"THE ANTIBODY RESPONSE TO
TUMOR SPECIFIC ANTIGENS"

by

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## ABSTRACT

Antibodies specific for tumor antigens have been successfully induced in syngeneic (A/J), and in H-2 compatible allogeneic mice (B10·A). Cultured cells of the spontaneous A/J lymphoma L1117 were used to immunize A/J and B10·A mice. Each animal received biweekly injections of $10^6$ mitomycin C-treated L1117 cells i.p. and was partially bled from the tail vein prior to each injection. In both strains the antibody response was shown to be specifically cytotoxic for L1117 cells, and no reduction in titre could be achieved by incubation with normal lymph node cells, normal thymus cells, 1509a fibrosarcoma cells or fetal cells of A/J mice. Antibodies from serum of both strains were shown to bind specifically to L1117 cells by using fluorescein-labeled rabbit anti-mouse $F(ab')_2$ for indirect cell membrane staining.

Antibodies were present at a low level in the first test bleeding at 2 weeks and continued to rise in titre until week 10 in the B10·A strain and until week 14 in the A/J strain. The response of A/J mice was markedly slower, the maximum titre reached in both strains being approximately the same. The classes of antibodies produced were somewhat unusual, both strains producing predominantly IgM antibodies even after 20 weeks immunization while IgG antibodies accounted for only a small part of the cytotoxic activity.

It is suggested that the tumor antigen (TA) may act as a "thymus-independent" antigen, thus the weak 19S response is the maximum possible in the absence of T cell cooperation. Comparison with results obtained elsewhere for the pneumococcal polysaccaride

type III antigen (SIII) further suggest that TA might activate "suppressor" T cells which actively prevent a normal antibody response.

The ability of normal A/J and B10·A mice to reject a challenge with graded doses of viable L1117 cells was taken as a measure of their capacity to mount a cell-mediated response to TA. No evidence of cell-mediated immunity was observed in strain A, all tumor cell doses tested being rapidly fatal to the mice. In contrast B10·A mice rejected the same challenge doses in all cases. The parallel between the ability of immunized B10·A mice to produce 7S antibody and the ability of normal B10·A mice to reject viable tumor cells may indicate that in this strain TA of L1117 cells activates helper T cells to a greater extent than in strain A mice.

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# CHAPTER I

# INTRODUCTION

Considerable evidence exists indicating that malignant cells possess specific antigenic determinants which appear as new antigenic moieties on cells transformed by viruses (6,7) or chemical carcinogens (4,5). Moreover it has been shown repeatedly that the tumor-bearing host is capable of recognizing these new antigens and producing a demonstrable response. As early as 1953 Foley (1) used tumor ligation and release to immunize the autochthonous host against subsequent tumor challenge, work that was substantiated by the classic experiments of Prehn and Main (2) and by the work of G. Klein and his associates (3). These experiments used rejection of tumor transplants to show immunity, consequently the antigens so identified became known as tumor-specific transplantation antigens (TSTA). Since that time a variety of in vivo and in vitro assays have been used, and have given rise to the more general designations tumor associated antigen (TAA) and/or tumor specific antigen (TSA). This report deals with the antibody response to an antigen present on murine lymphoma cells which cannot be detected on normal cells of the same strain, so the term tumor antigen (TA) has been chosen for use throughout as a general term including TSTA, TAA and TSA.

Thymus derived lymphocytes (T cells) have been regarded as primarily responsible for tumor rejection, and it has been shown that immunity to a chemically-induced tumor could be transfered with cells (4). Antibodies have traditionally been viewed as antagonistic to cell-mediated defenses because of their ability to enhance tumor growth or graft survival (35).

Reports of enhancement of tumor graft survival by antibody led

the Hellströms (29-32) to search for a similar effect protecting growing tumors *in vivo*. Initial reports seemed to indicate the existance of a "blocking antibody" in the circulation of tumor-bearing subjects which could specifically block their immune lymphocytes from destroying tumor cells. Later studies into the nature of the blocking factor suggest antigen (TA) or antigen-antibody complexes as being responsible.

Antibody directed to tumor specific antigens may also be cytotoxic under the appropriate conditions, and has been used to passively immunize mice against a syngeneic Gross virus induced tumor (38). In other systems a cytotoxic antibody response has been observed during progressive tumor growth, and no satisfactory explanation has been proposed for its lack of effectiveness (36).

Recent reports of antibody dependent cell-mediated cytotoxicity (ADCC) suggest another way in which antibody may be involved in the host response to tumors. It has been shown that antibodies directed to TA can potentiate tumor cell destruction by normal lymphocytes (43). Several cell types may be capable of participating in this phenomenon including macrophages and monocytes, possibly B cells, and a non-T, non-Ig-bearing cell tentatively designated a K cell (45).

Despite the potential for tumor destruction indicated by the presence of cytotoxic T cells, cytotoxic antibodies, and ADCC, neoplastic growths are often fatal to the host. Some progress is being made toward elucidating the processes regulating the immune response, notably in the area of regulatory T cells. Much work remains however, and the role of antibody in promoting or opposing tumor growth is not

yet clear.

