets in situ, resulted in inhibition of phagocytosis by autologous monocytes. This antibody could be eluted from senescent red blood cells, indicating that elimination of senescent platelets and red blood cells may be enhanced by the same antibody population.

## 5.2 Resistance to Bacterial Infection

A role for NAb in host resistance to bacterial infection is suggested by the fact that many of the specificities that have been identified are those of bacterial antigens (7,24,26,33,39,72-74). It has been argued that the bacterial species commonly associated with NAb reactivity are generally non-pathogenic. However, the relationship between their non-pathogenicity and NAb reactivity needs to be considered. In an elegant series of experiments, Briles and coworkers have demonstrated the importance of natural serum antibodies in resistance of mice to pneumococcal infections (133,168-170). S. pneumonia is non-pathogenic for most strains of mice, but xid mice are 1,000 to 10,000 times as susceptible to lethal infections by this bacteria, and this susceptibility is associated with their reduced NAb levels (168,169). Briles et.al. demonstrated that resistance in xid mice could be passively acquired with the transfer of normal

serum from non-xid mice (168). They extended these findings by passive immunization with monoclonal antibodies produced against this bacterial strain, and found that the most protective ones were those that were positive for the T15 idiotype, which is representative of the natural anti-pneumococcal antibody population (133).
Antibodies in human serum that have been implicated in a bacterial resistance function are those against S. mutans (24), an organism which is thought to play an important role in the etiology of dental caries. Antibodies to this strain are present in all humans, but the titer is significantly greater in individuals with low frequency of caries than in individuals with high caries frequency (77).

## 5.3 Natural Resistance to Tumors

That resistance to tumors could be mediated by natural (pre-existing) rather than immune mediated mechanisms was suggested when it became obvious that mice deficient in thymus-mediated immune responses were not more susceptible to a higher incidence of spontaneous tumors (171,172). Several groups have proposed that NAb may be active in this kind of resistance (12,14,15,19,71). Before discussing the evidence in support of this theory, the basic features of the natural tumor resistance mechanisms will be described, as a background for the discussion of the involvement of NAb.

Assays to measure natural resistance to tumors (NR) have included the small tumor cell inocula assay (90,71,173), in which the incidence of tumors, size of tumors, and time of death can be examined.

However, since this type of experimental design involves relatively long periods of time, a T-cell mediated influence cannot always be excluded. The use of nude mice, or AT X BM mice has been useful in overcoming this problem (19). The development of the in vivo radiolabelled tumor elimination assay (71,174-176) has provided a convenient system in which to study NR since the time of the assay is relatively short, ranging from hours (if the tumor is injected intravenously) (177) to 3-4 days (if the intraperitoneal or intra-footpad route is used) (71,178). These assays, in combination with in vitro assays measuring tumor cell susceptibility to various mechanisms have been the basic approach to the study of NR.

5.3.1. Natural Killer Cells: A "background" non-specific cytotoxicity had frequently been observed in the generation of cytotoxic T cells (179-183), and when it became apparent that this was a rather general finding, it was proposed that these cells (Natural Killer, or NK cells) may fulfill a function in tumor resistance (183), since they met the requirement for a non-T cell dependent mechanism. The amount of data that has become available on this cell type and its role in NR since its initial description is phenomenal. The field has now expanded to include a variety of NK related cells, such as lymphokine activated killer cells, anomalous killer cells, lectin activated killer cells. One of the problems in dealing with this cell type and the extensive literature describing it, is that its lineage and relationship to some of these other cell types is still controversial (184-187). The definition of NK cells has traditionally been a functional one, in that killing of NK

sensitive tumor targets but not of NK resistant targets has been the basis of defining an NK population. Subsequent to this, a morphological description was added to the definition: it was discovered that NK cells in human, mice and rats had a characteristic morphology termed large granular lymphocyte (LGL) (188-191). Unfortunately, neither definition has proven to be very useful. When attempts were made to clone NK cells, with the objective of obtaining sufficiently large quantities of cells for characterization and functional studies, it was frequently discovered that the cloned NK cells lost their typical NK target cell selectivity, and that they became what was termed "promiscous killer cells", since they killed NK resistant as well as NK sensitive target cells (192,193). This phenomena has also been observed when cytotoxic T cells are cloned (192) and shown to be dependent on the amount of IL-2 present in the growth media. LGL can be isolated on the basis of cell density differences between LGL and normal T cells (188-191), and if cell density fractionation is used in combination with adherence depletion of monocytes and nylon wool columns, relatively pure preparations of LGL can be obtained. Although this procedure does enrich NK activity as measured by functional assays, as a morphological criteria for defining NK cells it is not very useful, since activated T cell blasts also display this type of morphology (194).

Another method for defining NK cells has been the use of markers, a system which has been successfully used in the definition of various T cell subsets (195-198). In the human system, the marker defining NK cells is the HNK1, or Leu7. In addition, human NK cells display several monocyte and T cell markers, including OKM1 and OKT8

(199-201). In the murine system, the definition of NK cells using markers has proven to be more difficult. A marker commonly used is the asialo-GM1 (AGM1), but since T cells also bear this antigen (193), it is not very useful in the definition of NK cells. However, anti-sera against AGM1 is very efficient in depleting NK activity if injected into mice, and as such, has been a useful reagent in the depletion of *in vivo* NK cell activity (202).

The beige mutation in the mouse (bg/bg) has provided a model for an NK deficient host, which can be compared to bg/+ littermates in tumor resistance studies. These mice have impaired NK activity but retain normal T cell function (203).

In spite of the problems mentioned above in the definition of NK cell population, a considerable body of evidence has been accumulated in support of a functional role for NK cells in NR. The evidence from the murine system can be summarized as follows: 1) Elimination of NK sensitive ($NK^S$) tumors can be enhanced by NK activators such as poly I:C and interferon. On the other hand, interferon pretreatment of the target cell results in loss of NK sensitivity as measured *in vitro*, with a concommitant slowing of the *in vivo* elimination rate (177,178,204,205). 2) Tumor elimination in NK deficient hosts, such as α-AGM1 treated mice, or bg/bg mice is slower than in the normal counterparts (202,206-208). $NK^S$ tumors have an increased growth rate in bg/bg mice, as well as an increased metastatic behaviour when compared to NK resistant ($NK^R$) tumors (206,207). Treatment with cyclophosphamide which also depletes *in vivo* NK activity, results in increased lung metastases (209). 3) *In vivo* reconstitution of NK

deficient mice with NK cells restored resistance to the tumors, and decreased metastatic potential (208-210).

In the human system, a higher incidence of malignancies has been reported in individuals with NK deficiencies, such as the Chediak-Higashi syndrome, the X-linked lymphoproliferative disease, ataxia telangiectasia and severe combined immunodeficiency disease (211-213).

Although the evidence linking NK cell activity to natural resistance is impressive, there are exceptions, indicating that NK cells are not the only NR mechanism operating in vivo. Examples of these are lack of correlation between in vitro NK sensitivity and in vivo tumor elimination (177,204), and that in some studies, elimination of $NK^S$ tumors from C57Bl/6 bg/bg mice was as efficient as in normal C57Bl/6 mice (178). Compelling evidence indicating that NK activity on its own cannot account for natural anti-tumor resistance comes from a series of genetic studies using high and low NK activity strains, and their F1 hybrids and backcrosses as well as 23 BXD recombinant strains (214). Although an H-2 association was seen with resistance, in that $H\text{-}2^b$ were more resistant to tumors of $H\text{-}2^d$ origin than $H\text{-}2^d$ mice, this was not the case with in vitro NK activity.

These kinds of discrepancies have led to the investigation of other mechanisms which may be independently operative, or which may function in association with NK cells, but whose genetic regulation is independent of NK cells. An interesting model for this kind of relationship was recently put forward by Warner and Dennert (215). Although they were looking at allogeneic bone marrow resistance rather than tumor resistance, it is a useful model because NK cells

have also been implicated as effector cells in bone marrow resistance (216). Warner and Dennert were able to show, that although the effector cells were in fact NK cells, their function was modified by NAb, which in this case was specific for the allo-antigenic target cells. Resistance to bone marrow transplants required the presence of both NK cells and the appropriate antibodies. They postulated that antibody-dependent cellular cytotoxicity (ADCC) was the actual mechanism for the destruction of transplanted bone marrow cells.

5.3.2. Macrophages: Macrophages, when activated by agents such as Corynebacterium parvum (C. parvum), BCG, and lymphokines are cytotoxic for tumor cells (217-221). In addition, they can also kill tumor cells in antibody dependent cytotoxicity assays (222-225). The success of therapeutic use of IgG anti-tumor antibodies in some murine models has been attributed to this mechanism (225). They may also be involved in regulatory functions. For example, studies from this laboratory have shown that agents, such as silica, which deplete macrophage activity in vivo, also result in loss of NAb activity (20). They are also producers interferons and prostaglandins which regulate NK activity (226-228).

5.3.4. NAb: The hypothesis that NAb are involved in tumor resistance is supported by findings from our laboratory, as well as others. The initial evidence for the role of NAb came from studies showing correlations between serum NAb levels and tumor resistance. This has been shown to be the case with virally induced tumors (14), transplanted fibrosarcomas (19,146), and lymphomas (71). Further-

more, an inverse correlation was found between tumor sensitivity of cloned tumor lines to NAb and complement and host resistance (71). Analysis of the genetic and ontogenetic properties of NR to $NK^R$ tumors indicated that they correlated more closely with NAb activity than with NK activity (20). More recent studies by Chow et.al. (229-232) have extended these findings to in vivo and in vitro selection models. It was found that an $NK^R$ tumor which had been passaged in vivo, in the subcutaneous and intraperitoneal sites, exhibited a reduced sensitivity to NR as well as to NAb and complement (229,230). In vitro NAb and complement selection from generated variants were also more resistant as measured by the in vivo NR assay (231). These studies were interpreted to support the hypothesis that NAb may participate in in vivo tumor resistance, as well as in the selection of variants generated during tumor progression. More direct evidence for the role of NAb in resistance to a small tumor cell inoculum came from experiments where the tumor cells were pretreated with syngeneic NAb, resulting in lower tumor frequency (71). The conclusion from these studies was that NR is a heterogenous phenomenon, in which both NK cells and NAb may play a role. The model of bone marrow resistance developed by Warner and Dennert (215) which was described in the previous section, provides one explanation of how both mechanisms may be acting in combination. NAb mediated ADCC against syngeneic tumors has been demonstrated recently in studies carried out by Al-Maghazacki et.al. (233). They used nylon wool passaged spleen cells, and normal serum coated target cells. Although the levels of ADCC killing were low, they were in keeping with other studies in which immune antisera was used (234).

That NAb and NK cells can mediate tumor resistance independently of each other, cannot be ruled out however. This could possibly be the case in situations where the host is NK deficient or depleted, and yet able to mediate NR.

These on-going studies suggest a mechanism whereby NAb may mediate NR. Together with the evidence cited above, they support the hypothesis that NAb's do function in NR.

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CHAPTER 1

# INTERFERON TREATMENT OF A NATURAL KILLER-RESISTANT LYMPHOMA: AUGMENTATION OF NATURAL ANTIBODY REACTIVITY AND SUSCEPTIBILITY TO IN VIVO NATURAL RESISTANCE.

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ABSTRACT

Treatment of a natural killer cell-resistant ($NK^R$) DBA/2 lymphoma with L-cell interferon (IFN) enhanced its reactivity to serum natural antibody *in vitro* in cytolysis and absorption studies and increased the *in vivo* acquisition of natural and antitumor antibody in the peritoneal cavity. The IFN effects were both time- and dose-dependent. *In vitro* IFN-treated, [$^{131}$I]5-iodo-2'-deoxyuridine-labeled tumor cells, when injected intraperitoneally into normal syngeneic mice, were more rapidly eliminated than were untreated control cells. IFN treatment of the $NK^R$ tumor decreased "cold target" inhibition of NK lysis and did not alter binding or lysis by macrophages. These findings indicated that the enhancement of natural resistance to the IFN-treated tumor did not involve NK cells or macrophages and suggested that IFN may enhance host antitumor resistance by increasing tumor reactivity to antibody.

# INTRODUCTION

The presence on natural antibody in the sera of nonimmunized animals has been widely described (1-8). The specificities of the natural antibodies include antigens expressed on tumor cells, although the exact determinants have not been identified in most cases (9-14). Some of the specificities that have been identified include viral antigens (1,14), major histocompatible complex related antigens (MHC) (7,15), and thymocyte antigens (3).

The presence of murine tumor reactive natural antibodies has usually been detected using a complement toxicity assay. In this manner, we have been able to detect natural antibodies in the serum of DBA/2 mice which react with several syngeneic tumors, such as the T cell lymphomas, SL2-5 and the L5178Y (13,15). We have cloned several of these tumors and found variants differing in their susceptibility to natural antibody and complement, which were stable in their phenotype over long culturing periods (16).

On the basis of work from this laboratory and others, we have proposed the hypothesis that NAb participates in natural antitumor resistance. In support of this contention is the observation that the production of NAb is thymus independent (3,12,13). This has been shown in our laboratory using adult thymectomized mice and comparing their serum natural antibody levels to those of normal controls (16). Others have used nude mice and these studies have indicated that their levels of natural antibodies are equal to or higher than those of heterozygous littermates (11,12). Colnaghi et.al. (11,12)

have reported that NAb maybe under the influence of suppressor T cells, in that they found higher levels of natural antitumor antibody production in T cell depleted mice. We however, have not found higher NAb levels in T cell depleted mice in our systems. In addition, we have also concluded from our previous studies that the production of NAb is under genetic control (15,17). For example, low levels of natural antibody production are associated with the H-$2^d$ haplotype, whereas higher levels are associated with haplotypes such as the H-$2^b$ and H-$2^k$. These have been confirmed in F1 and backcross studies (15,18). Natural resistance against tumors is also thymus independent and under genetic control (19) therefore the above observations are important in the consideration of the contribution of NAb to this form of resistance.

In vivo studies from our laboratory and from other groups have yielded more direct evidence supporting the hypothesis that NAb may contribute to natural resistance mechanisms. In order to be convinced that the natural resistance mediated effects that we observed were in fact attributable to NAb rather than to natural killer cells, we have used tumor cells that were resistant to this latter effector mechanism. Thus we have shown a correlation between NAb sensitivity of NKR tumors and tumorigenicity, as well as between serum NAb activity and host resistance. This relationship has been demonstrated in assays measuring tumor frequency of transplanted tumors (12,20), and elimination of [131I]IdUrd labelled tumor cells (16,20-22). This latter assay is particularly useful in natural resistance studies because it allows the determination of tumor

elimination during the first three days after tumor inocculation, thus avoiding the involvement of a T cell mediated mechanism. This assay was originally established by Hofer et.al. (23,24) and control experiments have shown that the release of the radiolabelled iodine is due to cell death. Furthermore, the radiolabel remaining within the animal is due to intact tumor cells, and not due to reutilization of degraded material, because of rapid deiodination and excretion of degraded material (23).

Other studies which support our hypothesis are those of Aoki et.al. (1) and Ménard et.al., (12) which have shown that serum NAb levels correlate with lower tumor frequency in mice bearing endogenous murine leukemia virus, and with decreased growth of transplanted fibrosarcoma, respectivelly.

Although most tumor resistance studies have focused on cell mediated mechanisms, a role for antibody in tumor resistance is suggested by the observation that induced or passively acquired antibody reduced tumor growth and frequency as well as increased survival time (25-27). Other experimental systems in which antibody mediated in vivo killing has been implicated include enhanced resistance to tumors in semisyngeneic parabionts (28) and hybrid resistance to murine lymphomas (29,30). In addition, a recent study of Badger and Bernstein (31) has shown that anti-Thy1 antibodies can mediate resistance to a T cell lymphoma.

In the series of experiments reported here, we have made use of the membrane antigen-modulating properties of interferon to further study the role of NAb in in vivo natural resistance. The effects of

interferon on cell surface molecules are many, including increased expression of MHC antigens (32-34), FcGamma receptors (35), carcino-embryonic antigen (36), and concanavalin A receptors (37). In addition, $NK^S$ targets become resistant to NK mediated lysis when they are pretreated with interferon (38-42), making this a useful tool for looking at non-NK mediated natural resistance.

In the first series of experiments, we examined the ability of interferon to modify the in vitro and in vivo NAb binding capacity of $NK^R$ and $NK^S$ tumor cells. We then tested the hypothesis that NAb contributes to natural resistance by comparing the modification of interferon induced NAb reactivity of an $NK^R$ lymphoma to its altered tumorigenicity in the in vivo assay of natural resistance.

# MATERIALS AND METHODS

## Mice and tumors:

DBA/2 mice were obtained from the Jackson Laboratory Bar Harbor, Maine, and from the University of Manitoba Vivarium, Gunton, Manitoba. Mice were housed in laminar flow containment modules until 8-12 weeks of age. Inbred male 6 to 12 week old CBA/J mice were obtained from The Jackson Laboratory. The L5178Y-F9 (LY-F9), SL2-5, and YAC cells were described previously (16). The LY-F9 cells were shown to be $NK^R$ when tested as targets with normal and polyinosinic-polycytidylic acid (poly i:c) stimulated effector cells (cytotoxicity, $\leq$ 3%) at an effector:target ratio of 150:1 (21). The $L1210_S$ (interferon sensitive) and the $L1210_R$ (interferon resistant) lymphomas were obtained from Dr. B. Dalton, Medical College of Pensylvania, Philadelphia, Pa., and originated in the laboratory of Dr. I. Gresser, Institute de Recherches Scientifiques sur le Cancer, Villejuif, France.

Tumor cells were maintained in vitro in suspension cultures in Fishers Medium supplemented with 10% fetal bovine serum (10% FFBS). Only antibody-free fetal bovine serum was used in these experiments. All tumor lines were periodically mycoplasma screened and were consistently uncontaminated.

## Preparation of antisera.

Normal mouse sera: Normal 9 to 22 week old CBA/J mice were bled via the axilla into test tubes on ice. The blood was centrifuged

immediately at 200 x g for 10 minutes at 4°C to remove the clot. Serum was stored at -20°C.

Alloantisera: Anti-H-$2^d$ (B10.BR anti-B10.D2) antiserum was obtained by six intraperitoneal injections of B10.D2 splenocytes (1 spleen/3 recipients) every 2 weeks into B10.BR mice. Mice were bled via the axilla 6 days after the final injection.

Absorption of NAb: Normal CBA/J serum, diluted 1:4 with 50% FFBS, was absorbed with various concentrations of in vitro grown LY-F9 tumor cells at 4°C for 1 hour, with occasional mixing. In some experiments, serum was absorbed with 1 x $10^8$ thymocytes from 5 week old CBA/J mice per 300 µl serum for 1 hour at 4°C before absorption with SL2-5 tumor cells.

Interferon treatment of tumor cells:

Partially purified Newcastle disease virus-induced mouse L-cell interferon (obtained from Dr. C. A. Ogburn, Medical College of Pennsylvania) had a specific activity of 6 x $10^7$ mouse interferon reference units/mg protein. Mouse albumin was added at a 150:1 (mg albumin:mg interferon) ratio, and the interferon preparations were diluted in 10% FFBS and stored frozen (-80°C). Exponentially growing tumor cells were incubated at 1.0-1.5 x $10^5$ cells/ml with various doses of mouse interferon (see table I), for 20 hours in 10% FFBS with humidified 5% $CO_2$ at 37°C. Controls contained 1) no interferon, 2) interferon for a 1 hour incubation, or 3) mouse albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo.) at the same concentration as the interferon preparation.

NAb, NK and Macrophage in vitro cytotoxicity assays:

These assays were previously described (13,16). Briefly, for the NAb experiments, 4x105 $^{51}Cr$ labelled target cells were incubated with 200 µl of normal mouse serum for one hour at 37°C, after which they were washed once and 2.5x104 cells incubated with 40µl of a 1:10 dilution of rabbit complement (Cedarlane, Lowtox), which had previously been absorbed with the respective tumor cells ($10^8$ cells/ml of complement, for 1 hour on ice), in 96 V bottom plates. After a 1 hour incubation at 37°C, 160 µl of cold HBSS was added to each well, and 100 µl was removed for gamma counting after centrifuging the plates for 10 minutes at 150 x g to pellet the cells. Controls consisted of cells incubated with 10% FFBS in the first step, and serum treated cells as well as control cells incubated with complement dilution medium (10% FFBS) during the second incubation. Specific cytotoxicity values were calculated including complement toxicity values (derived by subtracting the percent $^{51}Cr$ release of control cells incubated in control media from the percent $^{51}Cr$ release of control cells incubated in complement containing media) as follows:

$$\frac{\text{Experimental release - background release - complement toxicity}}{\text{100\% - background release- complement toxicity}}$$

Pre-absorption of the complement ensured low complement toxicity levels (usually between 3 and 5%). All antibody toxicity assays were carried out in quadruplicate.

For the NK assay, 104 $^{51}Cr$ labelled target cells were incubated with the appropriate number of red blood cell depleted spleen cells in 96 V bottom plates in a total volume of 200 µl of 10% FFBS. The plates were centrifuged (50 x g) for 5 minutes, then incubated at

37°C for 4-5 hours. Before harvesting 100 µl, the plates were centrifuged for 10 minutes at 150 x g to pellet the cells. Percent specific cytotoxicity was calculated as follows:

$$\frac{\text{Experimental release - background release}}{\text{100\% - background release}}$$

All NK assays were carried out in triplicate. For obtaining activated macrophages, mice were injected with 1.5 mg pyran copolymer 7 days before the assay. Peritoneal cells were collected by washing with a total of 25 ml of cold HBSS. Cells were pooled and washed. The appropriate number of peritoneal cells were aliquoted into 96 well flat bottom plates, in 10% FFBS. After a 60 minute incubation at 37°C, the macrophage monolayers were washed two times with HBSS. 1 x $10^4$ $^{51}Cr$ labelled target cells were added to each well in 200 µl of 10% FFBS, containing 20 mM HEPES buffer, for an 18 hour incubation. Harvesting of supernatant, counting and cytotoxicity calculations were performed as described for the NK assay. These experiments were carried out in collaboration with Bill Pohajdak. The effect of IFN on NAb and NK sensitivity was found to be highly reproducible, although the results were not analyzed statistically in each case.

Detection of in vivo acquired NAb:

Complement lysis: Tumor cells were injected intraperitoneally at a dose of 5 x $10^6$ cells/mouse and were recovered by three peritoneal lavages in which a total of 20 ml cold HBSS was used. Cells were then washed and split into 3 aliquots of 2 x $10^5$ cells and were exposed to absorbed rabbit complement or diluent for 60 minutes at 37°C. The number of dead cells was determined by trypan blue

exclusion; lysis was expressed as the percentage of dead cells detected in the complement-treated group after subraction of the percentage of dead cells in the diluent-treated group.

$^{125}$I-labeled protein-A binding: Tumor cells were injected into and recovered from the peritoneal cavity as described above. They were then washed twice and incubated for 1 hour with 100 µl of a 1:200 dilution of rabbit anti-mouse F(ab')$_2$ for 60 minutes at 37°C, washed again, and incubated for an additional 60 minutes at 37°C in 100 µl (0.5 µCi) of $^{125}$I-labeled protein A (supplied by Dr. E. Rector, Department of Immunology, University of Manitoba). Cells were washed three times in HBSS and counted in an LKB gamma counter.

Natural Resistance Assay:

This assay has been described previously (21). Tumor cells were labeled in vitro with [$^{131}$I]-IdUrd, were washed three times with HBSS, and were injected intraperitoneally into groups of 5 KI-treated mice. After injection and at intervals of 18-24 hours the mice were whole body counted in a Beckman 8000 gamma counter.

## RESULTS

### IFN Modulation of Tumor Cell Lysis by NAb, NK and Macrophages

The prediction that interferon treatment of murine tumors would lead to increased expression of the MHC-linked determinants recognized by allogeneic NAb (15) was tested with two DBA/2 (H-2$^d$) tumors, the LY-F9 and SL2-5 lymphomas. After in vitro incubation of the cells with interferon for 20 hours, significant increases in

cytotoxicity mediated by C57BL/10 and CBA/J NAb, as well as by congenic anti-H-2$^d$ antiserum were observed (Figure 1).

Figure 2 shows a time course in which SL2-5 cells were incubated with 2,000 or 10 units of interferon/ml, and aliquots of the cells were tested for NAb, as well as for NK sensitivity, over a 24 hour period. NAb sensitivity increased as treatment time was prolonged, whereas the NK lysis decreased in a corresponding time-dependent manner. Although the LY-F9 is an NK$^R$ clone of the L5178Y lymphoma (21), it can act as a cold-target inhibitor in an NK assay with the NK$^S$ SL2-5 as a target cell. The inhibition of lysis was not as high as that produced by the SL2-5 as an inhibitor; however, interferon treatment of the LY-F9 also resulted in a suppression of its inhibitory capacity (Table 1).

To determine whether these effects were due to interferon or to other non-interferon proteins in the preparation that may have increased the cells' susceptibility to lysis, we performed a number of control experiments. Incubation of LY-F9 and SL2-5 tumor cells *in vitro* with mouse albumin, the major protein in the interferon preparation, did not enhance NAb or anti-H-2$^d$ cytotoxicity when these cells were compared to untreated tumor cells. In addition, treatment of tumor with interferon for 1 hour was without effect (data not shown). Further experiments, in which we used the L1210$_R$ cells originally described by Gresser *et.al.* (43), also supported the interpretation that we were observing an interferon mediated phenomenon. In vitro treatment of L1210$_R$ produced no modification

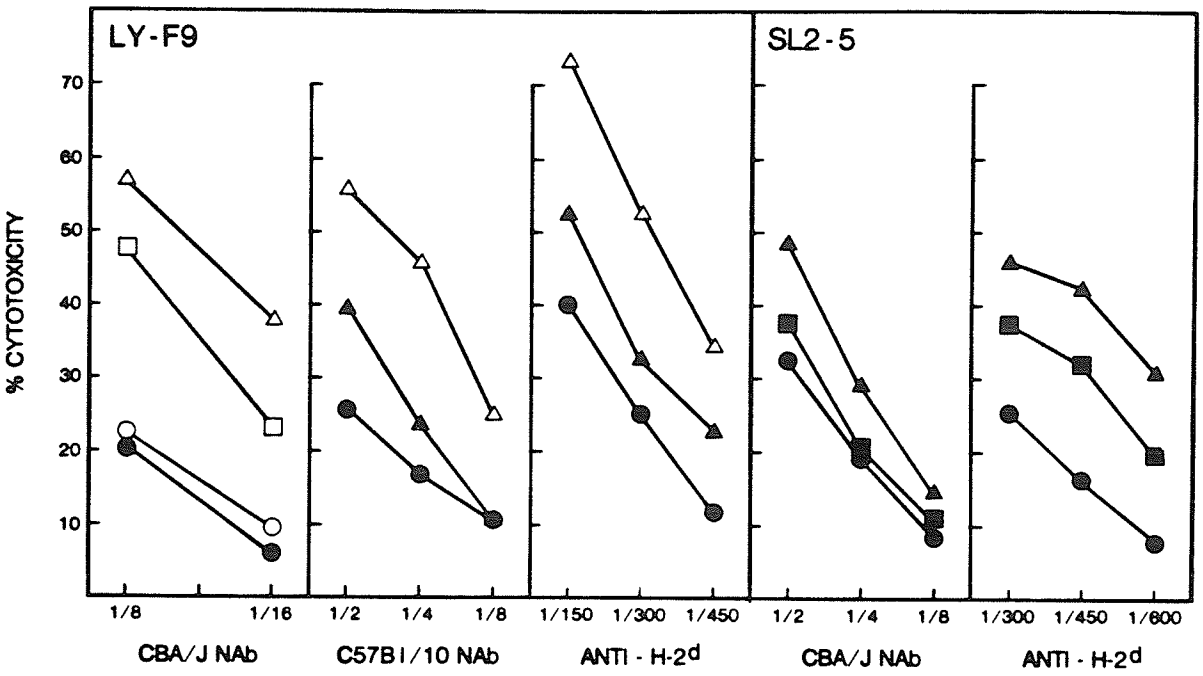


Figure 1: Effect of IFN on the lysis of LY-F9 and SL2-5 lymphomas by CBA/J NAb, C57BL/10 NAb, and congenic anti-H-2d antiserum. Tumor cells were incubated for 20 hr with 1 ( ○ ), 10 ( ■ ), 2,000 ( ▲ ), or 8,000 ( △ ) U IFN/ml or were left untreated ( ● ) before exposure to serum. Pools of serum from 4-10 mice were used in this experiment and in three subsequent experiments in which identical results were obtained.

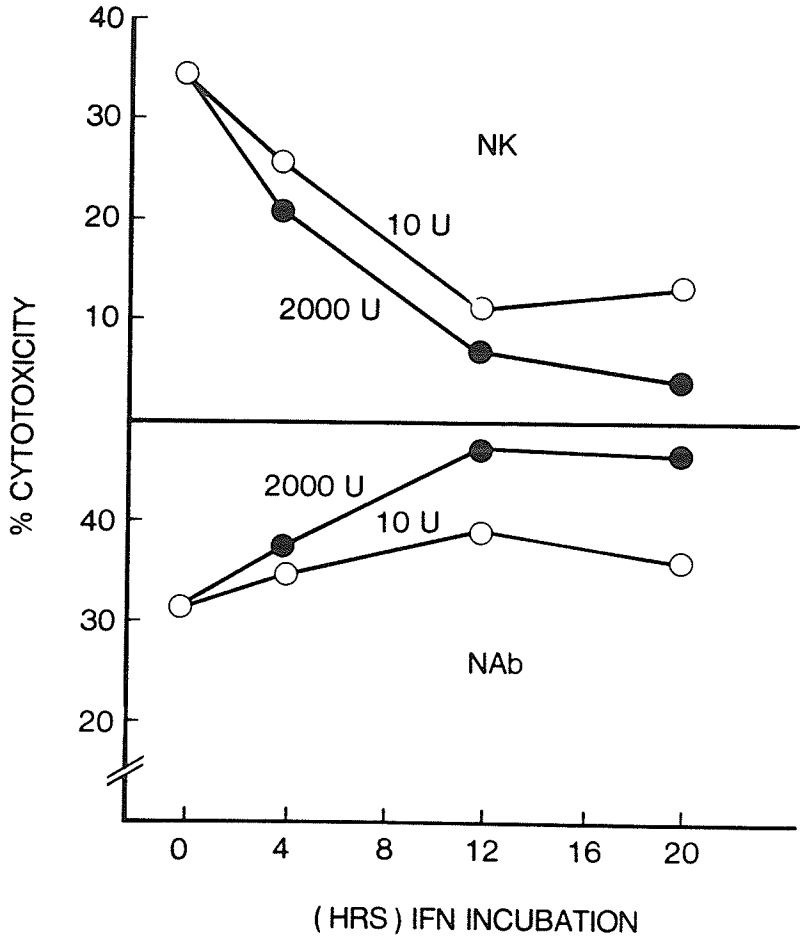


Figure 2: IFN modulation of SL2-5 sensitivity to lysis by CBA/J NK and NAb. IFN-treated SL2-5 cells were divided into aliquots and simultaneously were examined for sensitivity to lysis by normal CBA/J serum and splenocytes. The NK results are reported for the E:T of 150:1. CBA/J NAb was diluted to 50% whole serum. Tumor cells were incubated with 10 ( O ) or 2,000 ( ● ) U IFN/ml for the time periods indicated, and were washed and placed in assays.

TABLE 1. IFN suppression of NK cold-target inhibition

| Expt. | Cold-target inhibitor | Inhibitor: target ratio | IFN, U/ml[a] | Lysis, %[b] | Inhibition, % |
|---|---|---|---|---|---|
| 1 | - | - | - | 39.6 | - |
| | SL2-5 | 5:1 | 0 | 15.9 | 59.8 |
| | SL2-5 | 5:1 | 100 | 27.7 | 30.1 |
| | SL2-5 | 2.5:1 | 0 | 21.4 | 46.0 |
| | SL2-5 | 2.5:1 | 100 | 32.9 | 16.9 |
| 2 | - | - | - | 36.8 | - |
| | LY-F9 | 10:1 | 0 | 25.4 | 31.0 |
| | LY-F9 | 10:1 | 10 | 27.5 | 25.3 |
| | LY-F9 | 10:1 | 1,000 | 28.1 | 23.6 |
| | LY-F9 | 10:1 | 2,000 | 30.2 | 17.9 |
| 3 | - | - | - | 70.5 | - |
| | LY-F9 | 15:1 | 0 | 37.3 | 47.1 |
| | LY-F9 | 15:1 | 2,000 | 47.8 | 32.2 |
| | LY-F9 | 10:1 | 0 | 44.6 | 36.7 |
| | LY-F9 | 10:1 | 2,000 | 56.4 | 20.0 |
| | LY-F9 | 5:1 | 0 | 51.7 | 26.7 |
| | LY-F9 | 5:1 | 2,000 | 59.5 | 15.6 |
| | LY-F9 | 2.5:1 | 0 | 50.9 | 27.8 |
| | LY-F9 | 2.5:1 | 2,000 | 62.9 | 11.2 |

[a] Cold-target inhibitor cells were treated with IFN for 20 hr and then were washed and included in the assay. Effector cells were CBA/J splenocytes at an E:T of 150:1.

[b] In all experiments, $^{51}Cr$-labeled SL2-5 cells were used as targets.

CBA/J NAb, anti-H-2d, or NK reactivity, whereas the response of L1210S was similar to that of the other tumors (Figure 3).

We also compared the susceptibility of interferon treated and control cells to macrophage mediated cytotoxicity. As shown in Table 2, interferon treatment of two tumors, the SL2-5 and the YAC, did not result in enhanced lysis. When we compared the binding of IFN treated and control cells to macrophage monolayers, we observed some enhanced binding.

## Effect of Interferon on the Quantitative Expression of Membrane Antigens Reacting With NAb

The ability of interferon to increase MHC antigen expression on cells was demonstrated by quantitative absorptions of alloantisera by interferon treated and control cells (32,33). Similar experiments with CBA/J NAb indicated that the interferon treated LY-F9 cells were able to absorb more antibody than the untreated tumor cells (Figure 4). In a preliminary experiment we found that the absorption capacity of the SL2-5 lymphoma was not significantly affected by interferon. This result was unexpected in view of the earlier results in which interferon enhanced SL2-5 lysis by NAb. Since the SL2-5 tumor has an antigen that strongly cross-reacts with thymocytes and other experiments suggested that the expression of this thymocyte antigen may be reduced by interferon treatment (Pohajdak B, Greenberg AH: Unpublished data), we first absorbed the serum with CBA/J thymocytes and then restested the remaining NAb. As shown in Figure

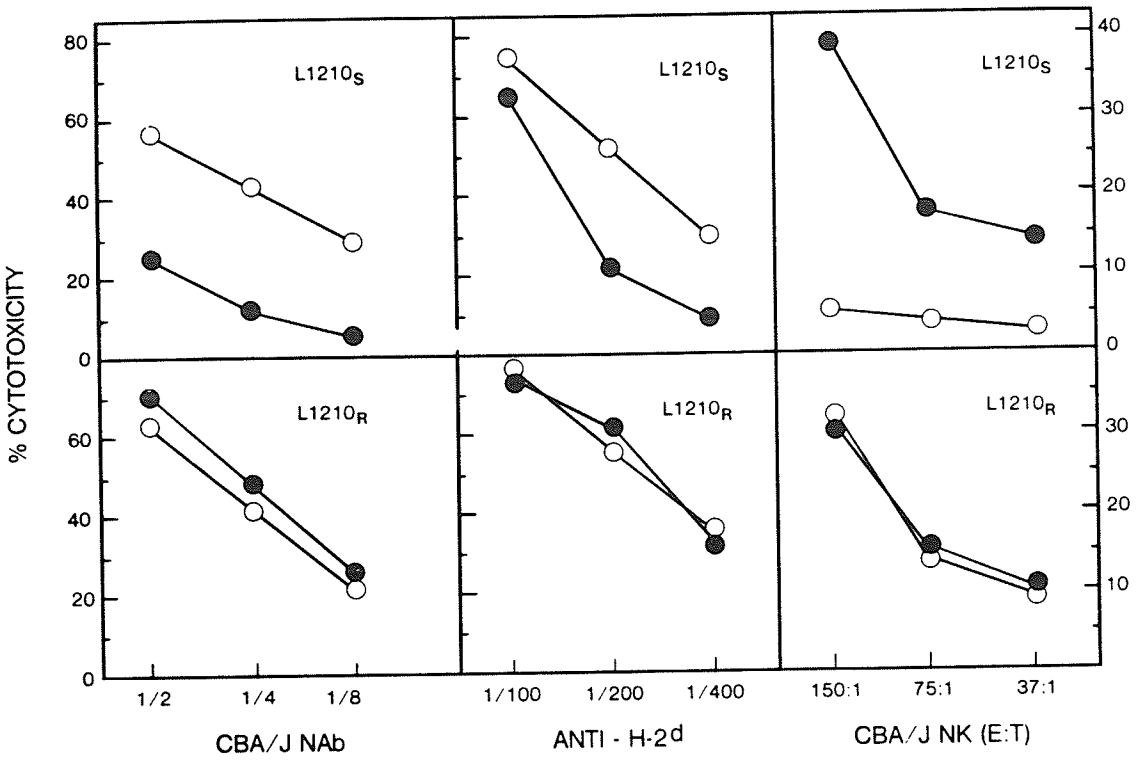


Figure 3: Lysis of IFN-treated $L1210_S$ and $L1210_R$ lymphoma cells by CBA/J NAb, anti-H-$2^d$ antiserum, and CBA/J spleen NK. Tumor cells either were incubated with 5,000 U IFN/ml for 20 hr (○) or remained untreated (●) before being placed in the respective assays.

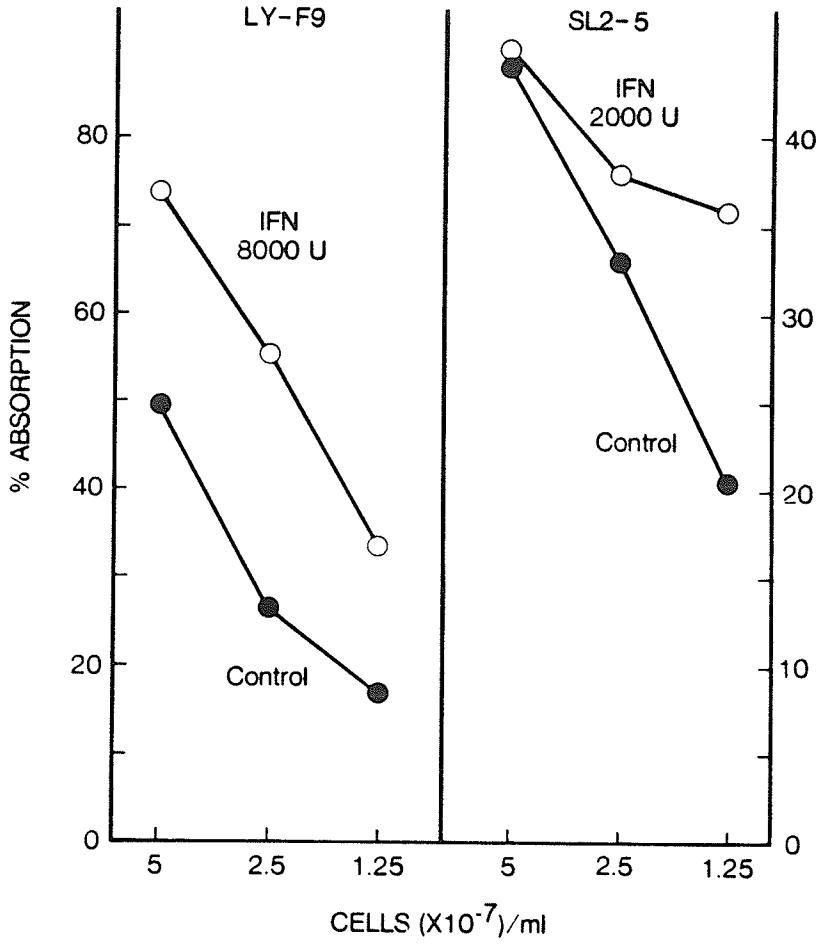


Figure 4: Quantitative absorptions of CBA/J NAb by IFN-treated murine lymphomas. Both LY-F9 and SL2-5 tumor cells when treated with IFN for 20 hr (○) showed quantitative increases in the capacity to absorb CBA/J strain NAb, as compared to the capacity of untreated control cells (●). Sera used in the SL2-5 experiments were first absorbed with CBA/J thymocytes to remove thymocyte autoantibodies.

TABLE 2. Effect of IFN treatment on sensitivity of lymphoma cells to macrophage cytotoxicity and binding to macrophage monolayers.

A.

| Expt. No. | Tumor | IFN Treatment | E:T Ratio | % Cytotoxicity Control target | % Cytotoxicity IFN-target |
|---|---|---|---|---|---|
| 1 | SL2-5 | 2000 U/ml for 20 hours | 60:1 | 21.7 | 0 |
| | | | 40:1 | 17.1 | 0 |
| | | | 20:1 | 13.5 | 0 |
| | | | 10:1 | 7.4 | 0 |
| | | | 5:1 | 1.8 | 0 |
| 2 | SL2-5 | 2000 U/ml for 20 hours | 50:1 | 18.4 | 5.4 |
| | | | 30:1 | 16.6 | 6.4 |
| | | | 20:1 | 15.1 | 1.5 |
| | | | 10:1 | 5.2 | 0 |
| 3 | YAC | 2000 U/ml for 20 hours | 50:1 | 40.2 | 35.8 |
| | | | 25:1 | 34.5 | 27.8 |
| | | | 7.5:1 | 6.2 | 8.8 |

B.

| Expt. No. | Tumor | IFN Treatment | E:T Ratio | % Binding Control target 5 min | Control target 30 min | IFN-target 5 min | IFN-target 30 min |
|---|---|---|---|---|---|---|---|
| 1 | LY-F9 | 4000 U/ml for 20 hours | 50:1 | 19.3 | 38.9 | 19.9 | 48.6 |
| | | | 30:1 | 13.6 | 43.2 | 23.0 | 43.9 |
| | | | 10:1 | 7.2 | 13.0 | 9.2 | 25.8 |
| | SL2-5 | 2000 U/ml for 20 hours | 50:1 | 11.3 | 23.4 | 12.6 | 20.4 |
| | | | 30:1 | 9.7 | 17.2 | 10.2 | 27.4 |
| | | | 10:1 | 6.9 | 7.1 | 9.5 | 8.1 |
| 2 | YAC | 2000 U/ml for 20 hours | 50:1 | 0.6 | 3.6 | 1.7 | 8.2 |
| | | | 25:1 | 0.8 | 6.7 | 1.6 | 8.5 |
| | | | 7.5:1 | 0.7 | 0.4 | 0.2 | 3.5 |

4, this procedure allowed us to detect a quantitative enhancement in antigen expression on this tumor as well.

In Vivo NAb Binding by Interferon Treated Tumor Cells

The experiments reported above were performed with allogeneic NAb. Syngeneic serum NAb ractivity with the LYF9 tumor was generally very low (<5%); therefore, there was insufficient antibody in this serum to detect the changes due to interferon treatment. However, previous work from this laboratory has shown that tumor cells inoculated intraperitoneally into syngeneic mice can acquire NAb within hours of implantation and that the level of lysis achieved is much greater than that with serum NAb (13). We postulated that the interferon enhancement of NAb binding may be more consistently evident in this in vivo environment. Therefore, tumor cells were incubated for 20 hours with 4,000 units of interferon/ml, were washed, and then were inoculated intraperitoneally into DBA/2 mice. Cells were recovered from the peritoneum by lavage and then were washed and exposed to absorbed rabbit complement (see "MATERIALS AND METHODS"). Figure 5 represents a summary time course of three experiments in which NAb binding to interferon treated and untreated LY-F9 cells was compared. By 6 hours, interferon treated cells were significantly more sensitive than were the controls to complement lysis ($p<0.02$), and this difference was maintained over a 26 hour period ($p<0.001$).

To further characterize this increase in NAb, we performed a second series of experiments with LY-F9 cells recovered after 18

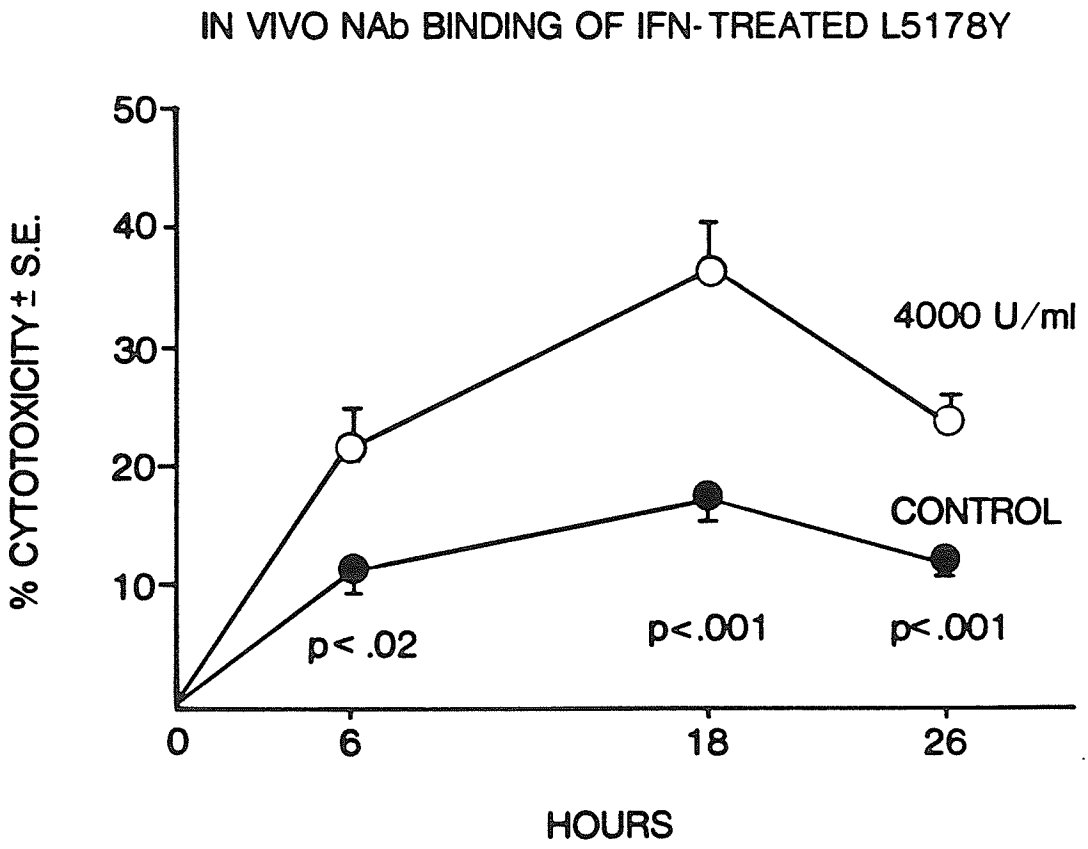


Figure 5: Complement-dependent lysis of IFN-treated and untreated LY-F9 cells after 6,18, and 26 hr of in vivo passage. Tumor cells were incubated with 4,000 U IFN/ml (○) or left untreated (●) before ip injection into mice. A total of 7-9 DBA/2 mice were used for each group.

hours of intraperitoneal passage. Figure 6 shows that the increase in NAb binding in vivo was dependent on both the dose and the time of incubation with interferon. Twenty hours, but not 1 hour of interferon preincubation resulted in increased NAb lysis, and 1,000 and 4,000 units/ml significantly enhanced antibody acquisition ($p<0.02$ and $p<0.001$, respectively), whereas 100 units/ml was ineffective. Controls incubated with mouse albumin, the major protein in our interferon preparation, did not modify the lysis of peritoneally passaged cells. Significantly enhanced in vivo NAb binding to interferon treated tumor cells was also detected by complement dependent lysis with the use of the SL2-5 tumor (Figure 7). Since interferon could have altered complement sensitivity rather than antibody binding, we proceeded with a second series of experiments that did not rely on complement activation. SL2-5 cells, recovered by peritoneal lavage, were washed and incubated with rabbit anti-mouse $F(ab')_2$ and $^{125}I$-protein A (See "MATERIALS AND METHODS"). Once again more antibody, this time detected by radiolabel binding, was found on the interferon treated tumor cells (Figure 8).