Two possible modes of tumor destruction, cytotoxic antibodies and antibody dependent cellular cytotoxicity, have already been mentioned. Promotion of tumor growth could occur through a blocking action similar to that envisioned by the Hellströms (29-32); or by alteration of the tumor cell itself through the mechanism of antigenic modulation (76); or by antibody mediated suppression of the immune response (35). The point of action of the latter is still unknown but several reports have appeared in which specific antibody, passively or actively induced, suppressed antibody formation (35), or delayed hypersensitivity (68) in a highly specific manner. High affinity antibody produced in response to tumor antigens may be present in quantities too small to destroy tumor cells yet sufficient to suppress further antibody production (67). The combined effects of antibody mediated suppression and antigen-mediated suppression may then in turn eliminate cellular cytotoxicity (70) allowing tumor growth.

Such hypotheses must be modified or discarded as new data become available, however the fact of antibody involvement in tumor immunology is evident despite uncertainty as to its role. The present study was undertaken in order to examine the nature of the antibody response to tumor antigens and some properties of the antibodies produced.

The study reported here examines the antibody response to tumor antigen(s) of a murine lymphoma in two strains of mice, one allogeneic and the other syngeneic to the tumor. The model chosen for study employs a lymphoma of A/J mice discovered in this laboratory and designated L1117, and examines the humoral immune response in mice of A/J

and B10·A strains. Mice of strain B10·A are congenic with those of C57B1/10J, but carry the H-2 locus of strain A. Viable tumor cells were treated to render them incapable of replication then used to immunize both strains of mice by identical protocols. The results indicate that both strains respond to the tumor cell antigen(s) in a highly specific manner, producing an antiserum which will lyse L1117 lymphoma cells in the presence of complement but having no activity against normal A/J cells nor against an unrelated tumor. Both antisera were analyzed as to kinetics of response, specificity of cytotoxic activity, immunoglobulin class(es) involved, and reactivity with antigens on fetal cells of the A/J strain.

# CHAPTER II

## REVIEW OF PERTINENT LITERATURE

# 1. TUMOR CELL ANTIGENS

The relevance of immunology to neoplasia hinges upon the existence of tumor antigens (TA), new cell surface antigens which did not exist on the tissue from which the tumor arose. The existence of such antigens was first proposed many years ago, but it did not gain general acceptance until the advent of inbred strains permitted transplantation of tumors between genetically identical animals. Foley (1) gave the first clear demonstration of tumor-specific immunity utilizing a methylcholanthrene-induced sarcoma in an established strain of inbred mice. Ligation and release of a growing tumor was followed by challenge with a graft from the same tumor. Animals rejecting this graft showed resistance to subsequent challenges with viable cells from the same sarcoma but not against unrelated tumors. Prehn and Main (2) confirmed and extended Foley's work. To eliminate any possibility that genetic differences were responsible for Foley's observations they repeated a similar series of experiments testing syngeneity by reciprocal skin grafting and demonstrating that preimmunization with tumor did not produce resistance to grafts of donor skin nor did skin grafts protect against subsequent tumor challenge. Any remaining doubts about minor antigenic differences due to heterozygosity of inbred strains were removed by the work of Klein and his coworkers (3) who immunized animals with irradiated cells from their own primary tumor.

Animal studies have indicated that spontaneous tumors, virus-induced and chemically-induced tumors all possess TA, but with one basic difference. Chemically-induced tumors each have a distinct antigen which does not cross-react even with tumors induced by the same

chemical (4,5). Virus-induced tumors also possess a distinct TA but in addition exhibit a virus-specific antigen which is cross-reactive for all tumors induced by that virus (6,7). Presumably "spontaneous" tumors would belong to one of these two groups.

Of particular interest in the present investigation are studies of viral and cell-surface neoantigens associated with virus-induced murine leukemias since preliminary work, published earlier, indicated a viral etiology for the L1117 lymphoma (8). Pasternak (9) studied virus-associated antigens in a myeloid Graffi leukemia. He found that Landschütz sarcoma I cells, which naturally harbour the virus, were capable of removing virus-neutralizing activity upon incubation with Graffi immune serum but did not decrease its ability to stain Graffi virus-infected cells in the indirect immunofluorescence test. In contrast, the original myeloid Graffi leukemia cells removed both virus-neutralizing and staining ability from Graffi immune serum. This would indicate that the original leukemia cells possessed two distinct neo-antigens, one of which was a viral antigen and the other a surface membrane antigen expressed by cells transformed by the virus.

Aoki et al. (10,11) found similar results in the case of Gross leukemia cells. Studying E ♂ G2 leukemia cells with anti-ferritin hybrid antibodies and electron microscopy they were able to show that Gross cell surface antigen, specified by Gross virus, was present on infected cells, but neither the Gross cell surface antigen nor a number of normal alloantigens were present on the viral envelope. They also noted that antiserum produced in the highly resistant C57Bl/6 strain reacts only with infected cells,

not viral envelope, and is deficient in virus neutralizing activity, probably indicating a specificity for Gross cell surface antigen. This is in contrast to the considerable neutralizing ability of antisera to Friend, Moloney, Rauscher, or Graffi leukemia virus prepared in a similar manner. The authors speculate that the lack of anti-virus antigen activity may be related to the fact that only Gross virus has been convincingly implicated in naturally occuring mouse leukemia.