## In Vivo Elimination of an Interferon Treated NAbS-NKR Lymphoma

Since interferon pretreatment significantly enhanced NAb binding to the LY-F9 cells in vivo, we reasoned that if this antibody plays a significant role in the elimination of LY-F9, the host's ability to kill the tumor should be correspondingly enhanced. Therefore, we injected interferon treated, [$^{131}I$]-IdUrd-labeled tumor cells into DBA/2 mice and followed the whole-body elimination of the radiolabel

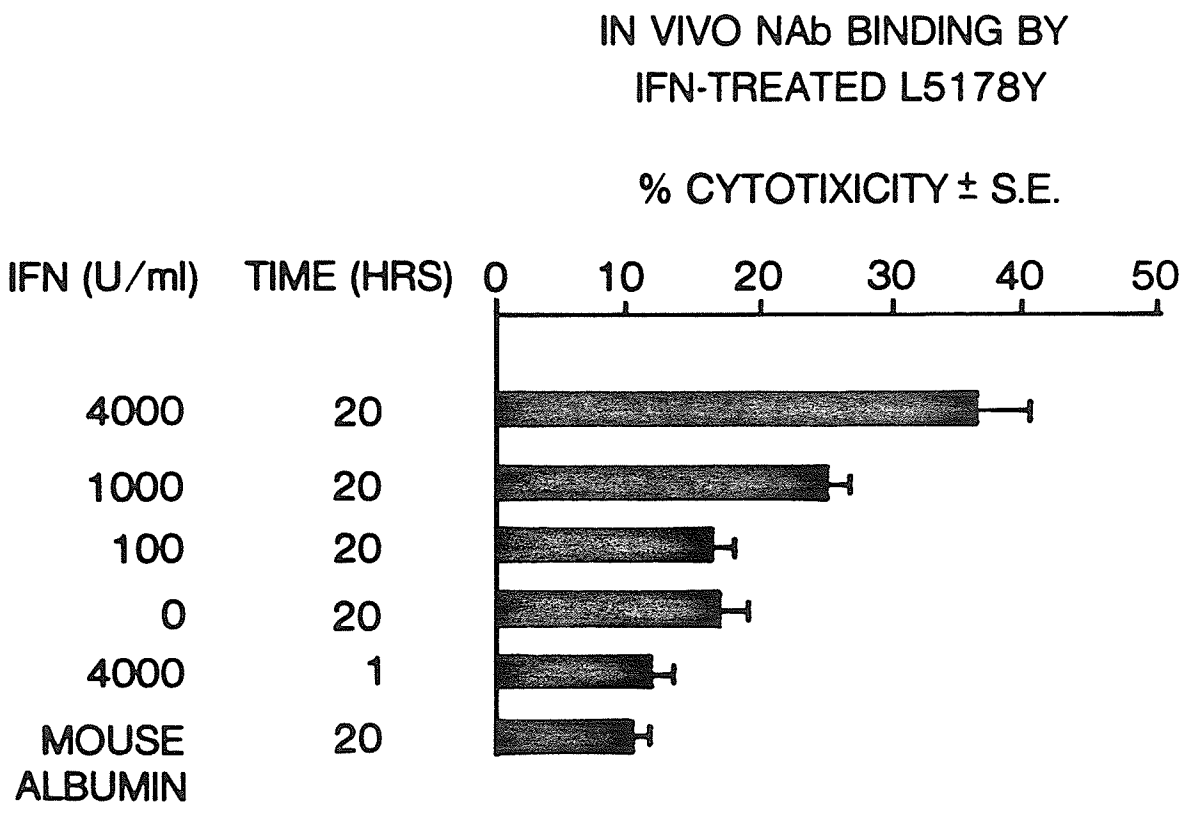


Figure 6: IFN dose titration for enhanced in vivo NAb binding. LY-F9 cells (5 x $10^6$), treated with IFN as indicated, were injected ip into groups of mice; cells were removed after 18 hr. The group receiving 4,000 and 1,000 U IFN/ml differed significantly in antibody acquisition. ($P<0.001$ and $P<0.02$, respectively) from the control groups.

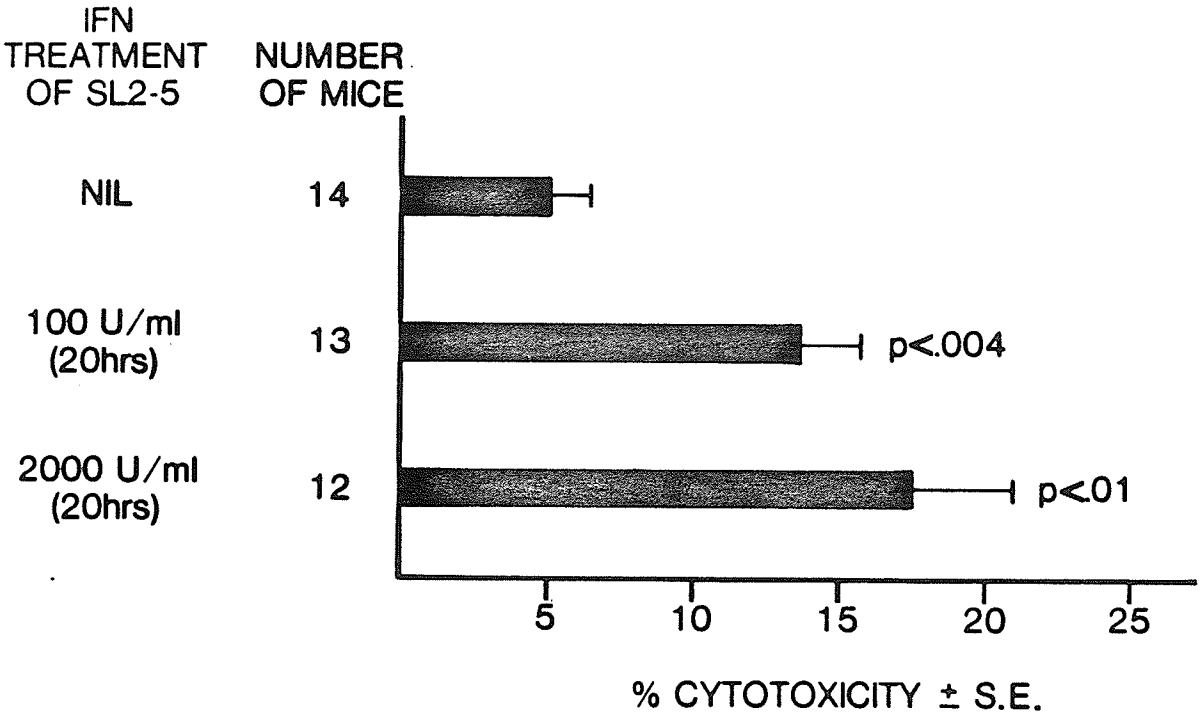


Figure 7: Effect of IFN on in vivo NAb binding to the SL2-5 lymphoma. Cells were treated with IFN as indicated, injected intraperitoneally for 4½ hours, then tested in a complement mediated assay as described in MATERIALS AND METHODS.

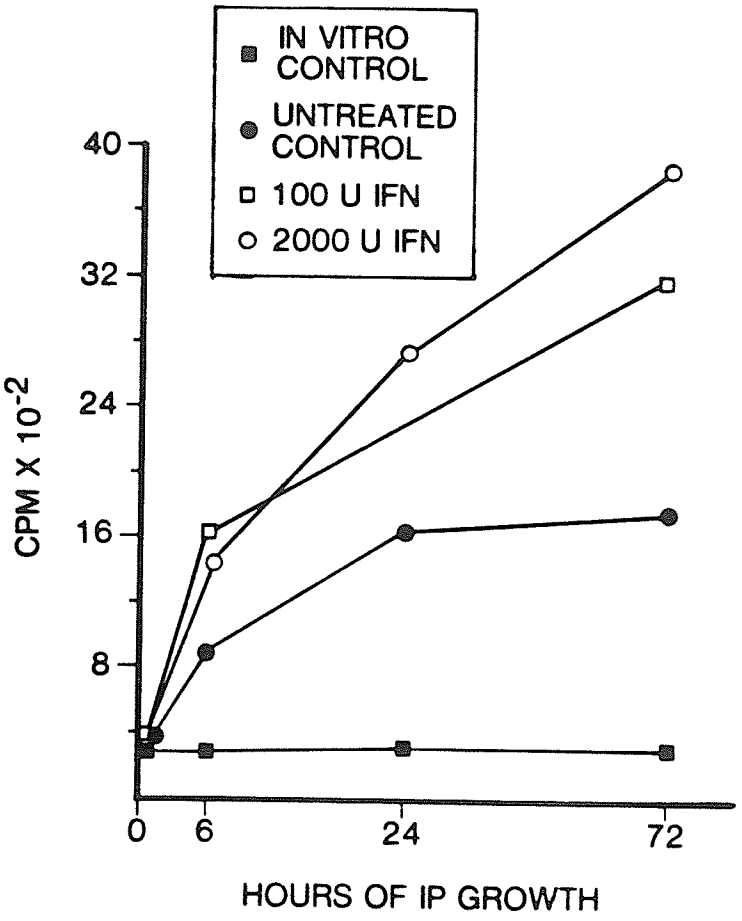


Figure 8: IFN enhancement of in vivo binding of NAb detected by rabbit anti-mouse F(ab')$_2$ and $^{125}$I-labeled protein A. Tumor cells were treated with 100 ( ▫ ) or 2,000 ( ○ ) U IFN/ml for 18 hr in vitro or were left untreated ( ● ), and then were injected ip into mice. Cells were recovered by peritoneal lavage at the time intervals indicated and were washed and incubated with anti-F(ab')$_2$ and $^{125}$I-labeled protein A. Each point represents the mean of triplicate counts on 2 x $10^6$ cells obtained from pooled lavages of 2 or 3 mice. ( ■ )= in vitro control cells.

over 3 days. An accelerated elimination rate was observed as compared to the elimination rate for untreated control tumor cells (Figure 9A). The enhancement was statistically significant on days 2 and 3. The effect was dose-dependent inasmuch as 1,000 and 100 units of interferon/ml resulted in intermediate elimination rates as compared to the rates with 8,000 units/ml. This enhanced resistance was also time dependent. Similar to the effect of interferon on in vivo antibody acquisition, 1 hour of exposure to interferon was not sufficient to affect tumor elimination (Figure 9B). These experiments were repeated a number of times, and although siginificance was not always reached in individual experiments, enhancement of elimination was usually observed. Table 3 is a summary of these experiments and shows that with the use of a range of cell inocula and interferon doses, enhancement was statistically significant on all 3 days of this assay.

We next inoculated different groups of mice with an increasing number of interferon treated and untreated tumor cells. Figure 10 illustrates such an experiment in which $10^6$ to $8x10^6$ cells after a 20 hour incubation in 4,000 units of interferon/ml were injected intraperitoneally into groups of 5 mice. In this experiment the enhanced elimination of the interferon treated group was statistically significant with the use of $10^6$ or $2x10^6$ cells, but this difference was not observed with larger cell doses.

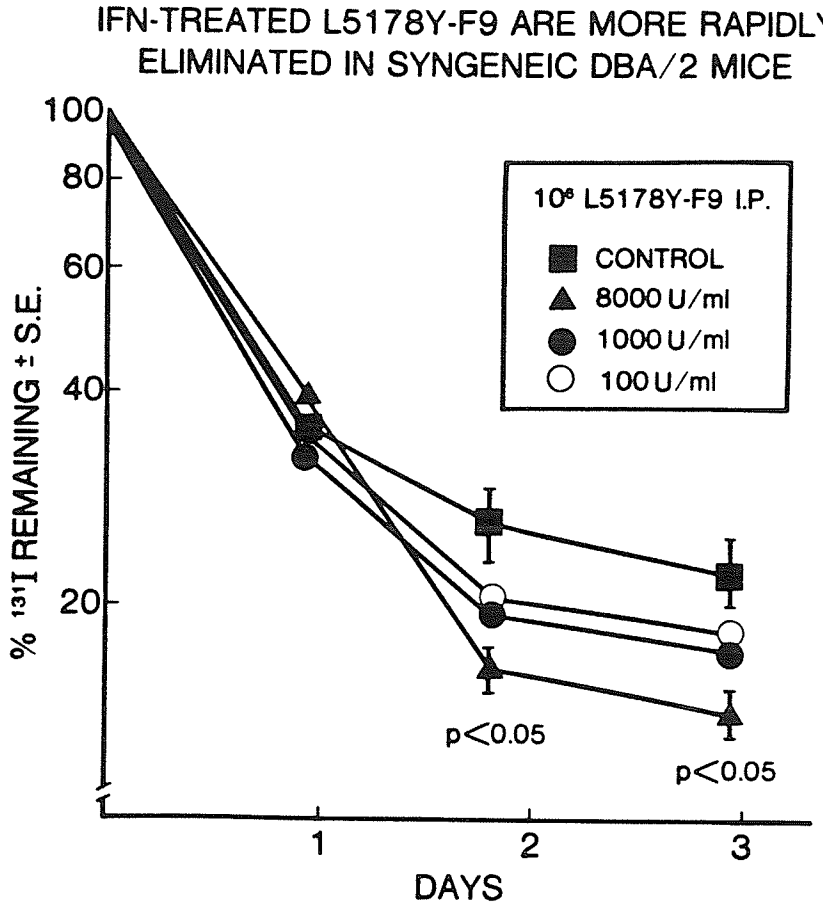


Figure 9: 131I elimination rates for IFN-treated and untreated LY-F9 tumor cells. [131]IdUrd-labeled cells (106) were injected into groups of 8- to 10-wk-old DBA/2 mice, 5 mice/group. A) The difference between the 131I remaining after injection of cells treated for 20 hr with 8,000 U IFN/ml (▲) and the 131I remaining after injection of untreated (control) tumor cells (■) was statistically significant on days 1 and 2. (●)= cells treated with 1,000 U IFN/ml; (○)= cells treated with 100 U IFN/ml.

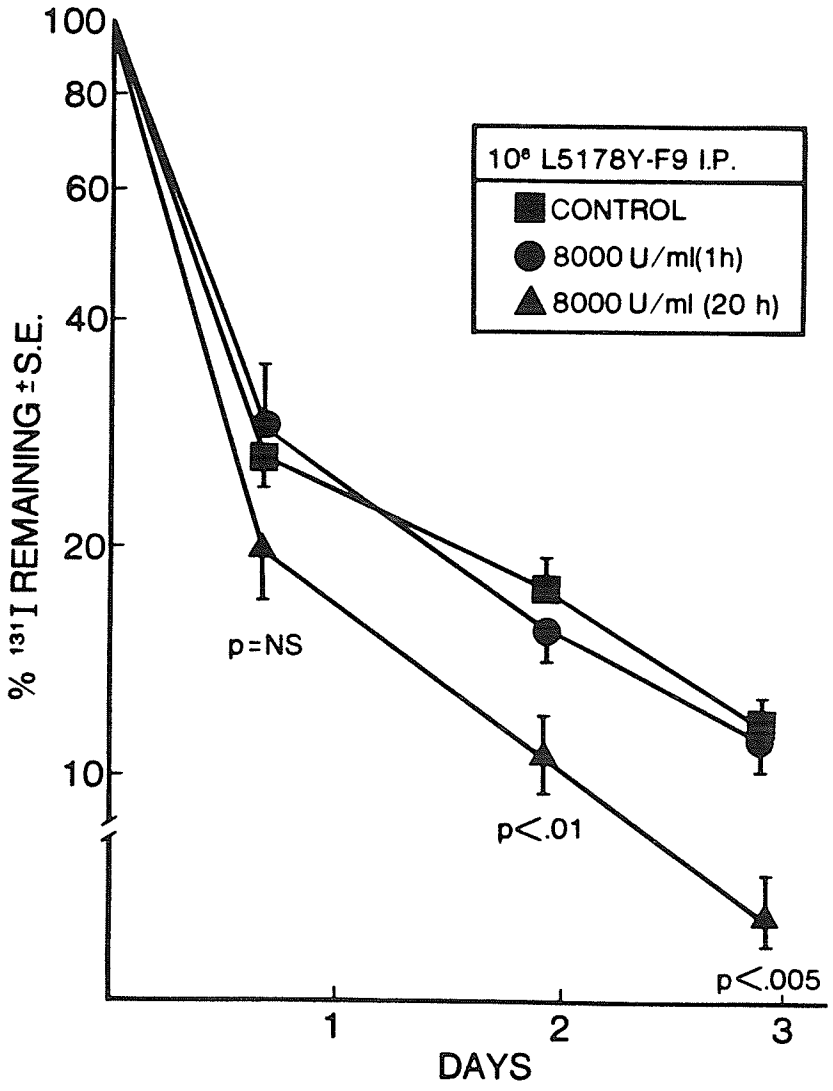


Figure 9: B) One-hour treatment with 8,000 U IFN/ml ( ● ) did not result in enhanced 131I elimination on any day. ( ▲ )= cells treated with 8,000 U IFN/ml for 20 hr; ( ■ )= untreated (control) tumor cells.

TABLE 3. Enhanced elimination of IFN-treated LY-F9 lymphoma cells

| Expt. No. | Cell dose | IFN treatment, U/ml, for 20 hr | $^{131}$I retained, % ± SE,[a] on: Day 1 | Day 2 | Day 3 |
|---|---|---|---|---|---|
| 1 | 5x10$^5$ | 8,000 | 20.5±0.8 | 7.9±0.3 | 4.05±0.1 |
| | | 0 | 51.7±7.4[b] | 16.9±2.7[c] | 6.94±1.1 |
| 2 | 10$^6$ | 8,000 | 25.1±4.3 | 12.0±2.6 | 9.3±1.8 |
| | | 0 | 38.0±5.5 | 20.7±3.4 | 15.4±2.5 |
| 3 | 10$^6$ | 8,000 | 20.1±3.4 | 10.9±1.1 | 6.6±0.8 |
| | | 0 | 26.8±2.9 | 17.6±1.7[b] | 11.8±1.0[b] |
| 4 | 10$^6$ | 8,000 | 39.4±3.7 | 16.2±1.2 | 14.3±1.0 |
| | | 0 | 35.1±3.2 | 26.0±3.2 | 22.6±2.6[c] |
| 5 | 10$^6$ | 8,000 | 27.2±2.7 | 14.5±1.3 | 9.5±2.0 |
| | | 0 | 40.1±5.0[c] | 24.3±2.8[d] | 18.6±2.1[c] |
| 6 | 5x10$^6$ | 8,000 | 62.3±5.5 | 41.4±4.9 | 32.6±4.1 |
| | | 0 | 80.6±3.6[d] | 56.9±4.9[d] | 45.6±4.4[c] |
| | 2x10$^6$ | 8,000 | 72.8±3.0 | 47.2±1.9 | 32.4±3.2 |
| | | 0 | 59.7±6.7 | 40.3±8.0 | 32.2±2.2 |
| 7 | 5x10$^6$ | 4,000 | 37.8±2.5 | 20.7±0.9 | 14.6±0.6 |
| | | 0 | 46.9±3.8 | 26.0±2.2[c] | 17.5±1.6 |
| 8 | 5x10$^6$ | 4,000 | 44.6±2.5 | 35.0±1.9 | 27.0±1.8 |
| | | 0 | 56.0±4.2[c] | 42.5±5.0 | 29.0±3.8 |
| 9 | 2x10$^6$ | 4,000 | 45.4±9.0 | 30.3±5.7 | 22.1±4.4 |
| | | 0 | 57.1±4.5 | 46.7±4.1[c] | 35.1±2.7[c] |
| 10 | 10$^6$ | 4,000 | 26.9±0.8 | 19.6±0.8 | 13.7±0.3 |
| | | 0 | 46.5±5.8 | 35.7±5.9[c] | 25.0±5.0[c] |

[a] Individual experiments were analyzed with Student's t-test; P-values for those experiments are given in footnotes b,c, and d. Analysis of the combined data by means of a paired t-test showed significant differences on day 1 ($P<0.02$), day 2 ($P<0.001$), and day 3 ($P<0.001$).
[b] $P<0.001$.
[c] $P<0.05$.
[d] $P<0.02$.

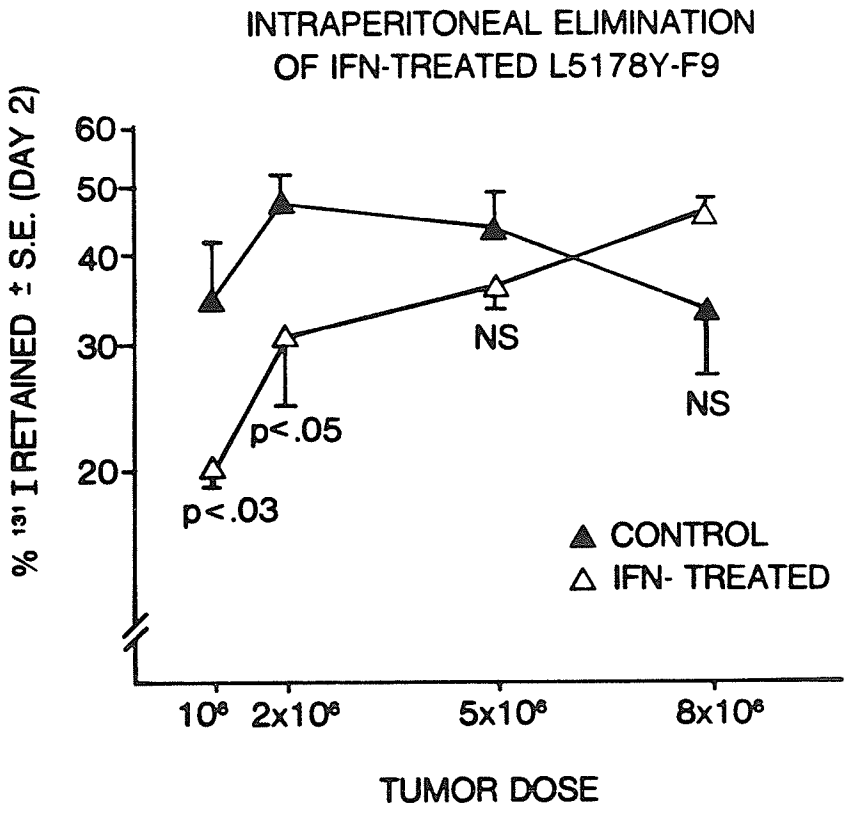


Figure 10: Percent 131I remaining on day 2 after different doses of IFN-treated and untreated tumor cells were injected ip into mice. Tumor cells were treated with 4,000 U IFN/ml for 20 hr ( △ ) or were left untreated ( ▲ ). They were labeled with [131I]IdUrd and injected ip into groups of 5 DBA/2 mice. The differences in the amount of 131I remaining on day 2 were statistically significant when 106 or 2 x 106 cells were injected. NS=not significant.

Effect of Interferon on In Vitro Growth of Tumor Lines

One of the many biological effects of interferon is growth inhibition of *in vitro* cultured cell lines (44). We therefore tested the growth inhibitory effects of interferon on the LY-F9 tumor line. Figure 11 shows that doses of 100 U/ml and 4,000 U/ml inhibited the growth to an equal extent, in contrast to our *in vivo* elimination studies, in which only the higher concentration of interferon significantly enhanced the elimination rate.

We examined the relationship between growth inhibition and *in vivo* tumor elimination in more detail, by treating tumor cells with mitomycin C. Table 4 shows that mitomycin C treated cells, which were 95% viable as determined by trypan blue exclusion, and incapable of division, were eliminated more rapidly at all tumor doses examined. This differed from the tumor-dose dependent enhancement which we showed in our previous *in vivo* elimination studies with interferon treated LY-F9.

## DISCUSSION

The ability of interferon to modulate cell surfaces depends on both the nature of the membrane antigen in question and the cell type (34,35). However, it has been a universal observation that interferon can increase MHC antigen expression on normal and neoplastic cells both *in vivo* and *in vitro* (32,33). Similarly, the treatment of target cells with interferon enhances both lysis of alloreactive, cytolytic T lymphocytes (40) and alloantibody induced, antibody dependent, cell-mediated cytotoxicity (39), probably by increasing

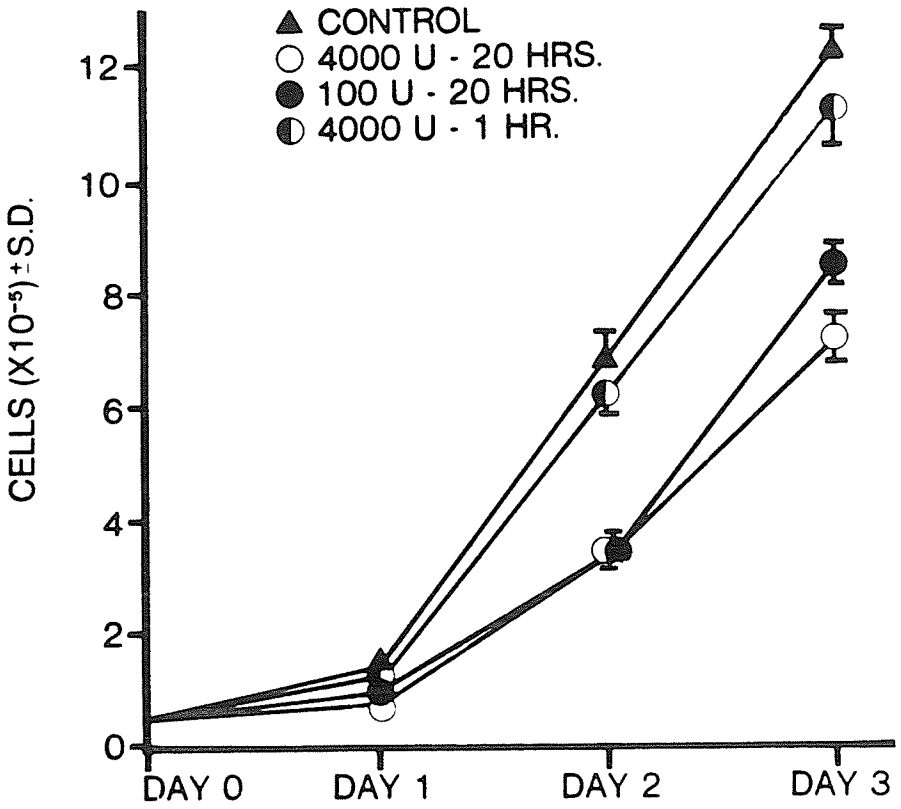


Figure 11: Growth inhibition of IFN treated L5178Y-F9. Tumor cells were treated with varying doses of IFN, for 20 hours or 1 hour, washed, counted, and cultured at 5 x 104 cells/ml.

TABLE 4. Effect of mitomycin C treatment on in vivo elimination of the LY-F9 lymphoma

| No of Cells Injected | Mitomycin C Treatment[a] | % $^{131}I$ Remaining ± SE Day 1 | Day 2 |
|---|---|---|---|
| $10^5$ | - | 19.9±2.9 | 4.3±3.8 |
| $10^6$ | - | 27.4±9.8 | 19.2±8.9 |
| $5x10^6$ | - | 34.3±5.2 | 25.4±3.5 |
| $10^5$ | + | 8.4±0.9 | not counted |
| $10^6$ | + | 10.9±1.0 | 6.5±0.4 |
| $5x10^6$ | + | 9.4±1.4 | 5.9±0.6 |

MHC antigen expression. Genetic studies from two laboratories have indicated that NAb may react with membrane components that are MHC antigen associated (7,15). This observation suggested to us that interferon may also affect the expression of NAb antigens.

The results presented in this paper have shown that three lymphomas, the LY-F9, SL2-5 and $L1210_S$ were more readily lysed by CBA/J and C57BL/10 NAb after in vitro incubation with interferon and that the lysis was associated with quantitative increases in membrane antigen. The ability of interferon to enhance in vivo syngeneic NAb acquisition may be somewhat more complex. The interferon enhancement of in vitro binding of allogeneic serum NAb suggests that this could be a result of the passive acquisition of a preformed antibody. However, we cannot rule out the possibility of rapid induction of antibody by tumor cells. In some experiments, the complement-mediated lysis of in vivo passaged tumor cells far exceeded the lysis of tumor cells with in vitro syngeneic serum-bound antibody (13). If rapid induction of antibody by tumor occurs in vivo, it would imply that the interferon treated tumor is more immunogenic.

We addressed another question in this study: What is the in vivo significance of the interferon modulation of the NAb reactive tumor cell antigen? To assess in vivo resistance, we measured the elimination of [$^{131}$I]IdUrd labeled tumor cells- a rapid assay for monitoring tumor cell death in vivo (23). This assay has been used to measure natural resistance (16,21,22) as well as the effect of passively transferred antibody on tumor rejection (25,26,40). For our studies we chose the intraperitoneal rather than the intravenous

route because these conditions would parallel the in vivo NAb-binding experiments. We have shown that a correlation exists between interferon enhancement of in vitro and in vivo NAb acquisitions and accelerated rejection of an $NK^R$ tumor. Increased NAb binding was observed at similar interferon doses and treatment times as those required to enhance tumor elimination. Although this observation is not direct evidence of NAb participation, it does point out the similar requirements of these two phenomena. Inasmuch as interferon can affect other membrane structures, modification of the in vivo activity of other effector mechanisms participating in natural resistance possibly could account for the enhanced elimination rate. We have ruled out, however, the direct participation of NK cells, both by selecting an $NK^R$ tumor for this study and by demonstrating that interferon suppresses NK lytic sensitivity and cold-target inhibition of $NK^S$ and $NK^R$ tumor lines. Therefore the contribution of NK cells in this system, if any, would be to slow the elimination rate, rather than enhance it.

A role for macrophage in the natural resistance phenomena has also been postulated (45-48). One of the effector mechanisms involves macrophage tumor cell binding (49,50), and although the macrophage target structure on the tumor cells has not been identified, conceivably, its expression could also be modified by interferon treatment, leading to enhanced macrophage tumor interaction. However, when we tested the in vitro sensitivity of interferon treated and untreated LY-F9 cells to activated macrophages, we found no enhancement in lytic sensitivity. In addition, we found that

interferon did not significantly enhance the binding of this tumor to macrophage monolayers. Therefore, we concluded that direct macrophage-tumor cell interaction is not likely to be responsible for the enhanced elimination of the interferon treated LY-F9 cells, although we cannot rule out macrophage participation where its activity is mediated through NAb.

Another interferon effect that we considered in our experimental design was accelerated elimination consequent to interferon induced growth inhibition. Under the exact conditions of interferon incubation used in the in vivo experiments, interferon growth inhibited the LY-F9 cells significantly at both 100 and 4,000 units/ml, whereas only the higher concentration significantly enhanced the in vivo elimination rate. To establish to what degree growth inhibition affected tumor elimination in this natural resistance assay, we examined the elimination rate of tumor cells treated with mitomycin C. These cells were eliminated significantly faster than were untreated controls at all cell doses. These results were quite unlike those of the interferon experiments, in which significant acceleration was noted only with $10^6$ to $5x10^6$ cells, whereas larger or smaller inocula were not eliminated more rapidly than were the controls. This suggested that the dose-response relationship of interferon treated cells was not the result of the growth-inhibiting effects of interferon.

In recent work both from this laboratory (51) and other laboratories (40), experiments have demonstrated that in vivo natural resistance to $NK^S$ tumors can be suppressed by pretreatment of

interferon and that this suppression corresponds to the loss of in vitro NK reactivity. It was our experience however, that this suppressed elimination was difficult to demonstrate intraperitoneally unless we used a highly interferon sensitive tumor and a host with high NK activity, such as the SL2-5 lymphoma, and young (5 week old) or poly I:C activated CBA/J mice. It has been shown by various groups that NK activity in the mouse peaks at 4-6 weeks of age, and declines as the mouse ages (52). Welsh et.al. (40) also found that loss of NK reactivity due to interferon pretreatment did not consistenly slow the elimination of radiolabelled tumor. If NK cells were the only effector mechanism responsible for the tumor elimination, then reduction of the NK sensitivity of the tumor should produce consistent abrogation of in vivo resistance. Since, as we demonstrated, interferon pretreatment promotes NAb acquisition, this could result in enhanced host resitance to the tumor via an NAb-mediated mechanism at the same time as NK cell lysis is suppressed. This suggests that host elimination of NKS tumors, which are also NAbS (6,16), will be paradoxically affected by interferon modulation of the membrane moieties responsible for these interactions, and that enhancement or suppression of host resistance will be observed, depending on the dominant mechanism operative in the particular host-tumor system under investigation. This hypothesis may also offer an additional explanation of why interferon has beneficial effects on the treatment of some malignant diseases but not on others (53).

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# CHAPTER 2

## THYMOCYTE REACTIVE NATURAL ANTI-TUMOR ANTIBODIES: STUDIES WITH MONOCLONAL NATURAL ANTIBODIES, AND A PHENYL-β-GALACTOSIDE REACTIVE MONOCLONAL ANTIBODY.

ABSTRACT

We have established murine hybridoma cell lines secreting monoclonal natural antibodies (mNAb) from LPS stimulated spleen cells and peritoneal cells. These were found to be predominantly thymocyte reactive, confirming previous serum NAb studies. One mNAb, PEC 3, however, reacted with a T cell lymphoma, but not with thymocytes. In addition, we examined the tumor and thymocyte reactivity of a monoclonal antibody (49H.8 mAb), specific for phenyl-β-galactoside (Ø-β-gal), which also crossreacts with the terminal disaccharide of the AGM1 molecule, (β-gal(1 3)β-GalNac. It binds to tumor cells of T cell origin, as well as to 65% of adult thymocytes, 12-15% of spleen cells and 8-12% of bone marrow cells. Like natural thymocytotoxic antibodies (NTA) it does not differentiate between Thy 1.1+ and Thy 1.2+ thymocytes. Binding to a panel of Thy 1+ and Thy 1- mutant T cell lymphomas did not correlate with the expression of the Thy 1 glycoprotein on the cell surface. The 49H.8 reactive antigen is a thymocyte differentiation marker, in that it is expressed to a higher degree on adult rather than fetal cells, but is absent on peripheal T cells. The 49H.8+ thymocyte population is found within the PNA+ population, but it is unlikely that these two reagents are detecting the same determinant. This conclusion is based on the finding that no blocking was observed when thymocytes were incubated with the two reagents simulataneously, and the difference found in the ontogeny of the appearance of PNA and 49H.8 reactivity.

INTRODUCTION

Our approach up to this point to the study of natural antibody (NAb) and tumor reactivity had been to look at serum or intraperitoneal NAb populations and analysing these against tumors either selected or treated for differences in NAb reactivity. Although a certain amount of information may be gained from this approach, in that absorption studies allow the differentiation of various NAb subpopulations, it has its limitations, especially in cases where the serum NAb activity is low, as for example, in the DBA/2-L5178Y-F9 combination (1). We therefore decided to establish hybridoma lines secreting monoclonal NAb (mNAb), a system which would provide the following advantages: 1) unlimited quantities of NAb specificities, some of which may have been represented infrequently in serum; 2) the possibility of analysing specificities in more detail; 3) the possiblitiy of using these mNAb in passive immunization experiments for analysis of tumor resistance. For the establishment of these hybridoma lines, we fused LPS stimulated spleen and peritoneal cells (PEC) from unimmunized mice with a non-secreting myeloma. Since we started these experiments, several other laboratories have used this approach to the study of NAb (2-10). These studies have included tumor reactive NAb (8), as well as NAb reactive with serum proteins (2-4). Colnaghi et. al.(8) obtained four monoclonal secreting hybridomas from C57BL/6 mice which reacted with the EL4 lymphoma of the same strain. They found that

their mNAb crossreacted with viral, fetal, or cell structure components. The laboratory of Avrameas et. al. has raised mNAb in several studies, using serum proteins as screening reagents. They were able to obtain mNAb secreting hybridomas from normal, adult mice, as well as neonatal mice (3,9). Others (10) have also been successful in obtaining self-protein reactive mNAb's from mice raised under germ-free conditions. Czerny-Provaznick et.al. (5) had been able to detect anti-MHC reactive NAb in serum of some mice, and confirmed these findings by the production of anti-MHC reactive mNAb (6,7). Most of these mNAb were reactive with public allogeneic specifities, but one mNAb from a BALB/by mouse (H-$2^d$) reacted with H-$2K^d$ determinants. Similarly, Longenecker et. al. confirmed earlier serum NAb finding by producing mNAb that were reactive with polymorphic chicken MHC determinants (11). These studies have established that it is possible to obtain mNAb with restricted specificity, although many are corssreactive with a wide variety of antigens.

A population of NAb's we were interested in were the natural thymocytotoxic antibodies (NTA). The presence of thymocyte reactive NAb is a very common occurrence in most strains of mice, including auto-immune and normal strains (12-14). Contrary to earlier suggestions, these antibodies do not appear to be responsible for some of the T cell abnormalities seen in autoimmune strains of mice (14,15).

Not much information is available regarding the nature of the NTA reactive antigenic determinant(s). Although serum from most strains

of mice display high thymocyte reactivity, the reactivity against spleen cells is usually low, unless these have been neuraminidase treated (16-19). This finding, together with other data indicating that natural antibodies recognize carbohydrate specificities (20-25), would suggest that the NTA population is also carbohydrate reactive. In an examination of the specificity of NTA in sera from NZB mice, Imai et.al. (26) found that the NTA could be divided into two types: those reactive with all thymocytes, and those reactive only with neuraminidase treated thymocytes and lymphocytes. This latter population could be inhibited by several sugars, including melibiose, lactose and phenyl-β-galactoside (Ø-β-gal). Another conclusion was reached by Parker et.al. (27) examining cell membrane binding of NTA. On the basis of co-capping of the antibodies, he hypothesized that NTA react with the Thy-1 glycoprotein or a closely related molecule.

NTA also display anti-brain activity (14). Brain tissue and thymocytes share antigenic markers, including Thy 1 (28,29), and AGM1 (30), although, in the case of the Thy 1, there are glycosylation differences between the thymocyte and brain forms (28), and the AGM1 is only a fetal thymocyte antigen (30). The antigen(s) responsible for the NTA thymocyte/brain crossreactivity have not been identified.

Studies carried out in our laboratory have demonstrated that a subpopulation of tumor reactive NAb of DBA/2 mice, a non-autoimmune strain of mice, could be absorbed with thymocytes (Appendix 1). To date there is no information as to what the NAb reactive common

adult thymocyte/tumor antigenic determinant may be. However, we did find that the tumor reactivity with NTA correlated with expression of the Thy 1 glycoprotein.

Because of our interest in tumor reactive NTA, we screened the fusion supernatants against tumor cells as well as thymocytes. Our results from these studies confirm our finding of serum NAb analysis that a high frequency of tumor reactive NAb are also thymocyte reactive. However, we were able to obtain one mNAb which reacted with a T cell lymphoma, but not with thymocytes.

In addition to the mNAb's obtained by this procedure, we analyzed another monoclonal antibody (mAb) made available to us. This mAb, 49H.8, was raised from a BALB/c mouse immunized with human neuraminidase treated red blood cells (31), and therefore cannot be classified as a true "natural antibody". It does, however, resemble NTA in several aspects: 1) It is specific for phenyl-β-galactoside, which is one of the NTA specificities identified by Imai et.al. (26); 2) It binds to neuraminidase treated lymphocytes and several murine tumors; in contrast, it did not bind to peripheral or ConA activated T cells (31); 3) It is an IgM antibody. In addition, it does crossreact with the terminal disaccaride of the AGM1 ganglioside, βGal (1 3)βGalNAc (31). We decided therefore to analyze its tumor reactivity in more detail by comparing the binding of the 49H.8 mAb to their NAb and complement sensitivity. We also describe 49H.8 thymocyte reactivity, in terms of its strain distribution, ontogeny and relation to PNA binding. In addition, we have addressed the question of the relationship of the 49H.8 reactive antigen(s) to the

Thy 1 glycoprotein by Flow Cytometry analysis of Thy 1+ and Thy 1- mutants.

## MATERIALS AND METHODS

### Mice and Tumors

BALB/c, DBA/2 mice and Sprague-Dawley rats were obtained from the University of Manitoba Vivarium. CBA/J and AKR mice were supplied by Jackson Laboratories, Bar-Harbor, Maine.

For fusion, three different myeloma lines were used: the MOPC315 obtained from Dr. B. M. Longenecker, Department of Immunology, University of Alberta, the NS-1 obtained from Dr. E. Rector, Department of Immunology, University of Manitoba, and the FOX-NY (32) obtained from HYCLONE, California. The BW5147 and S49 tumor lines were received from Dr. R. Hyman, Salk Institute, La Jolla (33). All other lines have been previously described (Chapter 1). Tumor lines were maintained by passaging in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), at concentrations between 5 x 104 and 6 x 105 cells/ml. We routinly screened all tumor lines for mycoplasma infection and have found them to be consistently mycoplasma free.

### Establishment of NAb secreting hybridomas

Spleens were removed asceptically from normal, non-immunized mice, and teased into single cell suspension. They were stimulated

with LPS (Sigma, Serotype 126:B6) at a concentration of 2 mg/ml, 106 cells/ml, for 48 hours, in RPMI media, supplemented with 10% fetal bovine serum (FBS), at 37°C and 7.5% $CO_2$. After two washes in serum free RPMI medium, they were mixed with either the MOPC315 or NS-1 myeloma cells at a 5:1 (spleen cell:myeloma) ratio. Fusion was carried out with a 40% polyethyleneglycol (PEG) solution as described previously (31). The cells were then plated in 24 well Linbro trays, containing fresh mouse red blood cells as feeder layers (107/ml), at a concentration of 1 x 106 spleen cells/ml. Selective media (HAT) was used from the onset, with the following drug concentrations: hypoxanthine, 100μM; methotrexate, 0.5μm (for fusions using the MOPC315); aminopterin, 0.4 μM (for fusions using the NS-1); and thymidine, 30μM. Ouabain (1.0 mM) was included in fusions using the MOPC315 myeloma, since this cell line was Oubain resistant.

PEC's were obtained by peritoneal lavage with 5 ml of sterile 11.6% glucose solution (34). These were stimulated with LPS as described for the spleen cell fusions. The fusion partner for this fusion was the FOX-NY cell line, which is APRT- as well as HPRT-. The fusions were performed as described by Oi and Herzenberg (35). The cells were then plated into 96 well flat bottom tissue culture plates at a concentration of 106 spleen cells/ml. Peritoneal macrophages were used as feeder layers for these cultures, and had been plated out in 100μl cultures 24 hours previously, at a concentration of 3 x 104 cells/ml. Selective media (AAT) was used from the beginning of the cultures, with drugs at the following

concentrations: adenine, 80μM; thymidine, 15μM; and aminopterin, 0.4μM.

Cultures were fed with fresh selective media on days 7 and 10 (day 0= fusion date).

Screening of supernatants

The Enzyme Immunoassay Kit available from Kierkegaard and Perry Laboratories (KPL, Gaithersburg, Maryland) was used for all screening. Thymocytes and tumor cells were fixed to flat bottom tissue culture plates as follows: The plates were incubated with 100μl of a poly-L-lysine/PBS solution (50 μg/ml) overnight at 4°C. After two washes with PBS, they were incubated with 0.1% gluteraldahyde in 0.1M $NaHCO_3$, for two hours at 37°C. The plates were washed twice with PBS before adding thymocytes ($10^6$ per well) or tumor cells ($10^5$ per well) in 100μl PBS containing 2% FBS. After centrifuging for 5 minutes at 400 x g, the plates were incubated for 1 hour at 37°C. The supernatants were flicked off, and replaced with a 0.05% gluteraldahyde/PBS solution for 5 minutes at room temperature. This solution was flicked off, the plates washed twice in PBS, and 200μl of PBS containing 10% FBS was added to each well, to block non-specific binding, for an overnight incubation at 4°C. Supernatants from hybridoma cultures were added, and screened following the procedure as outlined in the KPL manual. OD's were read with a Titertek Elisa plate reader.

Thymocytes, bone marrow and spleen cells

Thymocytes and spleen cells were obtained by removing thymus glands and spleens from mice or rats and passing them through a nylon mesh to obtain single cell suspensions in Hanks' Balanced Salt Solution (HBSS). These procedures were carried out at 4°C. Bone marrow cells were obtained by flushing the femur and tibia, using a 26 g needle and HBSS. When present, erythrocytes were removed by hypotonic lysis. All cells were washed three time prior to labelling experiments.

Natural antibody (NAb) sensitivity determination

This was measured using the NAb and complement assay previously described (Chapter 1).

Cell Surface Binding Studies

For radiolabelled antibody binding, the 49H.8 mAb was labelled with $^{125}I$ as previously described (31). Tumor cells were incubated with appropriate dilutions of $^{125}I$-49H.8 mAb in HBSS for one hour on ice, in triplicate, then washed three times with cold HBSS and counted in an LKB gamma counter. For Flow Cytometry, the 49H.8 mAb was bound to fluorescent latex beads (Polysciences, Warrington, PA), as described by Kieran and Longenecker (36). For our studies, 200 µg of mAb in 1 ml of PBS was bound to 100 µl of fluorescent latex beads. These were resuspended after washing to a final volume of 2 ml PBS (2% FCS, 0.1% $NaN_3$). PNA was bound at the same protein concentrations to red Covaspheres (Covalent Technology Corporation,

Ann Arbor, Michigan). These preparations were found to be stable at 4°C for several months. All preparations were sonicated before each experiment to ensure single bead suspension. For binding studies, 100µl of beads were mixed with either 100 µl of HBSS or 100µl of a 100mM Ø-β-gal solution and incubated at room temperature for 30 minutes in 96 well flat bottom plates. Cells (2 x $10^6$ in 50 µl of HBSS) were added to each well, the plates centrifuged at 400 x g for 20 minutes in a refrigerated centrifuge, then incubated on ice for a further 45 minutes. To remove cells from free beads, cells were resuspended, layered over 1 ml of FBS and centrifuged at 75 x g for 5 minutes. The pellet was then resuspended in 1 ml of PBS/2% FCS with 0.1% $NaN_3$ and analyzed on an EPICS IV Coulter Flow Cytometer. In all cases 20,000 cells were analyzed. Any free beads or nonviable cells were gated out on a two parameter (wide angle light scatter, 90° light scatter) setting.

For double labelling studies, 50 µl of 49H.8- and PNA-latex beads were incubated with 50 µl each of Ø-β-gal (100 mM) and D-galactose (400mM) before the addition of 2 x 106 cells. We compared the amount of thymocyte labelling when 50 µl rather than 100µl of 49H.8-latex beads were used, and established that there was no difference in the amount of labelling.

Further PNA binding experiments were performed with FITC conjugated PNA (Sigma). 2 x 106 thymocytes were incubated for 30 minutes with 30µg/ml FITC-PNA in HBSS containing 0.1% sodium azide at 4°C. Flow cytometric analysis was carried out immediately, without washing the cells.

# RESULTS

## Thymocyte/Tumor Reactive mNAb

Initial screening of fusions from BALB/c, CBA/J, and DBA/2 LPS stimulated spleen cells indicated that the majority of tumor reactive supernatants were also thymocyte reactive. The initial screenings were carried out against thymocytes, the SL2-5 lymphoma (which displays a high density of "thymocyte antigen" (TA) as determined by serum absorptions, Appendix 1), and the LY-F9, (which is not capable of absorbing thymocytotoxic antibodies from normal serum, and against which normal serum NAb activity is low). Since the supernatants used for initial screening were produced in 24 well Linbro plates and each well contained 5-15 colonies, we could not rule out the possibility that the production of several mNAb specificites in each well were responsible for the observed crossreactivity. All colonies from positive well were individually picked, transferred to fresh 24 well Linbro trays, expanded, and then cloned by limiting dilution. Supernatants were screened several times during this procedure, on plates coated with cells, as well as plates treated in the same manner as described in MATERIALS AND METHODS, with the exception that no cells were added. These provided a control for the detection of cellular rather than fetal bovine serum components. Table 1 is a summary of several mNAb derived from cloned hybridomas. In Table 1A we show that all of the tumor reactive mNAb were also thymocyte reactive. The thymocytes used in these screenings were syngeneic to the strain of the spleen cell donors. When we tested some of the

Table 1

Reactivity of mNAb with Thymocytes and Tumors

| mNAB | Strain of origin | OD(-Background) ± S.E. | | |
|---|---|---|---|---|
| A) | | Thymocytes[1] | SL2-5 | LY-F9 |
| 20N.2 | CBA/J | 0.550±0.06 | 0.340±0.05 | 0.420±0.06 |
| 20N.4 | CBA/J | 0.520±0.03 | 0.430±0.08 | 0.416±0.07 |
| 40N.1 | CBA/J | 0.600±0.01 | 0.030±0.01 | 0.061±0.02 |
| 40N.2 | CBA/J | 0.755±0.07 | 0.032±0.02 | 0.095±0.01 |
| 40N.3 | CBA/J | 0.711±0.06 | 0.044±0.02 | 0.100±0.05 |
| 37N.1 | BALB/c | 0.560±0.04 | 0.112±0.02 | 0.200±0.05 |
| 37N.4 | BALB/c | 0.412±0.06 | 0.125±0.05 | 0.075±0.05 |
| 37N.5 | BALB/c | 0.870±0.06 | 0.560±0.06 | 0.370±0.03 |
| | | | Thymocytes | |
| B) | | CBA/J ($H\text{-}2^k$) | C57B1/6($H2^b$) | BA/2($H\text{-}2^d$) |
| 10M.1 | CBA/J | 0.157±0.01 | 0.045±0.07 | 0.125±0.01 |
| 10M.2 | CBA/J | 0.155±0.02 | 0.252±0.01 | 0.155±0.01 |
| 10M.3 | CBA/J | 0.262±0.02 | 0.216±0.02 | 0.168±0.01 |
| 10M.4 | CBA/J | 0.194±0.01 | 0.214±0.02 | 0.156±0.02 |

Table 1 (continued)

| mNAb | Strain of origin | OD(-Background) ± S.E. | |
|---|---|---|---|
| C) | | Thymocytes (H-$2^d$) | LY-F9 |
| PEC.1 | BALB/c | 0.152±0.01 | 0.133±0.02 |
| PEC.2 | BALB/c | 0.242±0.03 | 0.145±0.02 |
| PEC.3 | BALB/c | 0.044±0.01 | 0.362±0.04 |

[1]Thymocytes were syngeneic to strain used for fusion

mNAb's against thymocytes of different strains we found that most of them reacted with thymocytes regardless of the strain of origin (Table 1B), confirming previous reports of serum NTA (12,27).