Thus it appears that virus-induced murine leukemias may express two distinct types of tumor-associated antigen: a virus-specified cell surface antigen; and a virion antigen. Antisera to these leukemias could recognize either or both of these antigens and would still be considered tumor-specific in that they recognized no antigens present on normal cells of the same mouse strain.

## 2. HOST RESPONSES TO TUMOR ANTIGENS

### A. CELLULAR RESPONSES

Cell-mediated cytotoxicity is primarily responsible for allograft rejection, and this also appears to hold true for tumor rejection. Thymus-derived lymphocytes (T cells) have been shown to be capable of specific cytotoxicity following sensitization in vivo to alloantigens (12,13). This killing is not known to require any accessory cells or factors (13) and thus corresponds well with the observations of Old, Boyse et al. (4) that immunity to chemically induced tumors could be transfered with cells. Similarly, Le Clerc et al. (14) have shown the presence of lytic T cells in a syngeneic model with a murine sarcoma virus tumor.

Controversy still exists over whether or not the lymphocytes of a tumor-bearing host are cytolytic in vivo for the growing tumor. The Hellströms and their coworkers have published numerous reports supporting the existence of such cytolytic cells (29,31) while Mikulska, Smith and Alexander (17) find active cells only if animals are examined three weeks after surgical tumor excision; spleen cells collected while the tumor was still in place showed no cytolytic activity. The difficulty in comparing and evaluating conflicting reports on this subject stems from the variety of assay systems employed by various authors. The Hellströms have used the colony inhibition assay (CI) (18) and the microcytotoxicity test (MA) of Takasugi and Klein (19) both of which require extensive cell culture periods. This has left their work open to the charge that lymphocytes are sensitized to the tumor cells in vitro but are

not sensitized in the tumor-bearing host. Mikulska et al. (17) used a neutralization assay in which spleen cells were mixed with tumor cells and re-injected into the experimental animals. Tumor cells mixed with spleen cells from an immune animal or an animal whose tumor was surgically removed 3 weeks earlier did not grow, while tumor cells mixed with spleen cells from a tumor-bearing animal did grow. In an attempt to resolve these difficulties Plata and Levy (20,21) have recently published a series of studies on the effector cells in the MA and chromium release test (CRT). Their data indicate a fundamental difference in the nature of the two tests. Microcytotoxicity assay requires a 48 hours incubation in vitro and measures a cytostatic, rather than cytolytic activity; it involves both T and non-T effector cells; it is inhibited by serum from tumor-bearing animals; and it shows a biphasic response with an intermediate period of no reactivity which corresponds to maximum tumor size. Chromium release test requires an 18 hour incubation in vitro and measures cytotoxic activity; it involves only T cells; it is not inhibited by serum from tumor-bearing animals; and it shows a monophasic response with maximum activity coinciding with maximum tumor size. Unfortunately, two factors make generalizations based on this data somewhat doubtful. In the first place the tumor chosen, a murine sarcoma virus induced tumor, regressed spontaneously starting approximately 15 days after injection and disappearing completely by day 25. All experimental subjects are thus capable of recognizing and rejecting the tumor, which may not be the case with progressively growing lethal tumors. Secondly it

was necessary to use two different target cell types for the two assays, neither of which were the tumor under study although all three carried the FMRGi antigen. The ascitic lymphoma used in CRT is non-adherent and hence not suitable for MA; the fibroblastic tumor cells used in MA could not be lysed by immune lymphocytes from MSV-tumor-bearing animals so were not suitable for CRT target cells.

However from this evidence, and some cell fractionation experiments using anti-Ig-coated glass bead columns or anti-$\theta$ treatment (20), it does seem clear that the two tests are measuring activities of separate cell populations. They conclude that MA detects T and non-T effector cells while CRT detects only effector T cells, but a different T cell population than that active in MA.

Cell-mediated cytotoxicity, effected by one or more T cell populations, thus is capable of destroying living tumor cells and can clearly effect rejection in an immune animal or upon transfer from an immune to a tumor-bearing animal. What is still unclear is the role played by these cells in the case of a progressively growing tumor which eventually destroys the host.

B. ANTIBODY-MEDIATED RESPONSES

(i) ANTIBODY

Cellular immunity, primarily T cell mediated cytotoxicity, has been regarded for some time as the principal anti-tumor defense while antibodies were regarded as ineffective or antagonistic, perhaps causing enhancement of tumor growth. Recent studies have shown this view to be an oversimplification.