Non-Thymocyte Reactive mNAb

Our results described above indicate that all the mNAb we had obtained by using LPS stimulated spleen cells as fusion donors reacted with thymocytes, which confirmed the high prevalance of NTA in many mouse strains (12-14). However, in previous studies, our laboratory had demonstrated the presence of what appeared to be MHC antigen reactive NAb in the serum of normal mice. This was detected by complement dependent lysis of the LY-F9 lymphoma, a tumor which does not bear the thymocyte antigen(s) (1, Appendix 1). We were therefore surprised at the lack of this specificity in our spleen cell fusion products. Since it had also been demonstrated that the peritoneum contained a substantial number of LY-F9 reactive NAb (Chapter 1), we decided to examine peritoneal B cell derived mNAb. We were able to obtain mNAb from one experiment where the peritoneal cells of 5 normal, 8 week old BALB/c mice were pooled, LPS stimulated, and fused with the FOX-NY myeloma. These were plated directly into 96 well plates rather than the previously used 24 well Linbro trays. Hybrid colonies were detected in approximately 25% of the wells, thus we could be reasonably confident, on a statistical basis that even during initial screening, we were exmaning monoclonal specificities (37). From this one fusion, (in which a total of 2 x $10^7$ peritoneal cells were fused, the equivalent of 1/5 of a spleen

cell fusion) we obtained three mNAb producing hybridomas. Two of these, PEC1.1 and PEC1.2 were thymocyte reactive, whereas the third, PEC1.3 reacted only with the LY-F9 tumor, and not thymocytes (Table 1). These clones were stored in liquid nitrogen for future studies.

Binding of 49H.8 mAb to tumor cells

We had shown previously that the 49H.8 mAb binds to various murine tumors, and that the amount of $^{125}$I-49H.8 bound varied within the lines tested (31). When the ability of the tumor lines to bind $^{125}$I-49H.8 mAb was compared to their sensitivity to NAb and complement lysis, a significant correlation was detected ($r=0.97$, $p<.002$). These results are illustrated in Figure 1. In a previous study, we had determined that the sensitivity of tumors to NAb and complement correlated with their susceptibility to lysis by anti-Thy 1 antisera and complement. In addition, Thy 1$^+$ tumors were more efficient at absorbing thymocytotoxic antibodies from normal and LPS stimulated DBA/2 serum (Appendix 1). Therefore, the 49H.8 antigen(s) appear(s) to be expressed to a higher degree on TA$^+$ tumors. Because of this correlation, and because it had previously been reported that Ø-β-gal is one of the specificities represented in the NTA population of NZB mice (26), we next examined 49H.8 mAb binding to thymocytes. As shown in Table 2, $^{125}$I-labelled 49H.8 mAb did bind to thymocytes, and this binding was inhibitable by the sugar Ø-β-gal, but not by Ø-β-gluc or Ø-α-gal. The 49H.8/thymocyte reactivity was characterized further using Flow Cytometry analysis.

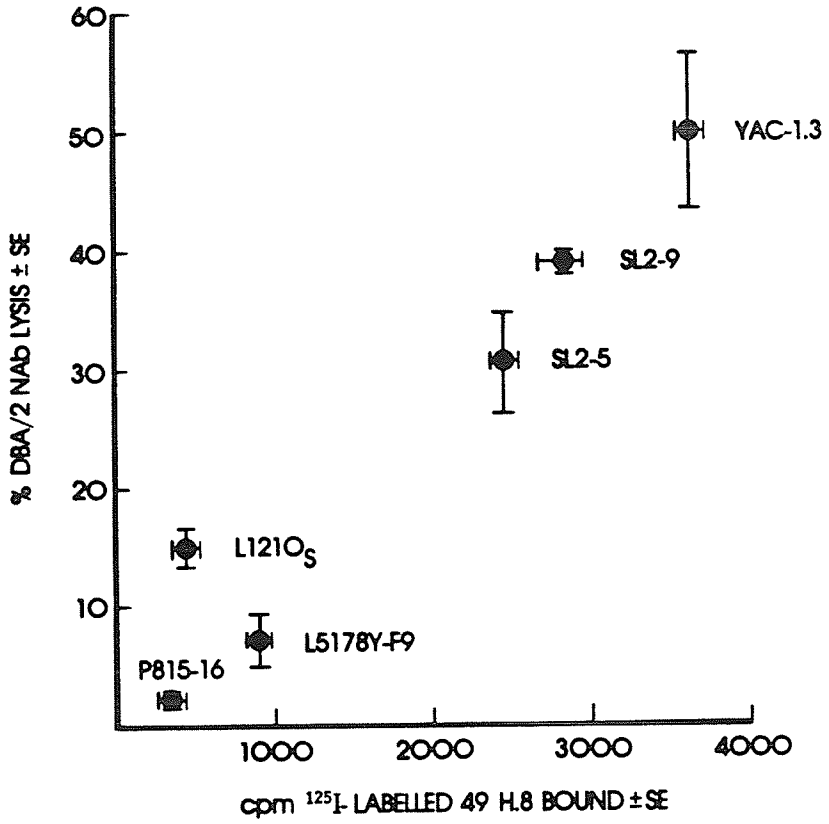


Figure 1: Correlation between DBA/2 NAb lysis of murine tumors and binding of 49H.8 mAb.

Table 2

Sugar Inhibition of Binding of $^{125}$I-49H.8 to Tumor Cells and Thymocytes

| | | cpm bound ± SD | |
|---|---|---|---|
| mAb | Sugar | SL2-5 | Thymocytes |
| 49H.8 | - | 2,371±2[illegible] | 12,484± 597 |
| 49H.8 | Ø-β-gal | 1,147± 68 | 1,887± 360 |
| 49H.8 | Ø-β-gluc | 2,619±110 | 12,309±3,463 |
| 49H.8 | Ø-α-gal | 2,218± 57 | 11,715±1,508 |

Reactivity of 49H.8 mAb with normal thymocytes, bone marrow and spleen cells

Figure 2 is a representative experiment, showing that 49H.8 mAb labelled fluorescent latex beads react with normal BALB/c thymocytes, to a much higher degree than either spleen cells and bone marrow cells, both in terms of intensity and percent positive cells. The amount of label bound in the presence of the inhibiting sugar, Ø-β-gal was always included as a non-specific control, and was found to be low or negligible in all cases. The number of positive cells in each cell population was calculated by integrating the number of positive cells, channel by channel, in the presence and absence of the inhibitor, Ø-β-gal (Table 3). The closely related, but not inhibitory sugar Ø-α-gal (31) did not affect the antibody binding to any of these cell types (not shown).

Rat and mouse thymocytes share several antigens, including Thy 1 determinants (38). When 49H.8 mAb binding to rat thymocytes was tested, we found that the expression of this antigen(s) is virtually identical on murine and rat thymocyte populations (Figure 3). In Chapter 3, we show that the antigen is also common to both murine and rat NK cells.

In order to determine whether the 49H.8 marker is related to the Thy 1 alloantigenic determinants, we compared binding of 49H.8-fluorescent latex beads to thymocytes from AKR (Thy-1.1) and BALB/c (Thy-1.2) mice. As shown in Figure 4, thymocytes of both

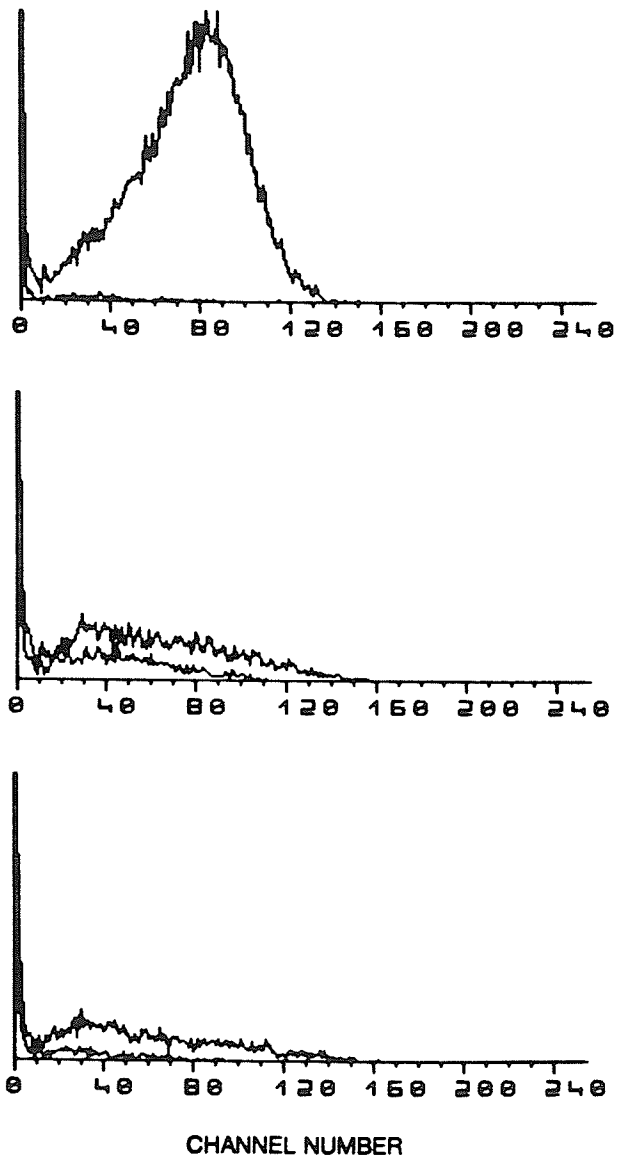


Figure 2: 49H.8-latex bead binding to BALB/c thymocytes (upper), spleen cells (middle) and bone marrow cells (lower). Bottom line in each histogram represents binding in the presence of Ø-β-gal. Vertical scale represents 250 cells.

Table 3

Tissue Distribution of 49H.8+ Cells

| | % of Total Cells | | |
|---|---|---|---|
| Cell Type | 49H.8 | 49H.8 + Ø-β-gal | Corrected Values |
| Thymocytes | 66.8 | 0.9 | 65.8 |
| Spleen cells | 19.0 | 7.1 | 12.4 |
| Bone Marrow cells | 12.5 | 2.0 | 10.3 |

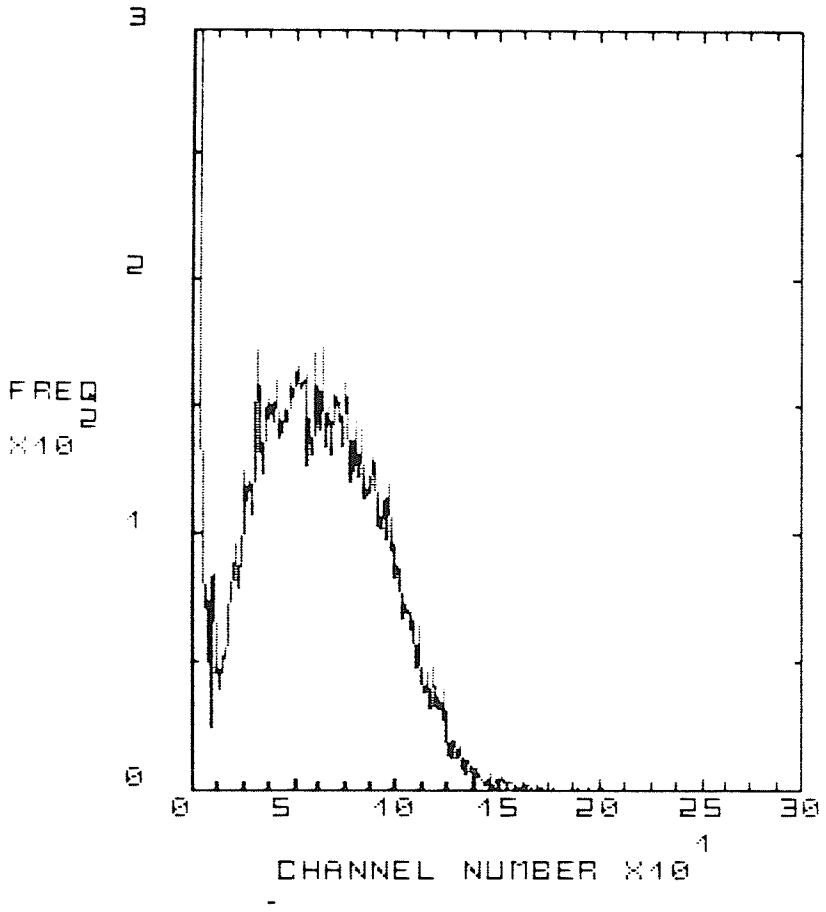


Figure 3: 49H.8-latex bead binding to rat thymocytes. Binding in the presence of Ø-β-gal was negligible (not shown).

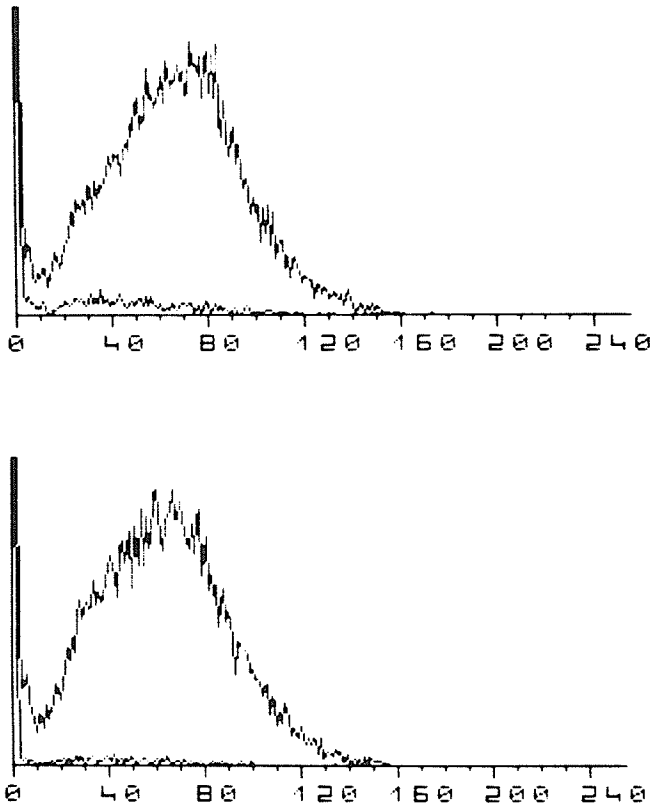


Figure 4: 49H.8-latex bead binding to BALB/c thymocytes (upper) and AKR thymocytes (lower). Lower line in each histogram represents binding in the presence of Ø-β-gal. Vertical scale: 250 cells.

strains bound identical amounts of this antibody. NTA also do not distinguish between these alloantigenic determinants, although there is some evidence that they may be recognizing a determinant on the Thy-1 or closely related molecule (27).

Reactivity of 49H.8 mAb with Thy 1- mutant cell lines

Previous studies indicated that the 49H.8 mAb reactive antigen(s) could be found in higher amounts on tumors that were Thy-1$^+$ as opposed to Thy 1$^-$ tumors (Figure 1). In order to examine the relationsip between the Thy 1 glycoprotein and the 49H.8 antigen(s) more closely, we compared binding of 49H.8-latex beads to a series of Thy 1$^-$ mutant T cell lymphomas to their parental Thy-1$^+$ lines. We obtained three BW5147 and two S49 lines from Dr. R. Hyman: the BW 5147 WT (Thy 1$^+$), BW 5147 A, a Class A mutant (Thy 1$^-$) and BW 5147 E, a Class E mutant (Thy 1$^-$) are derived from the AKR strain; the S49 WT (Thy 1$^+$) and S49 A, a Class A mutant (Thy 1$^-$) are derived from the BALB/c strain. The results of this experiment are illustrated in Figure 5. We found no correlation between the expression of the Thy 1 glycoprotein on the cell surface and the amount of 49H.8 binding. In fact, the Thy 1$^-$ BW5147 Class E mutant bound significantly higher amounts of 49H.8, than the WT or Class A mutant.

Ontogeny of the 49H.8 antigen(s) on murine thymocytes

Because the 49H.8 mAb crossreacts with AGM1, a fetal thymocyte marker (30), we tested its reactivity against thymocytes of different

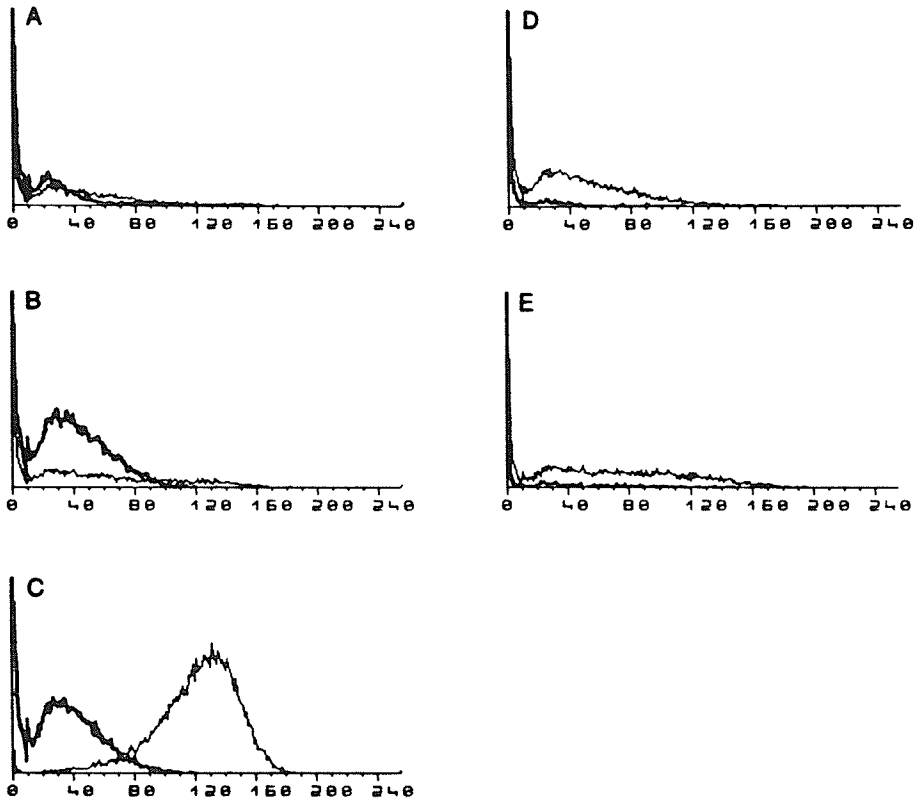


Figure 5: 49H.8-latex bead binding to BW 5147 WT (A), BW 5147 A (B), BW 5147 E (C), S49 WT (D), and S49 A (E). Bold line in each histogram represents binding in the presence of Ø-β-gal. Vertical scale: 550 cells.

ages. Our results, illustrated in Figure 6 indicate that it is clearly an adult thymocyte marker. The expression of the 49H.8 antigen(s) was lowest on fetal thymocytes, and increased as the age of the thymus donors increased, comparing newborn (1-3 day old mice), 2 week old and 5 week old mice. We also examined thymocytes from 1 week old and 3 week old mice, and found that the expression of the 49H.8 antigen(s) on these was intermediate to the levels for thymocytes from newborn, 2 week old and 5 week old donors. The fetal thymocytes used for this study were pooled from 15 to 20 day gestation fetuses, thus we have not investigated the possibility that this antigen is expressed in different amounts on thymocytes of increasing gestational ages. The histograms in Figure 6 represent binding of 49H.8-fluorescent latex beads without subtraction of non-specific binding measured in the presence of Ø-β-gal, but these background values were negligible in all cases, as shown in representative cases in Figure 7.

Comparison of 49H.8 and PNA binding

Approximately 85% of adult thymocytes express high PNA reactivity ($PNA^h$). These are the cortical (generally considered to be immature) thymocytes, whereas the medullary thymocytes (generally considered to be more mature) are low PNA binding ($PNA^l$) (39). On the basis of the structures recognized by both the 49H.8 mAb and PNA (ie. penultimate galactose residues), we expected to find a major overlap in the $49H.8^+$ and $PNA^h$ populations. We tested whether the 49H.8 positive thymocytes were among the $PNA^h$ population exclusively or whether some

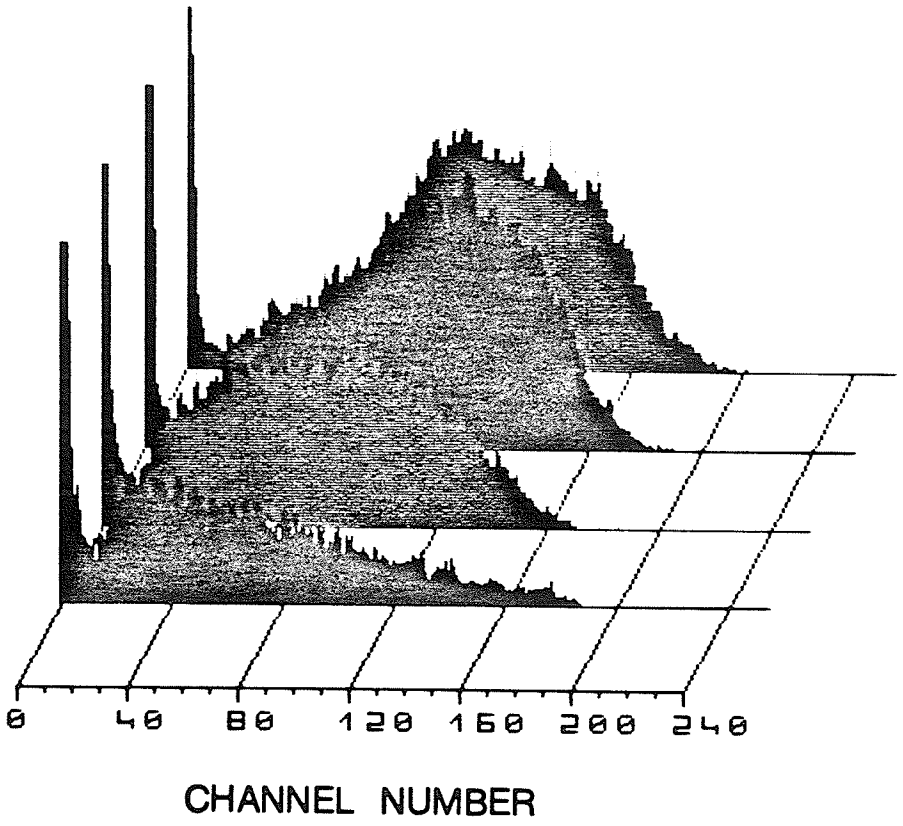


Figure 6: 49H.8-latex bead binding to thymocytes from BALB/c mice of different ages. From back to front: 5 week old, 2 week old, newborn (1-3 day old), and fetal. Vertical scale: 250 cells.

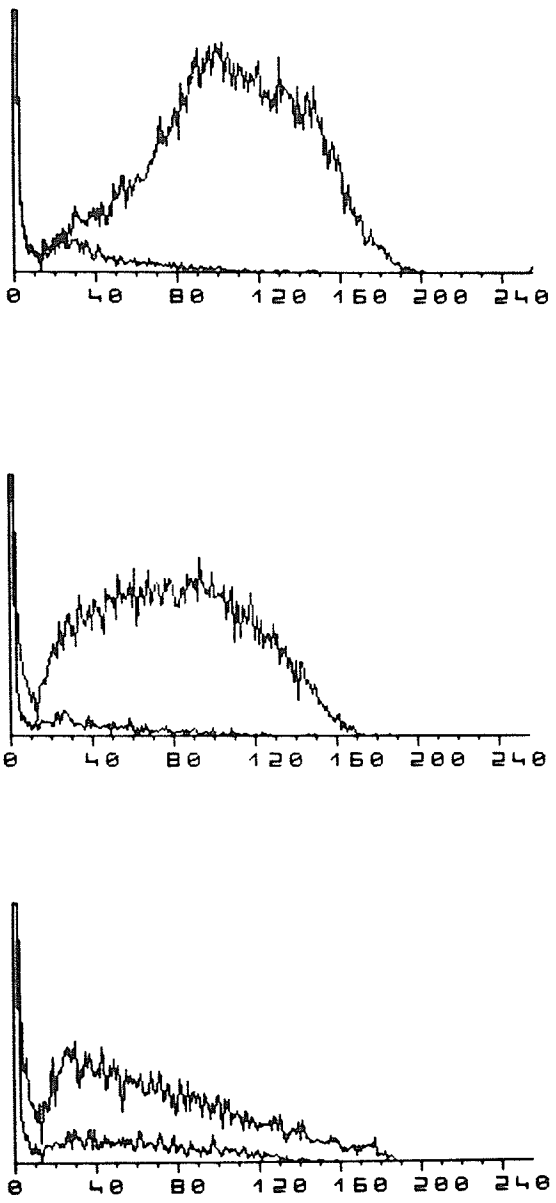


Figure 7: 49H.8-latex bead binding in the presence (lower line) and absence (upper line) of Ø-β-gal to thymocytes from BALB/c mice of different ages. Upper: 5 week old; middle: newborn; lower: fetal. Vertical scale: 250 cells.

of the 49H.8 positive cells were also to be found among the $PNA^l$ populations. Figure 8 represents a double labelling study, using PNA on red beads and the 49H.8 mAb on green latex beads. As is evident from the figure, the 49H.8$^+$ thymocytes were found primarily within the $PNA^h$ population, but there are some $PNA^h$ 49H.8$^-$ thymocytes. Since it was possible that the 49H.8 mAb and PNA were binding to the same cell surface molecule, we compared the amount of binding of 49H.8- and PNA-latex beads to thymocytes in where these two reagents had been mixed, to the amount of binding when the two reagents had been used separately. As shown in Figure 9, these binding curves were identical.

Since the 49H.8 mAb reacts preferentially with adult thymocytes we also compared PNA binding to fetal, neonatal, and adult thymocytes (Figure 10). In contrast to the 49H.8 mAb, PNA reacted equally well with newborn and adult thymocytes, and although the percentage of labelled fetal thymocytes was lower, the intensity of labelling on fetal PNA$^+$ thymocytes was not lower than on the adult cells.

## DISCUSSION

We have established several hybridoma lines secreting mNAb's from LPS stimulated spleen and peritoneal cells. We have confirmed our previous serum NAb findings that a large proportion of tumor reactive NAb are in fact NTA. In addition, we were able to obtain one hybridoma line secreting tumor reactive NAb that was not

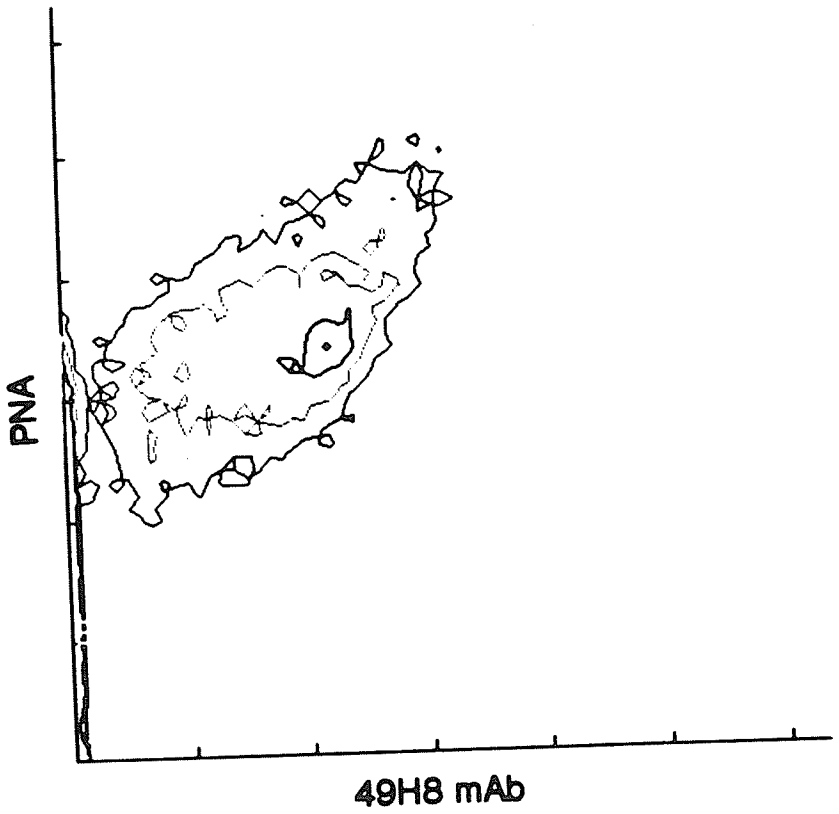


Figure 8: Two color analysis of thymocyte reactivity with 49H.8 (green latex beads) and PNA (red latex beads).

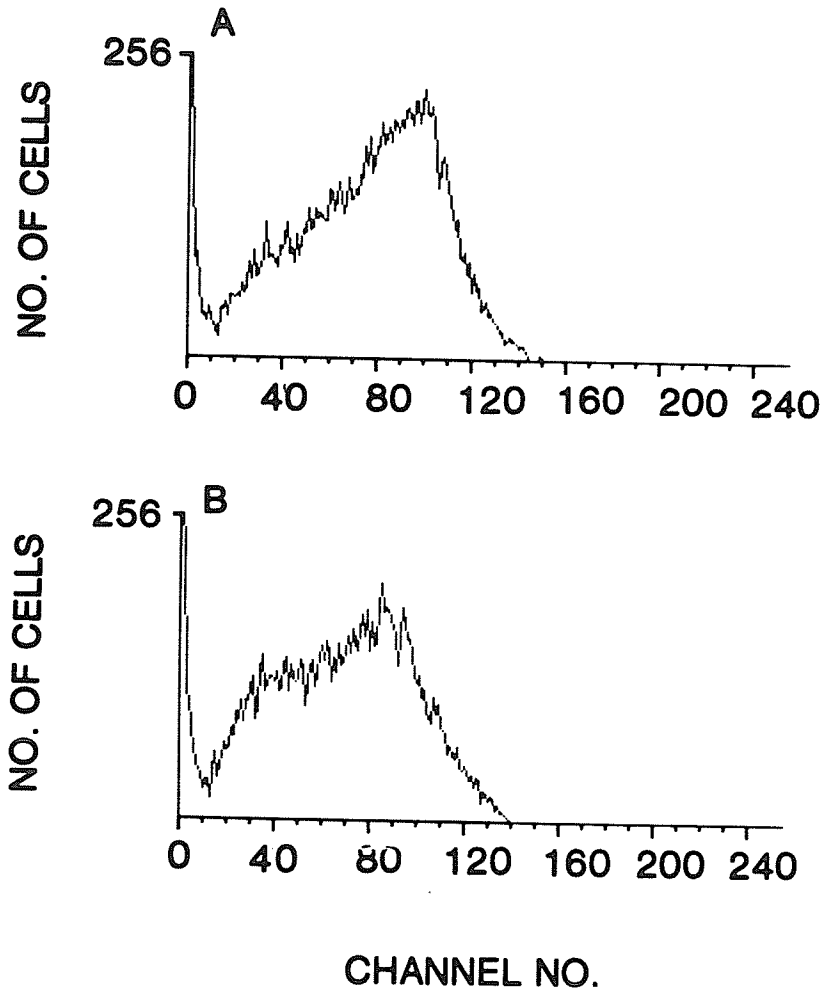


Figure 9: Binding of 49H.8 latex beads to thymocytes (green fluorescence analysis only). A) 49H.8-latex beads only. B) 49H.8 latex beads mixed with PNA-latex beads (red).

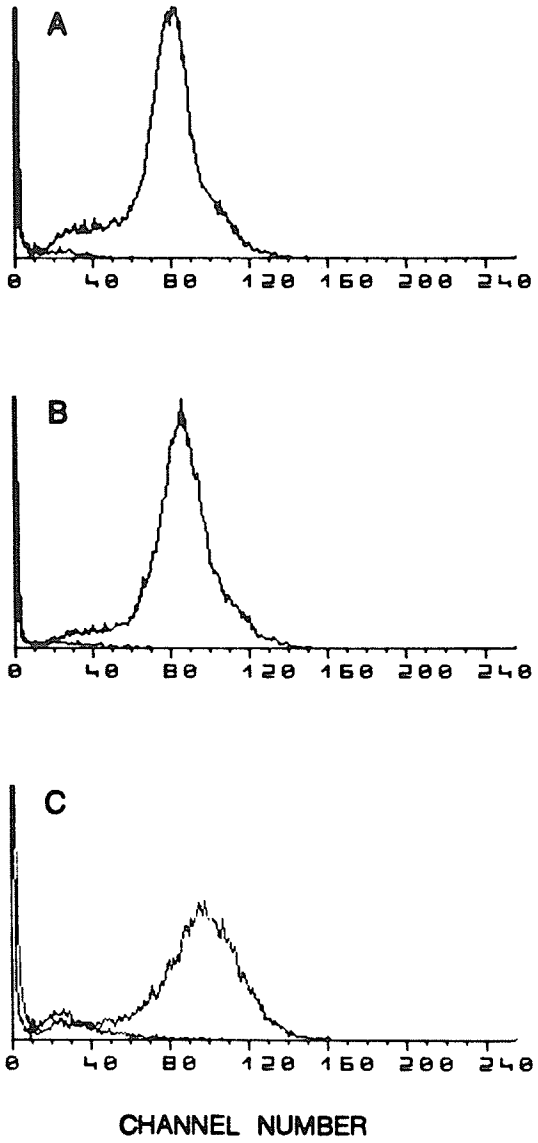


Figure 10: Ontogeny of thymocyte PNA reactivity. Thymocytes were obtained from 5 week old (A), newborn (B), or fetal (C) mice. Vertical scale: 250 cells. Lower lines in each histogram represent binding in the presence of 200mM D-Gal.

thymocyte reactive. This mNAb may be important in determining the NAb reactive determinant(s) on the TA- LY-F9 lymphoma.

We have also examined the thymocyte and tumor reactivity of a monoclonal antibody with a defined carbohydrate specifity. Our finding that the binding of 49H.8 mAb correlates with the tumor cells' sensitivity to NAb and complement further establishes the similarity of the 49H.8 mAb with serum NAb, discussed in the introduction.

In these studies, we have made the assumption that the mNAb we obtained were in fact representative of serum NAb populations. This assumption is supported by the study of Lymberi et. al. (40) which showed that anti-idiotypic antibodies, raised against mNAb, were reactive with serum NAb.

Others have not used LPS stimulation in their mNAb production procedure (3-7). We have included LPS in the production of our hybridomas in order to obtain B cell blasts, thus increasing the fusion frequency (11). LPS has also been used by several authors in the study of serum NAb (41-44). LPS may not be a true polyclonal B cell activator, in that it has been shown to activate B cells producing autoantibodies preferentially (44,45). Since we did not carry out frequency studies in which we looked at total number of immunoglobulin secreting hybrids compared with anti-tumor reactive antibodies, we cannot make any conclusions as to the preferential activation of tumor reactive NAb producing B cells in our system.

Although our results of this study are preliminary, in that we have not carried out any biochemical characterization of the antigens

reactive with the mNAb, they do indicate that these types of antibodies are easily obtained, and may be useful for differentiating various tumors, such as the TA+ SL2-5 and the TA- LY-F9.

We have used the 49H.8 mAb as a model of mNAb, and examined its reactivity against thymocytes further. The 49H.8 mAb is of interest in that it detects an adult rather than fetal thymocyte antigen(s). The antigen is also absent on peripheral T cells, but present on tumors of T cell origin (31). Several thymocyte differentiation antigens have been detected on tumors of T cell origin, but most of these are markers for fetal thymocytes. For example, the FT-1 marker, present on leukemia cells is recognized by a monoclonal antibody with binding specificity similar to the DBA lectin has been detected on 13 day fetal thymocytes, but is absent on adult thymocytes and peripheral T cells (46). Similarly, the YE1/7.1 antigen is present on a subpopulation of fetal as well as some mitogen activated adult thymocytes in addition to Thy-1 bearing lymphomas (47). Two other such antigens are the SC-1 (48) and the AGM1 (30) markers, the latter being expressed in an inverse relation to the Thy-1 glycoprotein. Like other thymocyte antigens, the 49H.8 is not thymocyte specific. It also binds to a smaller number of spleen cells as well as with approximately 10% of bone marrow cells. The Thy-1 glycoprotein, although commonly used as a T-lineage specific marker, is also not thymocyte specific. This antigen is also expressed on brain cells (28), and some bone marrow cells (49,50). In these cases, the Thy 1 marker does not delineate cells of the T cell lineage (49,50).

The correlation of 49H.8 and PNA positivity was predicted, because of their reactivity with penultimate galactose determinants on cell surface glycoproteins. Since these two reagents did not block each other's binding to the thymocytes cell surface, it seems unlikely that the 49H.8 antigen(s) and the PNA receptor(s) are identical. The fact that the 49H.8mAb reacted preferentially with adult rather than fetal or newborn thymocytes, in contrast to the results obtained with PNA, further supports this conclusion.

The biochemical basis for the Thy 1.1/Thy 1.2 difference is claimed to be a single amino acid change (51), but an alloantigenic difference has also been detected with carbohydrate reactive antibodies (52,53). Like NTA, the 49H.8 mAb does not distinguish between the Thy 1.1 and Thy 1.2 alloantigenic determinants. Although this means that it is not specific for the determinants responsible for Thy 1 alloantigenicity, it does not rule out the possibility that it may be reacting with other determinants on the Thy 1 glycoprotein. However, its strong reactivity with a Thy 1- mutant cell line does not support this conclusion. The BW5147 Class E mutant has been characterized further, and in contrast to the other Thy 1- mutants, it displays pleitropic changes in its glycosylation of cell surface glycoproteins (33,54). The Thy 1 molecule has both high mannose and complex oligosaccharide deficiencies, whereas other cell surface glycoproteins have only high mannose oligosaccharide deficiencies. In previous studies (55), using wild type and Con A resistant chinese hamster ovary (CHO) cell lines, we determined that the 49H.8 mAb bound in significantly higher amounts to the Con A

resistant clone, CR7. This line has been shown to have a defect in the glycosylation of N-linked olicogosaccharides, leading to lower mannose and higher fucose incorporation (56). There is also some indication that is has sialiation deficiencies, since it binds less Wheat Germ Agglutinin and more PNA than the wild type (55).

As described in Chapter 3, the 49H.8 antigen is also expressed on murine and rat NK cells, a property we originally thought was due to its crossreactivity with the terminal disaccharide of the AGM1 molecule. As we discuss in that report, we do not favor that interpretation, for the following reasons: 1) the difference in ontogeny of the 49H.8 and AGM1 markers on thymocytes, 2) the fact that binding of 49H.8 to spleen cells and thymocytes is not inhibitable by α-AGM1 antisera, 3) 49H.8 reacts significantly more with neuraminidase treated T and B cells, and AGM1 is not exposed by this treatment, and 4) 75 mAb's specific for the terminal disaccharide of AGM1, β-gal(1 3)β-galNac, were not able to deplete splenic NK activity when used in a complement depletion experiment.

The nature of the molecule(s) bearing the 49H.8 determinant(s) is currently being investigated. It could be an N-linked oligosaccharide on a glycoprotein, or a carbohydrate determinant on a glycolipid. Alternatively, in could be recognizing the same determinant being expressed on both types of membrane molecules. This is not an uncommon occurrence, as is the case with the human ABO blood group antigen system, where the sugar sequences responsible for the antigenic markers are found on red blood cell surface glycolipids, as well as on secretory glycoproteins (57). The low expression on fetal

thymocytes versus the high expression on adult thymocytes, and its absence on peripheral T cells could be due to differences in expression of the whole macromolecule or due to changes in the glycosylation pattern during differentiation. For example, the Thy 1 antigen appears on thymocytes at about 11 days gestation, is expressed in high amounts on thymocytes as they mature in the thymus, but is reduced on peripheral T cells (58,59). There are also qualitative changes, in that the carbohydrate portion of this glycoprotein undergoes changes during maturation. For example, Thy 1 from peripheral T cells has a more restricted molecular weight and charge heterogeneity and more sialic acid than Thy 1 extracted from PNA+ thymocytes, whereas Thy 1 from PNA- thymocytes resembles that of peripheral T cells (60,61).

At present we are unable to identify the 49H.8 reactive antigen. Although the 49H.8 reactivity on thymocytes and lectin resistant mutants resembles that of PNA (55), we have been able to dissociate the reactivity of these two reagents. The 49H.8 is also clearly different from another mAb with PNA binding specificity (53), which was able to distinguish surface carbohydrate epitopes of Thy 1.1 and Thy 1.2 thymocytes. We have also discussed the reasons for excluding Thy 1 and AGM1 as possible 49H.8 reactive antigens. In terms of its relationship to serum NTA, we can postulate that it represents one of the specificities previously identified (26), but it differs from the Thy 1 reactive serum NTA identified by Parker et. al. (27). The possibility that it represents an NAb specificity could be tested by

screening normal mouse serum with an anti-idiotypic antibody raised against the 49H.8 mAb.

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# APPENDIX I

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Table 1

Reactivity of murine tumors with Thy 1.2 monoclonal antibody and DBA/2 natural antibody

| Exp. | Tumor | Strain of origin | % cytotoxicity thy 1.2* | % cytotoxicity DBA/2 NAb+ |
|---|---|---|---|---|
| 1 | SL2-5 | DBA/2 | 70.7 | 30.7 |
| | SL2-9 | DBA/2 | 68.6 | 39.7 |
| | YAC-1.3 | A/Sn | 70.7 | 49.4 |
| | P815-16 | DBA/2 | 0.0 | 7.5 |
| | L5178Y-F9 | DBA/2 | 0.0 | 7.2 |
| 2** | SL2-clone 2 | DBA/2 | 35.4 | 21.5 |
| | SL2-clone 3 | DBA/2 | 35.4 | 25.9 |
| | SL2-clone 4 | DBA/2 | 27.5 | 34.9 |
| | SL2-clone 5 | DBA/2 | 49.2 | 27.3 |
| | SL2-clone 6 | DBA/2 | 38.5 | 29.4 |
| | SL2-clone 7 | DBA/2 | 8.3 | 11.1 |
| | SL2-clone 9 | DBA/2 | 25.2 | 36.5 |
| | SL2-clone 10 | DBA/2 | 24.5 | 10.8 |
| | SL2-clone R9V | DBA/2 | 0.7 | 0.6 |

* Monoclonal anti-Thy 1.2 (obtained from Dr. P. Lake, University College, London, England) was used at a dilution of $1/4 \times 10^4$ in experiment 1 and $1/10^5$ in experiment 2.

\+ In experiment 1 the DBA/2 cytotoxicity results are the mean of five experiments.

** All SL2 clones were tested with the anti-Thy 1.2 antibody and DBA/2 cytotoxicity in the same experiment. The correlation of anti-Thy 1.2 and DBA/2 NAb in experiment 2 was significant ($r = 0.688$, $p<0.05$). Combining the results of experiment 1 and 2 the correlation was highly significant ($r = 0.851$, $p<0.001$).

Table 2

Absorption of anti-thymocyte natural antibodies by murine tumors

| Exp. | Serum | Absorbing Cell | Numbers of* cells | DBA/2 Thymocytes % Cytotoxicity | DBA/2 Thymocytes % Absorption |
|---|---|---|---|---|---|
| 1 | Normal DBA/2 | SL2-5 | $10^6$ | 27.0 | 0.0 |
| | | | $10^7$ | 11.9 | 47.5 |
| | | L5178Y | $10^6$ | 21.1 | 7.0 |
| | | | $10^7$ | 30.4 | 0.0 |
| | | YAC-1 | $10^6$ | 32.7 | 0.0 |
| | | | $10^7$ | 17.6 | 22.4 |
| | | P815 | $10^6$ | 38.8 | 0.0 |
| | | | $10^7$ | 46.0 | 0.0 |
| | | Thymocytes | $10^8$ | 11.9 | 47.5 |
| | | | $2 \times 10^8$ | 0.0 | 100 |
| | | None | - | 22.7 | - |
| 2 | Normal DBA/2 | SL2-9 | $10^6$ | 29.9 | 14.6 |
| | | | $10^7$ | 16.6 | 52.6 |
| | | None | - | 35.0 | - |

Table 2 (continued)

| Exp. | Serum | Absorbing Cell | Numbers of* cells | DBA/2 Thymocytes % Cytotoxicity | % Absorption |
|---|---|---|---|---|---|
| 3 | LPS stimulated DBA/2 | SL2-5 | $4 \times 10^7$ | 16.6 | 72.2 |
| | | L5178Y | $4 \times 10^7$ | 62.4 | 0.0 |
| | | Thymocyte | $10^8$ | 42.4 | 38.1 |
| | | None | - | 61.6 | - |

* Serum was absorbed in 250 µl aliquots

Table 3

Absorption of anti-tumor natural antibodies by thymocytes and tumors

| Exp. | Serum+ | Target Cell | Absorbing Cell | Number of* cells | % Cytotoxicity | % Absorption |
|---|---|---|---|---|---|---|
| 1 | DBA/2 | SL2-5 ($TA^+$) | Thymocyte | $2 \times 10^8$ | 34.7 | 40.2 |
| | | | L5178Y ($TA^-$) | $10^7$ | 59.2 | 0.0 |
| | | | YAC-1 ($TA^+$) | $10^7$ | 50.6 | 13.8 |
| | | | P815 ($TA^-$) | $10^7$ | 55.7 | 4.9 |
| | | | SL2-5 ($TA^+$) | $10^7$ | 39.1 | 33.2 |
| | | | None | - | 58.6 | - |
| 2 | LPS-Stimulated DBA/2 | SL2-9 ($TA^+$) | Thymocytes | $3 \times 10^8$ | 38.4 | 55.7 |
| | | | None | - | 86.6 | - |
| 3 | LPS-stimulated DBA/2 | L5178Y ($TA^-$) | Thymocyte | $10^8$ | 56.4 | 6.9 |
| | | | None | - | 60.6 | - |
| | | Thymocyte | Thymocyte | $10^8$ | 38.1 | 38.0 |
| | | | None | - | 61.6 | - |

* Serum was absorbed in 250 µl aliquots

\+ Pooled sera from 6-12 mice was used in each experiment

TA = Thymocyte antigen defined by the ability to absorb anti-thymocytotoxic antibody.

# CHAPTER 3

## A PHENYL-β-GALACTOSIDE SPECIFIC MONOCLONAL ANTIBODY REACTIVE WITH MURINE AND RAT NK CELLS

ABSTRACT

The phenyl-β-galactoside (Ø-β-gal) specific monoclonal antibody (mAb) 49H.8 crossreacts with the terminal disaccaride structure of the AGM1 molecule. It was found to react with Ø-β-gal determinants on murine and rat splenic NK cells, as measured by complement depletion studies. Flow cytometry analysis identified the antigen on two IL-2 dependent cloned murine NK cell lines and the rat large granular lymphocyte leukemia, RNK. We have compared the 49H.8 reactivity to that of anti-asialo GM1 antisera (α-AGM1), on NK cells and a panel of NK related killer cells, including bone marrow derived killer cells, lymphokine activated killer cells (LAK) and anomalous killer cells (AK). We found that the 49H.8 specificity closely paralled that of α-AGM1. When tested against Con A reactive T cells, the 49H.8 mAb was less reactive than the α-AGM1, indicating that it may be a more specific marker for splenic NK populations than the α-AGM1.

INTRODUCTION

The 49H.8 monoclonal antibody (mAb) was raised against human neuraminidase treated erythrocytes, and reacted with a variety of human and murine tumors, as well as murine thymocytes (Chapter 3) and neuraminidase treated lymphocytes (1). Binding of the mAb to neuraminidase treated cells was completely inhibited by the carbohydrate phenyl-β-galactoside (Ø-β-gal) but not at all by the related sugar phenyl-α-galactoside (Ø-α-gal). In addition, it was partially inhibited by the terminal disaccharide of asialo GM1, (AGM1) βgal(1 3)βgalNAc (1). In tissue distribution studies we found that the 49H.8 antigen was expressed in 60-85% of adult thymocytes, 8-10% of bone marrow cells, and 12-20% of spleen cells (Chapter 2). Because the 49H.8 reactive antigen(s) (49H.8 Ag) is expressed on unfractionated spleen cells, but not on isolated T and B cells (1) and because it displays some crossreactivity with the terminal disaccharide of AGM1, a marker on murine and rat natural killer (NK) cells (2,3), we decided to test this antibody for reactivity with NK cells. AGM1 antisera has been found to be a useful reagent for depleting NK activity in vitro and in vivo (4). When spleen cells are treated with appropriate concentrations of α-AGM1 and complement NK cells are lysed preferentially (3), and on this basis it has been considered to be an NK cell specific marker. However, when used at higher concentrations, it does react with T cells and analysis of membrane glycolipids of T cell lines has revealed that these also express the AGM1 antigen (5). Since the

49H.8 is an AGM1 reactive monoclonal antibody whose specificity is more finely defined than the α-AGM1 antisera, we thought it may be useful to further characterize its reactivity with NK and NK-related cells. Our initial results, reported here, did indeed indicate that the 49H.8 mAb was reactive against murine and rat NK cells and that it could be used to deplete NK activity when used in vitro with complement. We also present evidence which indicates that the 49H.8 mAb can recognize lymphokine activated killer cells (LAK), bone marrow derived killer cells, and anomalous killer cells (AK) in a manner similar to α-AGM1.

## MATERIALS AND METHODS

### Mice and Cell lines:

CBA/J mice were obtained from the Jackson Laboratories, Bar Harbour, Maine. C57BL/6 mice and Sprague-Dawley rats were bred at the University of Manitoba Vivarium. All mice were used at 6-8 weeks of age. The L5178Y-F9 is a subclone of the L5178Y DBA/2 lymphoma and has been previously described (1). The YAC.1 tumor line was obtained from Dr. John Roder, Queens University, Kingston, Ontario and maintained in RPMI 1640 with 10% fetal bovine serum (FBS), at 37°C and 5% $CO_2$, in humidified air. All tumors were routinely tested for mycoplasma infection and consistently observed to be mycoplasma free.