Uhr and Möller (35) have defined immunological enhancement as "the prolonged survival of normal or neoplastic tissue grafts in histoincompatible recipients, which have been pretreated with antibodies directed against the graft or, alternatively, which have been presensitized with tissue of the graft genotype". Kaliss (22) showed that the active factor was circulating antibody directed against the incompatible antigens. By contrasting these results with those obtained for cell-mediated immunity it is readily apparent how the idea arose that immune lymphocytes, whether actively or passively induced, produced protection while specific antibody, again either actively or passively supplied, caused enhanced tumor growth. Initial studies were done with transplantation antigens, primarily H-2, but G. Möller (25) showed that anti-tumor antiserum could cause enhanced tumor growth in vivo. Gorer and Kaliss (23) had already shown that the result of passive administration of antiserum was not always enhancement, but depended rather upon the sensitivity of transplanted cells to lysis by antibody plus complement. However several papers by E. Möller (26-28) indicated that enhancement may have an important role in maintaining

autochthonous tumors and this led to the extensive studies by the Hellströms and their associates into the role of anti-tumor antibodies in tumor survival. In a review of their early work, Hellström and Hellström (30) describe a series of experiments done with mouse, rabbit, and human tumors in which it was shown that lymphocytes from subjects which had rejected a tumor were capable of significantly inhibiting tumor cell growth in the colony inhibition assay. Much to their surprise, however, lymphocytes from tumor-bearing subjects were equally effective. Prompted by the work on enhancement they examined the effect of serum from the various experimental subjects. Serum from normal subjects or those which had rejected the tumor showed no effect on either lymphocyte sample, however serum from subjects bearing a progressively growing tumor blocked the ability of lymphocytes to inhibit tumor cell colony growth. (29) This serum blocking factor showed specificity for tumor cell type, and could be removed by absorption with the corresponding tumor cells. Serum antibody was further implicated by the fact that activity could be removed by precipitation with anti-immunoglobulin antiserum, and that activity resides in the 7S fraction of serum.

Subsequent work indicated a more complex situation. Low pH eluates from human tumor cells (31), or fractions prepared by absorption and elution of tumor-bearing-mouse serum from corresponding tumor cells (32), could be separated into two fractions by ultrafiltration at low pH. If the fractions were added to the target cells for 45 minutes then removed, neither alone had any effect on cytotoxicity by sensitized lymphocytes while a 1:1 mixture

effectively blocked lymphocyte activity. This led them to propose that the blocking factor was an antigen-antibody complex rather than antibody alone.

Several facts could not be easily reconciled with this theory: blocking activity disappeared rapidly from serum following surgical removal of tumor, to be replaced by cytotoxic antibody (30); and the low-molecular weight eluate, but not the high molecular weight one, could block alone if allowed to remain in contact with lymphocytes and tumor cells for the duration of the assay.

An alternative explanation is that the active blocking factor is tumor antigen. According to this hypothesis, in the presence of an active immune response much of this antigen exists bound to serum antibody and consequently is localized in the 7S fraction of serum and can be bound to and eluted from tumor cells. Rapid disappearance of blocking activity following tumor removal would be due to loss of the source of antigen, antibody-bound antigen being rapidly removed from circulation leaving only cytotoxic antibodies whose production would continue for some time. Activity of the low pH eluates could be explained as follows: brief incubation of tumor cells with antibody or antibody-antigen complexes would allow binding and subsequent transfer to the microcytotoxicity assay where antigen from the complex blocks lymphocyte action but neither preincubation of tumor cells with antibody alone nor antigen alone would transfer antigen to the assay; if the 3 samples are added directly to the microcytotoxicity assay antigen alone or antigen-antibody complexes should both produce blocking, as was observed.

Arguments of this type have made "blocking factor" an unpopular

topic, despite widespread initial enthusiasm for the concept. It would be wrong however to assume that if blocking is due to antigen released by tumor cells, anti-tumor antibody is unimportant to the host response. Specific graft enhancement by antibody is well-documented; and Vánky et al. (33) have shown that preincubation of sarcoma cells with autochthonous serum completely abolished their ability to stimulate autochthonous lymphocytes as measured by uptake of radioactive thymidine. Even if antibody is shown not to be relevant to the blocking observed by the Hellströms, antibody or antibody-antigen complexes may have an important role in regulation of the immune response, a topic which is discussed in a later section.

The Hellströms also reported that following regression of a tumor cytotoxic antibodies could be detected in the circulation (30). Old, Boyse and Lilly (37) made similar observations in a mouse tumor system. Studying strains of mice normally susceptible to tumor induction by Friend virus they observed occasional tumor rejection. Serum from these mice could be shown to be cytotoxic for tumor cells in vitro in the presence of complement. In vitro cytotoxicity does not always correlate with protection in vivo, however in another study (38) they showed that an antiserum specific for the G antigen could be used to passively immunize mice against challenge with a syngeneic Gross tumor.

Herberman and Oren (36) also studied a Gross virus induced tumor in W/Fu rats. This tumor is strongly antigenic and regressed in 65 percent of the recipients within two weeks. In the other rats the tumor grew progressively or regressed after an extended period of

time. All rats produced specific, highly cytotoxic antibodies but these did not correlate with protection in vivo. The responses all showed a biphasic response in which the first peak could be shown to be 19S antibodies and the second peak 7S antibodies. The titre and duration of the secondary response was proportional to the duration of tumor growth, the highest titre being present in rats which died shortly afterward. It would appear that in this case cytotoxic antibody was produced in response to continued antigenic stimulus but conferred no protection on the host animal.