For assaying macrophage cytotoxicity, mice were injected with 200 µg of heat killed C. parvuum (Wellcome Laboratories, Beckenham, England), 5-6 days prior to the assay. Peritoneal macrophages were collected by peritoneal lavages with 20 ml of heparinized HBSS (100 units heparin/ml), pooled by centrifugation, washed three times with cold Hanks' Balanced Salt Solution (HBSS), then placed in sterile flat bottom 96 well microtiter plates, in triplicate, at appropriate concentrations. After a 60 minute incubation at 37°C, the adherent monolayers were washed once with RPMI/10% FBS. $^{51}Cr$ labelled target cells were then added to each well ($10^4$ cells in 200 µl), for a 16-18 hour incubation at 37°C, in humidified air and 5% CO2. 100µl of supernatant was harvested and counted, and cytotoxicity calculated as for the NK assay.

Antibody and complement depletion of effector cell population:

Spleen cells ($2 \times 10^7$) were incubated in 1 ml of the appropriate concentration of antibody diluted in HBSS, for one hour at 4°C. Cells were washed once, then incubated with 1 ml of complement (Cedarlane Lowtox) at 1/8 dilution in RPMI 1640 + 10% FBS. The complement had previously been absorbed with spleen cells and thymocytes ($10^8$ cells/ml), for one hour on ice to remove any cytotoxic activity. After the complement incubation, the cells were washed once, resuspended to the appropriate volume in RPMI 1640 and 10% FBS, then added to the $^{51}Cr$ labelled target cells. Lyophylized rabbit anti-AGM1 antisera was obtained from Dr. M. Sugi, Yamasa Shoyu Company, Chiba, Japan. The 49H.8 and 49H.24 mAb's have been described previously (1)

The ASP12.1 and SSP-2.3 are IL-2 dependent NK cloned lines, derived from A/J and SJL spleens respectively. Their isolation and maintenance has been previously described (6). The RNK tumor line was obtained from Dr. Craig Reynolds (NCI, Frederick, Md), and maintained by passaging in pristane-primed Sprague-Dawley rats (7). The 49H.8 and 49H.24 mAb secreting hybridoma cell lines were maintained in RPMI 1640 with 10% FCS or grown as ascites in pristane primed Balb/c mice (1).

Cytotoxicity assays:

The NK assay has been described previously (8). Briefly, the appropriate number of spleen cells was incubated with $10^4$ $^{51}Cr$ labelled target cells, for a duration of 4-6 hours at 37°C in 96 well V bottom microtiter plates, after which the plates were centrifuged, and half the supernatant was removed from each well for gamma counting. Cytotoxicity values for NK as well as other lytic assays are calculated as follows:

$$\frac{\text{Experimental cpm - Background cpm}}{\text{Total cpm added - Background cpm}} \times 100$$

Lytic units/$10^7$ cells were calculated by dividing $10^7$ by the number of effector cells required to produce 20% or 30% lysis of $10^4$ target cells. These calculations are based on a regression line constructed for eack experimental point from three different E/T rations. Experiments showin depletion of NK activity by antibody and complement were repeated a minimum of three times, and the results reported in LU were highly reproducible.

For poly I:C stimulation, 100 µg poly I:C in 100 µl HBSS was injected intraperitoneally 18-24 hours prior to sacrifice.

Affinity purified preparations (1 mg/ml) were used throughout this study.

Anti-Thy 1.2 antisera was produced by immunizing AKR mice with six weekly injections of $10^7$ C3H thymocytes. Mice were bled seven days after the last injection.

In Vitro Interferon Stimulation:

2 x $10^7$ spleen cells were incubated with 1 ml of 1000 U/ml murine β-IFN (Lee Bio Molecular, San Diego, California) for 12 hours, then washed and added to $^{51}Cr$ labelled targets in appropriate concentrations.

Generation of Lymphokine Activated and Bone Marrow IL-2 Activated Killer Cells:

PHA conditioned medium (PCM) from human tonsils was generously provided by Dr. John Wilkins, University of Manitoba. For spleen cell and bone marrow cell activation, we followed the protocol described by Koo et.al. (9). Briefly, 5 x $10^6$ spleen or bone marrow cells were incubated in 1 ml of media containing 10% PCM, 20 mM Hepes and 10% FCS in 24 well Linbro trays. Cultures were split at 2-3 days, and thereafter fed every 3 days. 5 x $10^5$ irradiated (1500 R) syngeneic bone marrow cells were added as feeder layers to the bone marrow cultures. No feeder layers were used for spleen cell cultures. After the appropriate incubation time, cells were washed three times in HBSS. For antibody depletion studies, effector

cells were treated with antibody and complement before or after PCM treatment as described above.

Generation of cytotoxic T lymphocytes (CTL) and Anomalous Killer (AK) Cells:

Mixed lymphocyte cultures were established as described by Kärre et.al. (10), using C57/BL6 anti-BALB/c cultures. AK cell populations were harvested after three days in culture. For in vitro generation of CTL's, C57/BL6 anti-BALB/c cultures were established as above, and restimulated after 5 days with fresh irradiated BALB/c cells. CTL's were harvested and used three days after secondary stimulation.

Lectin Activation

RBC depleted C57BL/6 spleen cells were incubated at a density of 2 x 105 cells/well in 96 flat bottom microtitration plates in RPMI 1640 containing 10% FCS, and 0.5 µg/ml of Con A, or 10 µg/ml LPS (serotype 026:B6, Sigma) in a total volume of 200 µl. After 48 hours, 1 µCi 3H Thymidine (Amersham), specific activity 5 Ci/mM, was added to each well, and cells harvested 24 hours later with a Titertek harvester. Incorporation of 3H Thymidine was determined by counting triplicate samples in a Beckman Scintillation Counter.

Flow cytometry analysis of NK cell lines and RNK tumor

50 µl of a 1/50 dilution of 49H.8 mAb in PBS was incubated with 50 µl of either Ø-β-gal or Ø-α-gal (25 mM final concentration) for 30 minutes before being added to 106 cloned ASP-12.1 or SSP-2.3 clones

cells in 50µl of PBS. After a 60 minute incubation on ice, the cells were washed twice with PBS, then incubated with 50 µl of a 1/20 dilution of FITC labelled sheep anti-mouse IgM $F(ab')_2$ fragments (Cappel Laboratories) for 30 min on ice. After two washes, the cells were processed for flow cytometry. For binding to the RNK line, we used 49H.8-fluorescent latex bead conjugates, prepared as described in (11). We found that the bead conjugate method yielded more consistent results in previous studies of 49H.8 tissue distribution and used it for all subsequent studies. 200 µl of the stock preparation of 49H.8 mAb was bound to 200 µl of green fluorescent beads (Polyscience) which had previously been activated with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, (Sigma), (EDCI), by incubating at 4°C overnight. The beads were washed free of unbound antibody by washing twice with a 2% FCS/PBS solution in a Beckman microfuge, and resuspended to a total volume of 2 ml of 2% FCS/PBS, with 0.1% $NaN_3$. These preparations were stable for several months if kept at 4°C. For flow cytometry, 50 µl of beads were incubated with 50µl of either Ø-α-gal or Ø-β-gal in flat bottom 96 well micro-titration plates for 30 minutes at room temperature. 2 x $10^6$ cells in 100 µl of HBSS were added to each well, and the plates were centrifuged at 400 x g for 20 minutes. After a further incubation of 45 minutes on ice, the cells were separated from unbound beads by carefully layering over 1 ml of FBS, and spinning at 75 x g for 5 minutes. After aspiration of the supernatant, the cell pellets were resuspended in 1 ml of 2%FBS/PBS with 0,1% $NaN_3$, and 20,000 cells

analyzed by flow cytometry. The results were plotted on a logarithmic fluorescence intensity scale.

## RESULTS

### Depletion of in vitro NK activity by 49H.8 mAB

Table 1 shows that the 49H.8 mAB can be used to deplete splenic NK activity, similarly to the α-$AGM_1$ antisera, in both CBA/J and C57BL/6 mice. Although there is some inhibition if effector cells are pretreated with antibody alone, the addition of complement resulted in much higher depletion. The 49H.24 mAb was used as a control. It originated from the same fusion as the 49H.8 mAb and is specific for the T-hapten (1). It does not crossreact with the βgal(1 3)βgalNAc disaccharide nor with Ø-β-gal and Ø-α-gal.

It has previously been reported that AGM1 is also present on rat NK cell membrane (3). The results presented in the last section of Table I show that 49H.8 mAb, like α-AGM1 antisera, also depletes rat NK activity. In contrast, neither α-AGM1 nor the 49H.8 mAb were able to deplete NK activity from human LGL preparations (data not shown).

Because binding of the 49H.8 mAb to neuraminidase treated RBC's can be inhibited by the hapten Ø-β-gal, we tested this sugar, as well as the closely related but non-inhibitory sugar Ø-α-gal, for their ability to inhibit NK depletion by both 49H.8 mAb and anti-AGM1 antisera. Table 2 shows that Ø-β-gal can inhibit NK depletion by the 49H.8 mAb in a dose dependent manner (Experiment 1), whereas it

Table 1

In vitro depletion of NK activity by 49H.8mAb and α-AGM1

| Effector Cells | Effector Cell Treatment Ab | C[1] | Lytic Units/$10^7$ cells |
|---|---|---|---|
| CBA/J Spleen Cells | | | |
| | - | - | 27.0 |
| | - | + | 22.7 |
| | 49H.8 (20μg/ml) | - | 13.3 |
| | 49H.24 (20μg/ml) | + | 22.2 |
| | 49H.8 (40μg/ml) | + | 1.4 |
| | 49H.8 (10μg/ml) | + | 5.9 |
| | 49H.8 (1μg/ml) | + | 11.1 |
| | α-AGM1 (1/100) | + | 0.20 |
| C57Bl/6 Spleen Cells | | | |
| | - | - | 47.6 |
| | - | + | 48.8 |
| | 49H.8 (20μg/ml) | + | 0.03 |
| | 49H.8 (10μg/ml) | + | 0.11 |
| | 49H.8 (1μg/ml) | + | 1.1 |
| | α-AGM1 (1/100) | + | 0.01 |
| Rat Spleen Cells | | | |
| | - | - | 16.9 |
| | - | + | 16.4 |
| | 49H.8 (10μg/ml) | + | 0.5 |
| | α-AGM1 (1/100) | + | 4.2 |

Table 2

Inhibition of 49H.8Ab Activity By Ø-β-GAL

Exp. 1

| Effector Cell Treatment Ab | SUGAR | C[1] | Lytic Units per $10^7$ cells |
|---|---|---|---|
| - | - | - | 16.6 |
| - | - | + | 14.2 |
| 49H.8 | - | + | < 0.001 |
| 49H.8 | Ø-β-GAL 10mM | + | 13.3 |
| 49H.8 | Ø-β-GAL 5mM | + | 11.7 |
| 49H.8 | Ø-β-GAL 1mM | + | 4.3 |
| 49H.8 | Ø-α-GAL 10mM | + | < 0.001 |
| - | Ø-β-GAL 10mM | - | 17.5 |
| - | Ø-α-GAL 10mM | - | 17.8 |

Exp. 2

| | | | |
|---|---|---|---|
| - | - | - | 16.7 |
| α-AGM1[b] | - | + | < 0.001 |
| α-AGM1 | Ø-β-GAL 20mM | + | < 0.001 |
| α-AGM1 | Ø-β-GAL 10mM | + | < 0.001 |
| α-AGM1 | Ø-β-GAL 5mM | + | < 0.001 |
| 49H.8 | Ø-β-GAL 20mM | + | 14.3 |
| 49H.8 | Ø-α-GAL 20mM | + | 0.001 |

[a] Concentration of mAb used for this experiment: 10μg/ml

[b] Concentration of antisera used for this experiment: 1/100

does not affect the toxicity of the α-AGM1 antisera (Experiment 2). Ø-α-gal does not inhibit either one of the antibodies and neither sugar has any effect on NK activity in the absence of antibody and complement.

We next tested whether spleen cells depleted of endogenous NK activity by the two antibodies could be activated by interferon (IFN). IFN is an NK cell activator (12) and this is due to its ability to activate pre-NK cells, as well as increasing the turnover rate of activated NK cells (13-15). When α-AGM1 or 49H.8 mAb treated spleen cells were exposed to 1000 U/ml of β-IFN for a period of 12 hours, no further NK activity was generated, as shown in Table 3. In contrast, control spleen cells were typically enhanced by IFN pretreatment (lines 4 and 5). Thus, α-AGM1 and 49H.8 deplete both active endogenous NK cells as well as any inactive NK precursors (13-15).

Effect of α-AGM1 on the Binding of 49H.8 mAb to Spleen Cells and Thymocytes.

Because the 49H.8 crossreacts with the terminal disaccharide of the AGM1 molecule, we tested the ability of α-AGM1 antisera to block the binding of 49H.8. Figure 1 shows that, binding of 49H.8 mAb coupled to fluorescent latex beads to spleen cells and thymocytes is not inhibited by preincubation with α-AGM1.

Table 3

Interferon Treatment of 49H.8 + $C^1$ Treated Spleen Cells

| Effector Cell Treatment Ab | $C^1$ | IFN* | Lytic Units per $10^7$ cells |
|---|---|---|---|
| - | - | - | 4.3 |
| - | + | - | 2.3 |
| - | - | + | 8.3 |
| - | + | + | 16.7 |
| α-AGM1 | + | - | 0 |
| α-AGM1 | + | + | 0 |
| 49H.8 | + | - | 0 |
| 49H.8 | + | + | 0 |

* 1000 U/ml - 12 hours.

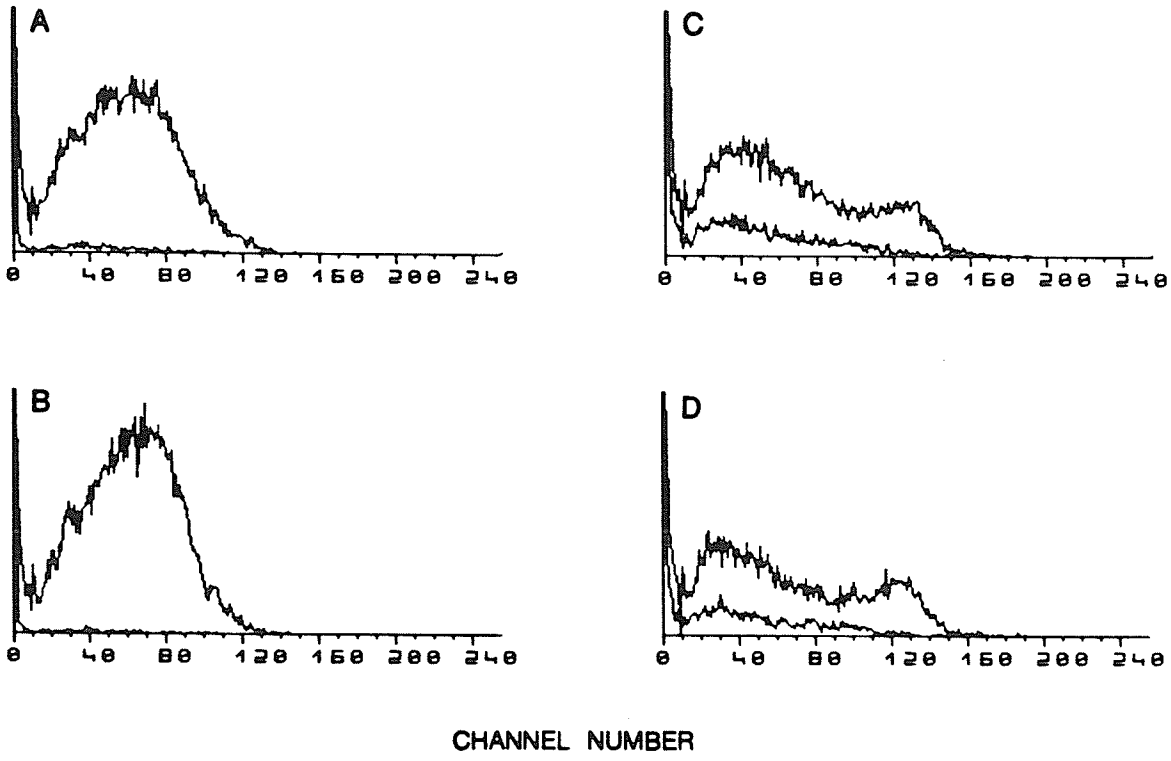


Figure 1: Effect of α-AGM1 antisera on binding of 49H.8-latex beads to thymocytes and spleen cells. A) Control thymocytes. B) α-AGM1 pretreated thymocytes. C) Control spleen cells. D) α-AMG1 pretreated spleen cells. Lower line in each histogram represents binding in the presence of Ø-β-gal. Vertical scale: 250 cells.

49H.8 mAb reactivity with cloned IL-2 dependent murine NK cells and a rat LGL leukemia

In order to determine whether the expression of the antigen recognized by the 49H.8 mAb, was limited to endogenous NK cells only, we examined the reactivity of two IL-2 dependent, murine NK lines (6) and the rat NK tumor line, RNK by flow cytometry. Figure 2 shows that the 49H.8 mAb binds to both the ASP-12.1 and the SSP-2.3 lines and that the binding is inhibitable by Ø-β-gal, but not the closely related sugar, Ø-α-gal. Similarly, the RNK tumor also bound the 49H.8 mAb (Fig 3), which in this case had been coupled to fluorescent latex beads.

Reactivity of 49H.8 mAb and α-AGM1 with other types of killer cells

Over the past years, several different kinds of NK-like or NK-related killer cells have been described, and attempts have been made to ascertain the origin of these types of cells in relation to NK cells. Various markers have been used for these studies, including AGM1, Thy-1, and the NK alloantigens, NK-1 and NK-2. We have examined several types of NK-related cells for 49H.8 mAb and α-AGM1 reactivity, including bone marrow derived killer cells, lymphokine activated killer cells, anomalous killer cells as well as cytotoxic T cells. In these studies we measured reactivity by antibody and complement depletion of cytotoxic activity. Depletion was examined at the level of effector cells, as well as precursor cells. The definition of "precursor cells" in this study is an

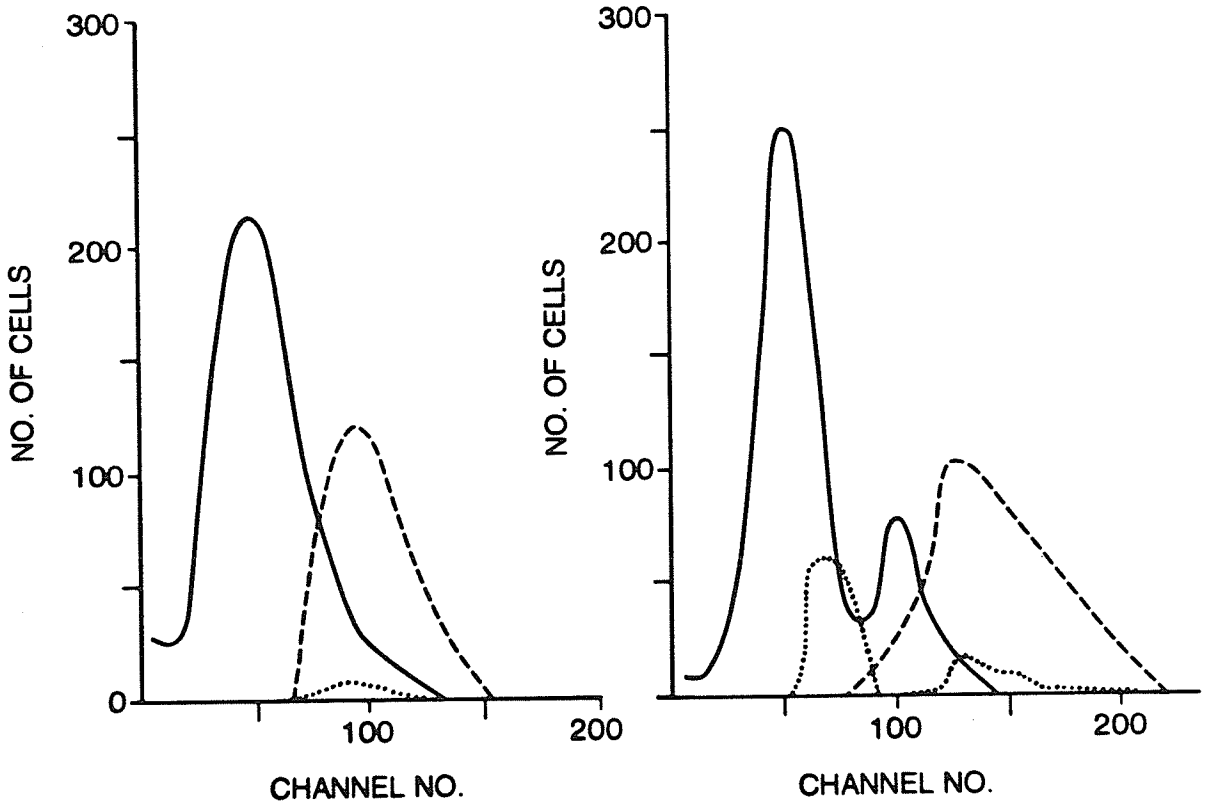


Figure 2: 49H.8 mAb binding to murine NK clones ASP-12.2 (A) and SSP-2.3 (B), in the presence of Ø-α-gal (----), and Ø-β-gal (....). Solid line represents sheep anti-mouse IgG F(ab')2-FITC background binding.

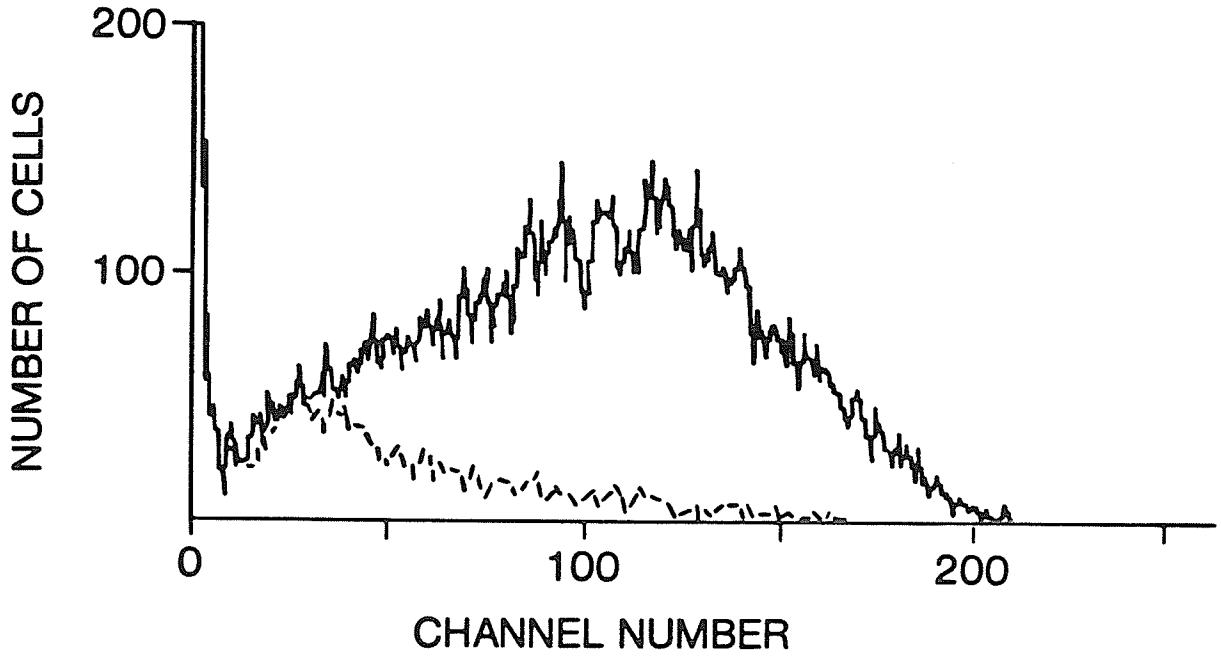


Figure 3: 49H.8-latex bead binding to the RNK leukemia in presence (lower line) and absence (upper line) of Ø-β-gal.

operational one, that is, any loss of effector cell cytotoxicity due to antibody and complement treatment prior to the generation of effector cells was attributed to the loss of "precursor cells." These results are all summarized in Tables 4 and 6.

a) Bone Marrow Derived Killer Cells: It has previously been reported that bone marrow derived killer cells can be obtained by culturing bone marrow cells in IL-2 containing media (9). When bone marrow cells were pretreated with 49H.8 and complement (Table 4) no inhibition of effector cell activation was observed. Treatment with α-AGM1 and complement resulted in a slight inhibition, but it was never a large reduction in repeated experiments. Similarly, bone marrow effector cells were not sensitive to treatment with these antibodies and complement. Since we performed these experiments, other investigators have also reported that both bone marrow precursor and cultured cytotoxic effector cells express only low levels of AGM1 (16,17). The concentration of Ab used for these and subsequent experiments (1/20 for 49H.8 and 1/100 for α-AGM1) was higher than required for depletion of splenic NK cells. These and all subsequent experiments included splenic NK depletion controls for antibody activity (see last section, Table 4).

b) Lymphokine Activated Killer (LAK) Cells: LAK cells are potent cytotoxic effector cells generated by incubating splenic or peripheral blood lymphocytes in (IL-2) containing media, and they kill NK resistant as well as NK sensitive tumor targets, but not

normal cells (18,19). As shown in Table 4, both 49H.8 mAb and α-AGM1 antisera were able to deplete some effector activity if precursor cells were treated with antibody and complement prior to activation. Effector cells were also sensitive to antibody and complement treatment, but in neither case was the antibody as effective as in NK depletion experiments (Table 1).

c) Anomalous Killer (AK) cells: These type of effector cells were described by Kärre et.al. (10). They are produced in certain strain combination of MLR cultures, appearing before the antigen specific cytotoxic T cells peak. Kärre et al (10) demonstrated that NK cells are required in the responder population by depletion of NK cells with NK alloantisera and soya bean agglutinin adherence. Our AK system consisted of C57BL/6 responders and irradiated BALB/c stimulator cells. At three days of culture, we found AK cell activity, directed at H-$2^d$ and H-$2^k$ bearing targets whereas if irradiated C57BL/6 cells were used as stimulators, no AK activity was generated (Table 5). However, our AK cells differed from those described by Kärre et.al. (10) in that they were effective against both $NK^S$ targets (YAC) and $NK^R$ targets (L5178Y-F9). If C57Bl/6 responder spleen cells were pretreated with α-AGM1 antisera, when tested against the YAC tumor, the cytotoxic activity of the AK effector cells was eliminated almost totally (Table 6), whereas the 49H.8 mAb was able to deplete the activity approximately 50%. When tested against the L5178Y-F9 tumor both antibodies resulted in equal (>50%) depletion of AK precursors. When effector cells were treated

with antibody and complement both antibodies were able to deplete AK activity, although not to the same extent as NK cell activity.

d) Cytotoxic T lymphocytes (CTL): The same strain combination used for AK cell generation was also used for generating in vitro CTL's, except that the cells were cultured for 5 days, then restimulated with fresh irradiated BALB/c splenocytes for an additional 3 day culture period. The specificity of CTL activity was confirmed to be against H-2$^d$ by testing cytotoxic activity against a panel of tumors of H-2$^d$ and H-2$^k$ origin (Table 5). We found that both α-AGM1 and 49H.8 mAb had some effect on both precursor and effector cell populations (Table 6), which was more pronounced at the lower effector:target ratios.