Several explanations have been offered to account for the apparent ineffectiveness of such antiserum which is so strongly cytotoxic in vitro yet provides no protection in vivo. Gorer and Kaliss (23) noted in an early study on enhancement of murine sarcoma that the ultimate effect often depended upon the amount of antiserum given; while small doses enhanced tumor growth, larger doses could be cytotoxic. Similar results were obtained by Möller (39) with leukemia cells. The resistance or susceptibility of cells to lysis by antibody plus complement is also a factor to be considered. Growth of cells highly resistant to lysis by antibody due to decreased surface antigen (40) would presumably be more readily enhanced, while more susceptible cells would be destroyed by the presence of the same antiserum.

Results obtained in the AKR strain of mice, which is highly susceptible to leukemia, point up the importance of factors external to the tumor cell — antibody system. It was found that infusion of normal serum into AKR mice bearing spontaneous leukemias caused rapid destruction of leukemia cells. Evidence points to a complement

component deficiency as being responsible, in this case probably C5 (41). The authors also refer to preliminary experiments which indicate that complement may be the limiting factor in determining the effectiveness of antibody-mediated tumor cell destruction in the mouse.

These explanations may help to account for the ineffectiveness of antibody is some situations but they do not exclude the possibility that cytotoxic antibody has a role in tumor cell destruction, especially with susceptible cells such as leukemias (40), in situations where the accessory systems, such as complement, are adequate.

(ii) ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY

Antibodies may exert a third effect, in addition to the cytotoxic and enhancing properties already discussed. Recent reports have indicated that specific antibody is capable of potentiating the destruction of corresponding target cells by non-immune lymphocytes, a phenomenon known as antibody-dependent cellular cytotoxicity (ADCC). Two excellent reviews by Perlmann, Perlmann and Wigzell (46), and by MacLennan (47) appeared in Transplantation Reviews in 1972 covering the important work in this field to that date, a brief summary of which follows below.

Perlmann and Holm first showed clearly that non-immune lymphocytes could lyse target cells in the presence of anti-target cell antibodies. This required IgG antibodies with an intact Fc piece (IgM was ineffective) and was specific for the cells bound by the antibodies. It could also be shown to occur with antibody concentrations much lower than those required for complement-mediated lysis. The effector cells possessed receptors for Fc, and could be removed from cell suspensions on anti-Ig coated glass bead columns. This could indicate the presence of surface immunoglobulin or may be due to binding by the Fc receptors, however it is clear that they are not identical with antibody-producing B cells although a B cell subpopulation was not ruled out.

It was later shown (42,47) that depletion of T cells, either by treatment with anti-$\theta$ plus complement or by using spleen cells from thymectomized, irradiated, bone marrow reconstituted mice, had no effect on the ability of spleen cells to mediate ADCC.

Synergistic effects were noted when spleen cells of immune rats were used for ADCC instead of normal lymphocytes. (43) Serum from W/Fu rats immunized with a Gross virus induced lymphoma (C58NT) was capable of mediating ADCC with non-immune rat lymphocytes, but the spleen cells of these immune rats were also capable of antibody-independent cell-mediated cytotoxicity (CMC). If immune serum and immune spleen cells were combined the total effect was greater than the sum of ADCC with non-immune cells and immune CMC. This phenomenon lasted only a short time after the initial immunization, later reverting to a value equal to the sum of CMC and ADCC.

The same authors studied the cell types involved in this system in a second paper (44). Addition of excess unlabeled cells did not affect ADCC of $^{51}$Cr-labeled antibody-coated cells, nor did addition of excess antibody-coated cells decrease the lysis of $^{51}$Cr-labeled cells by CMC, indicating two distinct effector cell populations. The cells responsible for CMC could be selectively removed by treatment of immune spleen cells with anti-θ plus complement or by incubation with monolayers of target cells; while those responsible for ADCC could be selectively removed by depletion of Fc receptor-bearing cells on antibody-coated sheep erythrocytes.

Macrophages and monocytes have also been reported to be capable of immunologically specific target cell destruction. Temple et al. (48) found that peritoneal exudate (PE) cells from immunized guinea pigs could kill target cells even in the presence of cytochalasin B which inhibits phagocytosis. Cytophilic antibody

could be detected on the surface of these PE cells by rosette formation and by immunofluorescence. Antibody eluted from these cells or provided in the form of immune serum could "arm" normal PE cells rendering them specifically cytotoxic for the corresponding target cells.

Specific destruction of tumor cells by macrophages from immunized mice has been reported by Evans and Alexander (49). Specifically cytotoxic macrophages can be obtained from the peritoneal cavity of immunized mice; in vitro by contact of non-immune macrophages with spleen cells from hyperimmunized mice; or in vitro by exposure of non-immune macrophages to the cell-free supernatant obtained when spleen cells from immunized mice are cultured with the specific antigen. When added to tumor cell cultures bearing the corresponding antigen, such macrophages destroy the target cells by membrane contact, not phagocytosis. No killing is observed if the macrophages are added to unrelated tumor cells, however if macrophages are made immune by one of the three methods described above, then exposed briefly to the corresponding antigen, they became non-specifically cytotoxic. Presumably the sequence of events involves "arming" normal macrophages with specific antibody which binds via the Fc receptor. Binding of specific antigen to antibodies on "armed" macrophages produces "activated" macrophages which are non-specifically cytotoxic.