Reactivity of 49H.8 mAb and αAGM1 antisera on Con A reactive T cells

In a previous study (1) we had shown that $^{125}$I-labelled 49H.8 mAb did not bind significantly to peripheral T cells and Con A blasts. We have further confirmed the lack of reactivity with peripheral T cells by attempting to deplete T cells proliferating in response to Con A. Table 7 shows anti-Thy 1.2 antisera completely eliminates proliferation as measured by $^3$H-TdR uptake in Con A containing culture, whereas it does not affect B cell proliferation in response to LPS. In contrast, treatment with 49H.8 mAb and complement does not affect the proliferation to either Con A or LPS, and α-AGM1 antisera only partially depletes T cell proliferation.

Table 4

Effect of 49H.8 mAb and α-AGM1 on Activated Bone Marrow Cells and LAK Cells

| Ab Treatment Ab | C[1] | Cell Type[a] | % Cytotoxicity (YAC) 150:1 | 75:1 | 37.5:1 | 19:1 |
|---|---|---|---|---|---|---|
| - | - | Bone Marrow Precursor[b] | NT | 52.5 | 45.4 | 31.7 |
| 49H.8[c] | + | Bone Marrow Precursor | NT | 52.9 | 49.9 | 35.0 |
| α-AGM1[d] | + | Bone Marrow Precursor | NT | 41.9 | 33.1 | 24.9 |
| - | - | Bone Marrow Effector | 64.8 | 58.7 | 53.4 | NT |
| 49H.8 | + | Bone Marrow Effector | 66.5 | 61.2 | 55.4 | NT |
| α-AGM1 | + | Bone Marrow Effector | 59.6 | 57.0 | 43.2 | NT |
| - | - | Fresh Bone Marrow | 5.4 | 4.6 | 6.3 | NT |
| - | - | LAK Precursor | 69.6 | 55.0 | 44.1 | NT |
| 49H.8 | + | LAK Precursor | 46.8 | 33.1 | 23.3 | NT |
| α-AGM1 | + | LAK Precursor | 54.9 | 44.2 | 26.8 | NT |
| - | - | LAK Effector | 69.6 | 55.0 | 44.1 | NT |
| - | + | LAK Effector | 61.1 | 43.0 | 34.3 | NT |
| 49H.8 | + | LAK Effector | 28.2 | 22.1 | 16.2 | NT |
| α-AGM1 | + | LAK Effector | 20.2 | 12.6 | 8.3 | NT |
| - | - | NK | 30.5 | 22.9 | 15.1 | NT |
| 49H.8 | + | NK | 1.5 | 1.6 | 1.0 | NT |
| α-AGM1 | + | NK | 3.2 | 2.8 | 1.6 | NT |

See footnotes next page.

Footnotes for Table 4

[a] See MATERIALS AND METHODS for culture conditions

[b] See text for definition of precursor

[c] Concentration of mAb used for these experiments: 10 µg/ml

[d] Concentration of antisera used for these experiments: 1/100

Table 5

Generation of AK cells and CTL in mixed lymphocyte cultures

| | Responder | Stimulator | Culture Period[a] | Cytotoxicity (E/T:50:1) L5178Y-F9 ($H\text{-}2^d$) | YAC ($H\text{-}2^a$) | RI ($H\text{-}2^k$) |
|---|---|---|---|---|---|---|
| Exp. 1 | C57B1/6 ($H\text{-}2^b$) | BALB/c ($H\text{-}2^d$) | 3 days | 62.3 | 46.1 | 31.7 |
| Exp. 2 | C57B1/6 | BALB/c | 3 days | 73.2 | 32.6 | NT |
| | C57B1/6 | C57B1/6 | 3 days | 14.2 | 4.8 | NT |
| | BALB/c | C57B1/6 | 3 days | 4.5 | 11.9 | NT |
| | BALB/c | BALB/c | 3 days | 0 | 4.6 | NT |
| | C57B1/6 | BALB/c | 3 days + 5 days | 46.9 | 20.4 | 6.3 |

[a] See MATERIAL AND METHODS for details.

Table 6

Effect of 49H.8 mAb and α-AGM1 on AK and CTL

| Ab Treatment Ab | C[1] | Cell Type[a] | % Cytotoxicity (YAC) 50:1 | 25:1 | 12.5:1 | % Cytotoxicity (L5178Y-F9) 50:1 | 25:1 | 12.5:1 |
|---|---|---|---|---|---|---|---|---|
| - | - | AK Precursor[b] | 35.5 | 26.6 | 16.2 | 85.6 | 81.7 | 56.0 |
| - | + | AK Precursor | 46.7 | 33.3 | 20.5 | 80.7 | 79.4 | 69.2 |
| 49H.8[c] | + | AK Precursor | 20.4 | 14.6 | 10.5 | 28.7 | 17.0 | 10.7 |
| α-AGM1[d] | + | AK Precursor | 5.4 | 3.5 | 2.3 | 21.4 | 12.7 | 4.2 |
| - | + | AK Effector | 35.5 | 26.6 | 16.2 | 85.6 | 81.7 | 56.0 |
| 49H.8 | + | AK Effector | 24.2 | 10.9 | 9.2 | 72.6 | 58.3 | 35.7 |
| α-AGM1 | + | AK Effector | 21.7 | 14.5 | 6.4 | 67.4 | 52.4 | 27.0 |
| - | - | CTL Precursor | NT | NT | NT | 78.1 | 65.5 | 48.3 |
| - | + | CTL Precursor | NT | NT | NT | 80.2 | 69.2 | 63.8 |
| 49H.8 | + | CTL Precursor | NT | NT | NT | 71.9 | 55.3 | 36.4 |
| α-AGM1 | + | CTL Precursor | NT | NT | NT | 62.3 | 46.8 | 21.1 |
| - | - | CTL Effector | NT | NT | NT | 78.1 | 65.3 | 48.3 |
| 49H.8 | + | CTL Effector | NT | NT | NT | 52.7 | 32.5 | 19.0 |
| α-AGM1 | + | CTL Effector | NT | NT | NT | 55.7 | 43.0 | 23.1 |

[a] See MATERIAL AND METHODS for culture conditions

[b] See text for definition of precursor cells

[c] Concentration of mAb used for these experiments: 10 μg/ml

[d] Concentration of antisera used for these experiments: 1/100

Table 7

$^{3}$H-TdR Incorporation in 49H.8mAb and α-AGM1

Treated Spleen Cells

| Treatment Ab (concentration) | C[1] | DPM - Background (± S.D.) CON A | LPS |
|---|---|---|---|
| - | - | 302,874 ± 7111 | 282,588 ± 8778 |
| - | + | 318,533 ± 6510 | 326,834 ± 15366 |
| α Thy 1.2 (1/100) | + | -4,343 ± 2006 | 221,966 ± 15873 |
| α-AGM1 (1/50) | + | 177,763 ± 5326 | 295,226 ± 16827 |
| 49H.8 (10/μg/ml) | + | 304,860 ± 7337 | 360,434 ± 17833 |

Reactivity with tumoricidal peritoneal exudate macrophages:

We next tested the 49H.8 mAb against C. Parvum activated peritoneal macrophages and found that neither antibody and complement, complement alone or antibody alone affected the levels of macrophage cytotoxic antitumor activity (Table 8).

## DISCUSSION

We have presented evidence showing that the Ø-β-gal reactive 49H.8 mAb reacts with murine and rat NK cells. Although it cannot be considered to be a strictly NK specific marker since it also binds to thymocytes and a small percentage of bone marrow cells (Chapter 3), in splenocytes it does show preferential NK cell reactivity. In addition, it binds to IL-2 dependent cloned NK lines and to the rat NK tumor, RNK.

We have compared its reactivity pattern to that of α-AGM1 antisera, testing both antibodies on a variety of NK-related killer cells and Con A reactive T cells. Although these two antibodies appear to behave similarly when tested against a panel of different types of killer cells, they are different in that the α-AGM1 antisera depletes Con A reactive T cells more efficiently than the 49H.8 mAb. This confirms earlier findings that the 49H.8 antigen is absent on peripheral T cells (1). We have no biochemical evidence to substantiate this at present, but our results would indicate that the

Table 8

Effect of 49H.8 mAb on C. parvum Activated Macrophages

| Macrophage Treatment | | % Cytotoxicity (L5178Y-F9) | | |
|---|---|---|---|---|
| Ab | C' | 50:1 | 30:1 | 20:1 |
| - | - | 23.2 | 17.5 | 10.4 |
| 49H.8 (1/20) | - | 19.7 | 13.9 | 6.7 |
| 49H.8 (1/20) | + | 24.6 | 16.8 | 7.8 |
| 49H.8 (1/50) | - | 24.3 | 16.1 | 10.7 |
| 49H.8 (1/50) | + | 21.9 | 16.8 | 10.3 |
| - | + | 30.4 | 22.2 | 9.9 |

49H.8 may be a more specific NK marker compared to α-AGM1, since this marker is also present on peripheral T cells (5).

Another important difference between the two markers is the ontogeny of their appearance on thymocytes. The 49H.8 is an adult thymocyte marker (Chapter 3) and the AGM1 a fetal thymocyte marker (20), although both are present on approximately the same number of bone marrow cells (Chapter 3,20).

Although maximum depletion of splenic NK activity required the presence of complement, some inhibition of lysis was observed when murine spleen cells were incubated in higher concentrations of antibody without complement. We have not examined this finding in detail, but it could be an indication that the membrane antigen(s) recognized by the 49H.8 mAb is directly involved in NK-target cell interaction.

The relationship between cloned NK lines maintained in IL-2 containing growth media and endogenous NK cells is somewhat controversial at present, but since these lines displayed a broader target specificity than their endogenous splenic counterparts, and were also Thy-1 positive, it could be argued that they are more representative of the LAK cell type. The rat leukemia line which we examined for binding by flow cytometry is more representative of endogenous rat NK cells, both phenotypically and in terms of target cell specificity (7), and our binding studies with the RNK tumor and complement depletion experiments with rat splenic NK cells indicates that the mAb recognizes both cell types.

For the comparison of 49H.8 and α-AGM1 reactivity with other types of killer cells we used previously established cell culture systems to generate the killer cells, and some data on characterization of cell surface antigens on these various types of cells is already available. Bone marrow derived killer cell systems include those cultured in the presence of lymphokines (9) and culturing in normal growth medium only (16,21). Studies of NK markers on bone marrow killer cells and bone marrow derived (cultured) cytotoxic cells have examined the Thy 1 (16,21), Qa $5^+$, (21) AGM1 (16), and NK alloantigens, (9,16). Thus, Minato et.al. (21) found that bone marrow cytotoxic cells (4 day cultures, in the absence of lymphokines) were Thy $1^-$, Qa$5^-$, and that these did not respond to IFN or IL-2. Klimpel et.al. (16) cultured bone marrow cells for 6 days (no lymphokine) and in their system, precursor cells were Thy$1^-$, but cytotoxic effector cells were Thy$1^+$, expressing only low levels of AGM1 and NK alloantigen. This system depended on the C57/BL6 origin of the bone marrow cells. Koo et.al. (9), were able to detect an NK$1^+$ lytic cells in normal bone marrow which was repsonsive to IFN and lymphokine containing media. Upon 5-6 days of culturing in Con A conditioned media, their effector cells were NK$1^+$ and Qa$5^+$. Thus, the heterogeneity of NK like killer cells is also clearly evident within the bone marrow. Other studies have focused on splenic NK cell precursors within the bone marrow. One study has shown that splenic NK repopulating bone marrow precursor cells are NK2.$1^-$, AGM$1^-$, Qa$5^-$ and Thy$1^-$, but Qa$2^+$ and H-$2^+$ (17), and another study showed that splenic NK cells, recently arrived from bone marrow

precursors (Thy1-) are Thy1+ (22). Whether the spleen repopulating cells are the same as the precursors for in vitro derived killer cells has not been established.

Although we also used C57/BL6 bone marrow cells, we found that neither fresh bone marrow cells, nor cells cultured in media without lymphokine displayed any cytotoxicity against YAC cells in contrast to Klimpel et.al. (16), but our lymphokine activated bone marrow killer cells also expressed only low levels of AGM1, both at the precursor and effector level. This marker was not examined in the lymphokine culture system described by Koo et.al. (9), but it does confirm the findings of Klimpel et.al. (16) that cultured bone marrow killer cells express only low levels of this antigen. In addition, the 49H.8 antigen, although present on a subpopulation of normal bone marrow cells, was not associated with this type of killer cell. We have not established whether the 49H.8+ and the AGM1+ bone marrow cells represent the same or different populations.

Splenic lymphokine activated killer (LAK) cells have been described both in the human and murine system (18,19,23). According to Suzuki et.al. (18) the generation of Il-2 dependent killer cells is dependent on AGM1+ NK cells, and the cytotoxic effector cells are also Thy1+, Ly5+, AGM1+, Lyt1-, Lyt2-. In Mulè's et al (19) system, the effector cells were also Thy1+, but Lyt2+. This latter group also indicated that in the human system, at least, NK cells are not required for the generation of LAK cells (19). In an earlier study, Minato et.al. (21) reported that both Thy1+ Lyt2+ and Thy1+ Lyt2- cells were regulated by IL-2, therefore it is possible that this

discrepancy can be explained on the basis that the two groups were looking at different cell populations, that is, Suzuki et.al. examined cloning efficiencies of fractionated LGL in IL-2 whereas, whereas Mulé et.al. used whole spleen cell suspensions incubated with recombinant IL-2. Our results, with α-AGM1 antisera and the 49H.8 mAb would indicate that the NK population normally depleted by these two antibodies are not totally responsible for the generated LAK cells and supports the hypothesis that both NK cells and T cells contribute to LAK cell activity.

The AK cells generated in our experiments differed somewhat from those described by Kärre et.al. (10), in that they did not retain NK target cell specificity. This could be explained on the basis of the data of Brooks et.al. (5) which indicate that cloned antigen specific T cells can "differentiate" into NK-like cells (lysing only $NK^S$ targets) or into promiscuous cells, (capable of lysing $NK^R$ targets) depending on the amount of T cell growth factor (IL-2) in the culture conditions. Thus, it is conceivable that under our conditions the cells developed into the latter type of killer cells. Like Kärre et.al. (8), we found that $AGM1^+$ NK cells were required for this generation, as measured by complement depletion, confirming their results with NK alloantisera and soya bean agglutination depletion. The smaller effect seen with 49H.8 mAb and complement depletion could be explained on the basis that the 49H.8 mAb recognizes a somewhat different population of cells (ie - has less T cell reactivity than α-AGM1), and that both the $AGM1^+$ NK cells and T cells may be required for the generation of AK cells. In terms of CTL depletion, our

results are in agreement with earlier reports that α-AGM1 does react with CTL's, although a higher concentration is required for this depletion compared to splenic NK depletion. The 49H.8 mAb also was effective in partially depleting CTL activity.

Our initial interest in testing the 49H.8 mAb against NK cells was based on its crossreactivity with the terminal disaccharide of the AGM1 molecule, however, it now seems less probable that the two antibodies are reacting against the same determinant. This interpretation is based on the difference in ontogony on thymocytes noted above, as well as several other findings: 1) While Ø-β-gal blocks 49H.8 binding it does not inhibit the α-AGM1 reactivity. Although this does not rule out that the two antibodies are reacting with the same macromolecule, this data is consistent with the view that two different determinants are being recognized. 2) We tested 75 hybridoma supernatants which contained antibodies against the $AGM_1$ terminal disaccharide (βGal(1 3)βGalNAC) as tested by an ELISA to this antigen, and none of them were able to deplete splenic NK activity in the presence of complement, although several of these supernatants did bind to the RNK tumor line. 3) The 49H.8 mAb reacts with both neuraminidase treated B and T cells (1); certainly this increase in binding cannot be attributed to AGM1 binding, since this antigen is not found on B cells, and is also not exposed by V. cholera neuraminidase treatment. 4) α-AGM1 antisera did not block 49H.8 binding to spleen cells.

The reactivity of 49H.8 mAb with mature thymocytes and NK cells should not be taken as evidence linking NK cells to the T cell

lineage. Markers such as the Thy-1, commonly accepted as T cell lineage markers at least within murine species, have also been found to be expressed on bone marrow cells which bear no relationship to the T cell lineage (24,25).

The biochemical nature of the 49H.8 reacting antigen on thymocytes and NK cells is currently being investigated. It could potentially be a determinant present on both a glycolipid and a glycoprotein. Carbohydrate determinant sharing between glycoproteins and glycolipids is a common occurrence, as evidenced by the human ABO blood group antigens (26) and as well by the human NK reactive HNK-1 (Leu 7) antigen (27). Changes in carbohydrate sequences on glycoproteins and glycolipids during differentiation are also common, as exemplified by changes in sialic content on the Thy1 glycoprotein (28) and branching during i-I differentiation on erythocyte glycolipids (26). Thus, the 49H.8 mAb possibly recognizes a differentiation antigen on thymocytes which is absent on peripheral T cells. Its reactivity against NK cells could be due to the presence of the same cell membrane molecule, or the same carbohydrate determinant on a different molecule. Biochemical studies currently underway will help to resolve this question.

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DISCUSSION

In Chapter 1 we have shown that NAb binding to tumors correlates with their in vivo elimination when injected intraperitoneally, making use of the tumor cell surface modulating properties of interferon. Although we have not examined the actual NAb reactive antigens whose expression is increased by interferon, we suggested that it may in fact be the MHC related antigens. The availability of monoclonal NAb (mNAb), from our laboratories, as well as those obtained recently by Czerny-Provaznik et. al. (1,2), which have been made available to us, will provide an opportunity to look at this question in more detail. One of their mNAb's was shown to react with syngeneic H-2D and H-2K determinants, and it can now be tested for in vivo effect with normal and interferon treated cells. We were not able to produce the H-$2^d$ NAb specificity found in serum absorption studies in mNAb from spleen cell populations, possibly because of the low frequency of the B cell population producing this specificity. In their studies, Czerny-Provaznik et. al. (1,2) found that these specificities were not easily obtained.

The fact that the peritoneal cavitity is a rich source of NAb reactive with the tumor used for the in vivo studies may be due to the fact that this site is characterized by a larger population of the Ly 1 B cells (3). Hayakawa et.al. (3) have established that the spontaneous production of autoantibodies is predominantly produced by Ly $1^+$ B cells, which account for 2% of splenic B cells and 10% of peritoneal B cells. We do not know if all tumor reactive antibodies

are in fact produced by Ly $1^+$ B cells, but the difference in B cell composition in the spleen and peritoneum may result in the preferential production of different sets of specificities. We performed only one experiment with peritoneal cells, but the fact that we were able to obtain one mNAb that was tumor reactive and not thymocyte reactive, a finding not obtained with splenic B cells, would support this possibility. The fusion approach would also allow us to look in more detail at the predominant B cell population producing tumor reactive NAb of different specificities by separating different B cell populations before the fusions are performed.

In terms of NAb specificities and the origin of these, it would be of interest to compare our tumor reactive NAb to those obtained by others reactive with serum proteins and intracellular structures (4-6). It was suggested by Dr. Avrameas (personal communication) that a mNAb bank be set up so that mNAb's from different laboratories could be compared.

The recent findings obtained in the laboratory of Dr. D. A. Chow (7) that NAb may mediate ADCC against tumors, and those to Warner and Dennert (8) using bone marrow, are of great interest in that they provide an effector mechanism whereby NAb may be functioning in vivo, which is not complement dependent. Previous studies from our laboratory had shown that tumor elimination was not diminished in complement depleted mice, using cobra venom factor (a C3 activator which produces C3 depletion) and C5 deficient mice (9). The mNAb will also be useful in the study of NAb mediated ADCC.

We have focused our attention in the last two chapters on a

monoclonal antibody mAb, 49H.8, which we have used as a model of murine NAb's. We have not determined as yet the nature of the cell surface antigens which it recognizes, although we have ruled out the Thy 1, PNA receptor and AGM1. Current studies in the laboratories of Dr. R. McGarry (University of Calgary) and Dr. R. Hyman (Salk Institute) are investigating this question in more detail. In terms of its thymocyte reactivity, the 49H.8 mAb is of interest in that it detects a differentiation marker on adult thymocytes, which is absent on pheripheral T cells. Collaborative studies with the laboratory of Dr. B. Mathieson (NIH, Frederick, Md) are currently underway, in which the thymocyte reactivity of this antibody is being examined in terms of other thymocyte markers.

Although we were initially interested in this mAb as a model of thymocyte reactive NAb, we have also pursued its reactivity with a subpopulation of spleen cells. Other studies, using NTA of autoimmune NZB mice did indicate that a subpopulation of peripheral T cells (in this case, suppressor T cells) was also recognized by NTA (10). We have not looked at 49H.8 and suppressor T cell reactivity, but have found that it recognizes the NK cell subset. Studies requiring the isolation of NK cells in murine strains have been hampered by the lack of good markers. The NK reactivity of the 49H.8 mAb is currently being examined by Dr. B. Mathieson and Dr. J. Ortaldo (NIH, Frederick, Maryland), to confirm our findings, as well as extend them to purified LGL fractions from different sources, such as liver and spleen. In addition, Drs. P. Ernst and J. Bienenstock are examining the reactivity of the 49H.8 mAb with

intestinal epithelial NK cells. Since they have identified both AGM1$^+$ and AGM1$^-$ NK cells (11), it will be of interest to see if the AGM1 and 49H.8 reactivities can be dissociated. We are currently examining the usefulness of the 49H.8 mAb for the isolation of LGL/NK cells in murine strains.

In summary, we have provided additional evidence supporting the hypthesis that NAb participate in tumor resistance, and established hybridoma lines secreting mNAb. In addition, we have detected a nes thymocyte differentiation antigen, as well as another murine NK cell marker. These studies have led to several collaborations, which will seek to confirm and extend these findings.

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## CLAIM TO ORIGINALITY

The work presented in this thesis represents original contributions to scientific knowledge in the following areas: 1) A tumor cell's sensitivity to natural antibodies in vitro and in vivo may be enhanced after in vitro interferon treatment. 2) This interferon induced inhancement correlates with in vivo tumor elimination. Although previous studies had demonstrated a relationship between natural antibody sensitivity and natural resistance, our studies were the first to show this in a system where the natural resistance was restricted to one mechanism by the slection of a cloned tumor line. 3) A monoclonal antibody (49H.8) was identified whose specificity was similar to natural thymocytotoxic antibodies. 4) The 49H.8 monoclonal antibody detects a thymocyte differentiation antigen which is different from previously identified markers. 5) This same antibody also identifies a splenic natural killer cell population with a reactivity pattern which is more restricted than the commonly used anti-asialo GM1 polyclonal rabbit antisera, although the antibody does crossreact with the terminal disaccaride of asialo GM1.