Reporting the results of a workshop at the Second International Congress of Immunology, MacLennan and Harding (45) proposed the following designations for antibody-mediated cellular cytotoxicity:

K cells

1. Non-adherent, non-phagocytic cells without easily demonstrable immunoglobulin, which are not identifiable with T cells and which separate with cells showing a morphological appearance of small to medium sized lymphocytes. These cells have receptors for the Fc fragment of IgG and at least some of these cells have receptors for both C3b and C3d.
2. As in (1) but showing glass adherent properties.

B cells

Some preliminary evidence implicates immunoglobulin bearing cells.

Macrophages and Monocytes

These cells can kill a variety of antibody-sensitized target cells by intra-cellular or extra-cellular lysis. The phagocytic receptor is clearly different from that on K(1) cells and the Fc receptor may also be different.

These categories are still tentative, and considerable work remains to be done on the nature of effector cells and their mechanism of action. It has been shown however that antibodies are capable of rendering normal cells of several types specifically cytotoxic for tumor cells. This suggests an important alternative to the conventional anti-tumor defenses of cytotoxic T cells and cytotoxic antibody, and a new role for antibody in tumor immunology.

# 3. ROLE OF THE IMMUNE RESPONSE IN TUMOR GROWTH

## A. GENERAL CONSIDERATION OF MECHANISMS

Host defenses against neoplastic cells have traditionally been regarded in terms of the theory of immunological surveillance originally proposed by Thomas (50) and later refined by Burnet (51). Here the primary function of the immune system is maintaining the integrity of the host by elimination of "non-self", such as invading microorganisms or neoplastic cells. Growing tumors would represent instances of failure of surveillance and a number of theories were put forward to account for this.

It had been observed (4) that a very small inoculum of antigenic tumor cells would occasionally grow while a larger dose was rejected. This was described as "sneaking through" the host defenses by avoiding detection until tumor growth outpaced the host immune system, after which time recognition was of no consequence. This seems an unlikely explanation as many observations have been made of spontaneous regressions of established tumors.

Low levels of surface antigen could render a tumor less effective at eliciting a response and less susceptible to immunological destruction. Such "immunoresistant" tumor cell lines have been reported (54), but although this may be an important factor in a few cases it is not a general observation among successful tumor cell lines.

Host factors provide another explanation. Immunodeficiency diseases, immunosuppressive therapy, and declining immune responsiveness in old age have all been correlated with an increased

incidence of neoplasms (52), but again these appear to be special cases rather than the general rule.

Voisin (55) made a major departure from this type of antagonist concept when he proposed that the immune system be regarded as a complex interaction of effector mechanisms capable of a complete spectrum of responses ranging from tolerance to rejection, for which he coined the terms "facilitation reaction" and "rejection reaction". The fate of a particular cell would therefore depend upon the extent to which it stimulated these two reactions.

Neither Voisins's theory, nor immunological surveillance provide an explanation of the fundamental processes of antigen recognition and regulation of the immune response. Consequently considerable interest has been generated by recent reports of a regulatory mechanism operating at the T cell level which can suppress both humoral (56-58) and cellular (59-64) manifestations of the immune response.

Gershon and Kondo (57) found that transfer of spleen cells from mice tolerized by large doses of sheep erythrocytes (SRBC) to thymus deprived mice (via adult thymectomy) prevented the latter from responding to a challenge dose of SRBC. A similar effect was observed with the IgE response to DNP-ascaris antigen, however in this case T cells from hyperimmune mice suppressed an ongoing anti-DNP response (56). It appears that in addition to the helper effect known to be exerted by T cells in the antibody response, there exist cells with the ability to act in the opposite manner and turn off the antibody synthesis by B cells.

Similar effects have also been observed with delayed

hypersensitivity (DH) responses to BGG in rats (59) and to picryl chloride in mice, (60) in which thymocytes from tolerized animals transfered to normal subjects inhibit the immune response to subsequent challenge with the same antigen. Also, suppression of tumor rejection, presumably a cell mediated immune reaction has been reported by Fujimoto et al. (62-64). Thymus or spleen cells from mice bearing a methylcholanthrene-induce sarcoma (1509a) can specifically suppress the immune rejection of a tumor even in hyperimmune mice. That this is due to a T cell population was shown by abrogation of the suppressive effect by treatment of cells with anti-θ or anti-thymocyte serum (ATS) plus complement prior to transfer.

It was noted above that suppressor T cells could be found either in immune (56) or tolerant animals (57). This was extended by work in the tumor system in which it was shown that two factors could be separated from spleen cells of tumor bearing mice which produced suppression or enhancement of immune rejection when used separately, but which produced suppression when used together in their original ratio. It would appear from these results that antigen stimulates both suppressive and reactive immune responses, the net result <u>in vivo</u> being determined by the degree to which each is stimulated, which in turn must be controled by such factors as the nature of the antigen and its mode of presentation.

The following section will examine some of the ways in which anti-tumor antibody may participate in tumor growth, including the possibility of a feedback-type mechanism operating on this central regulatory system.

B. ROLE OF ANTIBODY

(i) PROMOTION OF TUMOR GROWTH

Section 2B covers various aspects of the antibody response to TA and suggests several possibilities for participation in tumor growth *in vivo*. The most obvious would be through the agency of blocking factor, which was last reported to be an antigen-antibody complex (32). While the very real possibility exists that the blocking activity may be due entirely to antigen, a requirement for antibody has not been completely ruled out.

Immunological enhancement is another mechanism through which anti-tumor antibody may promote tumor growth. Enhancement has often been shown with passively administered antibody and in preimmunized subjects (35), and it is the latter especially that suggests a possible role in natural tumorigenesis and/or maintainance of established tumors. Although enhancement can be effected by precoating tumor cells *in vitro* with specific antibody (65), it appears that the effect must be on the immune response of the host rather than a change in the properties of the tumor cell itself. One of the major pieces of evidence in this regard is the requirement that enhancing antibodies be produced in the recipient strain. Tumor-specific antigens from another strain are ineffective (65). This leads to the possibility that tumor enhancement occurs through antibody mediated suppression of the immune response.

Suppression of antibody formation by passive administration of specific antibody simultaneously with antigen or shortly afterward is a well-known phenomenon (35). The suppression is highly specific and can be effected with relatively small amounts of hyperimmune

serum for the primary response, although the same quantity of antibody has little or no suppressive effect on a secondary response, much larger amounts then being required. (70)

A recent report by Birnbaum et al. (69) suggests that antibody mediated suppression also may produce tolerance in adult animals. A single intravenous dose of DNP-BGG induced a high degree of tolerance in adult mice in which a small residual population of cells produced very high affinity antibody. This effect could be duplicated in terms of both residual high affinity antibody production and the carrier specificity of the tolerant state by antibody mediated suppression induced by injections of anti-DNP-BGG. The authors propose that the initial i.v. injection of small amounts of antigen selectively stimulates production of high affinity antibody which in turn suppresses further antibody production. This induction of an antibody mediated suppressed state by injection of antigen may be relevant to the following studies on suppression of delayed hypersensitivity.

Axelrad (68) found that the primary delayed hypersensitivity response to sheep erythrocytes in rats could be partially suppressed in two ways. One was a direct suppression due to the presence of i.v. antigen and only lasted for a short time after immunization. The other was due to the presence of anti-SRBC antibodies resulting from earlier antigen injection or from passive transfer of hyperimmune serum. Neither antigen nor antibody alone could induce complete suppression while the two procedures together produced complete suppression of the delayed hypersensitivity response.

This success led to attempts to promote renal allograft survival

by a similar combination of the two procedures (70). Lewis (L) and Brown Norway (BN) rats have major histocompatibility differences. A kidney from an $F_1$ LBN donor is invariably rejected by a Lewis recipient. Treatment with L anti-BN antiserum at the time of surgery or with BN cells one day before surgery resulted in prolonged graft survival. Even when long term survival occured in individual rats considerable kidney damage usually occured. Combination of the two treatments however, produced a survival rate comparable to that for syngeneic grafts and showed no evidence of renal damage.

These studies are particularly interesting in the light of the study by Birbaum et al. (69) discussed above. If i.v. injection of antigen is capable of producing very high affinity antibody, residual antigen plus antibody might in turn suppress delayed hypersensitivity producing a state of sensitization, but non-reactivity, not unlike that observed in tumor bearing hosts. (29,30)

(ii) INHIBITION OF TUMOR GROWTH

Two possibilities exist for destruction of tumor cells by antibody-dependent processes. Serum which produced complement dependent lysis of tumor cells *in vitro* has been obtained from animals whose tumors have regressed (30,37) and from animals immunized with cells incapable of division (15). While cytotoxicity *in vitro* does not necessarily correlate with protection *in vivo*, potential for such action does exist.

Antibodies have also been shown to be capable of causing tumor cell destruction through ADCC. Macrophages, monocytes, and "K" cells may all be capable of participating in this type of cytolysis (45) in which target cell destruction is apparently effected by the cell while specificity is supplied by the antibody. (46,47)

In addition to direct destruction of tumor cells, antibody may indirectly inhibit tumor growth by promoting development of the host anti-tumor response. Studies by Dixon et al. (71) showed that the immune response to injection of soluble protein antigens is characterized by a rapid elimination of antigen from the circulation followed by the appearance of circulating antibody. For a brief interval before free antibody appears antigen-antibody complexes can be detected in the circulation and these cause no apparent suppression of the appearance of free antibody. In fact on the basis of evidence reviewed by Uhr and Moller (35) such complexes can have a positive effect on the subsequent antibody response, although this effect may be indirect, in which the antibodies act as opsonins to produce a more highly immunogenic form of the antigen.

(iii) ANTIGENIC MODULATION

The discovery by Boyse, Old and coworkers of the phenomenon of antigenic modulation introduces the possibility that specific antibody may protect tumor cells by inducing the removal of surface TA. Antigens of the TL system were discovered to exist on the thymus cells of some strains of mice (TL+) but not on other (TL-), and on leukemia cells including some from TL- strains. TL- mice were immunized with allogeneic TL+ leukemia cells on the assumption that this would make them resistant to syngeneic TL+ tumors. However if the immune mice were challenged with syngeneic TL+ leukemia cells the tumor grew progressively, killing the host (72). Examination of cells from such a tumor showed that they no longer expressed the TL antigens, and subsequent experiments showed that this was a reversible phenotypic change which occured only in the presence of anti-TL antibodies (73-75). This phenomenon was termed "antigenic modulation" by the authors and since then has been demonstrated for H-2 antigens on mouse peritoneal exudate cells (78); for Ig on mouse lymph node cells (77); for H-2 antigens on EL4 leukemia cells (77,79); and for tumor specific antigens on rat leukemia cells. (76)

Despite the diversity of systems from which these results were obtained, some generalizations can be made regarding antigen modulation:

1. A number of cell types, including normal thymus cells, lymph node cells, peritoneal cells and some neoplastic cells, have the ability to modify their surface antigens in the presence of specific antibody.
2. This is an active metabolic process inhibited by certain

drugs and by low temperatures.

3. Loss of surface antigen is specific and is reversible once the specific antibody is removed.
4. Antigen loss is a phenotypic change occuring within the lifetime of a single cell.

It seems unlikely that modulation plays a role in maintaining established tumors as these are known to express surface antigens recognizable by the host, as noted in section 1 of this review. The possiblity remains that modulation is involved in tumorigenesis at which time a weakened antigenic stimulus may alter the direction of the host response. Lost of circulating antibody following the establishment of a tolerant state, as hypothesized earlier, would then allow re-expression of TSA by the tumor cells.

# 4. SUMMARY

Since the initial work of Foley, Prehn and Main, and Klein, tumor specific antigens have been demonstrated repeatedly with both virus induced and chemically induced tumors. Today it is generally accepted that most, if not all tumors have antigens distinct from those present on the tissue from which the neoplastic growth arose, and that under the appropriate conditions the host is capable of recognizing and responding immunologically to these antigens. The response can take the form of specifically cytotoxic T cells, complement-dependent lytic antibodies, or antibody dependent cytotoxic cells, all of which have been shown to be capable of specific destruction of tumor cells. There is also the possiblity that the antibody response may produce enhancement of tumor growth, perhaps in the form of antibody-antigen complexes.

The interplay between these effector mechanisms and their net effect on tumor growth is only partially understood. Studies into the fundamental regulatory mechanisms of the immune response have revealed a level of T cell - T cell and T cell - B cell interaction through which regulatory T cells may selectively enhance or suppress each aspect of the immune response depending upon the nature of the antigenic stimulus.

Only T cell cytotoxicity is considered to be a purely cell-mediated phenomenon. All the other potential responses to a tumor antigen are antibody mediated, and there is some evidence that even the delayed hypersensitivity response can be suppressed by antibody. Despite its potential importance relatively little is shown about the antibody response to TSA. They are known to be capable

of immunologically specific cell lysis in the presence of complement; may mediate target cell lysis by non-immune lymphocytes; may enhance tumor growth when present in pre-immunized or passively immunized animals; possess the curious ability to protect target cells against antibody mediated lysis through the mechanism of antigenic modulation; and are able to suppress in a highly specific manner both delayed hypersensitivity and continued antibody production.

The aim of this study was to examine some of the aspects of the antibody response to TA in two systems, one syngeneic and the other allogeneic but H-2 identical. It is shown here that both systems produce antibodies specific for the TA although with some difference in the response kinetics. The results of studies on the class of antibody produced present some interesting questions about the immunological recognition of TA and suggest several possibilities for future research with this system.

# CHAPTER III

## NATURE OF THE PROBLEM

While *in vitro* studies have shown that anti-tumor-cell antibodies can effect tumor cell destruction *via* cell-mediated or complement-mediated mechanisms and that they are capable of tumor enhancement in passive transfer experiments, study of their actual role *in vivo* has been hindered by the fact that circulating anti-tumor antibodies are usually detectable only in animals whose tumors have regressed but not in tumor-bearing animals (30,37). It is possible that fundamental differences may exist between neoplasms which are ultimately rejected and those which grow successfully, consequently the results of studies utilizing antisera from "tumor-regressed" animals may not reflect the situation existing in a tumor-bearing subject.

The general aim of this study was to examine the nature of the humoral response to tumor antigens in order to clarify the role of the antibody response in a tumor-bearing host. Antisera from animals which had rejected a tumor were considered unsuitable for the reasons mentioned above, as an alternative a model system was chosen in which cells from a murine lymphoma (L1117) which is uniformly fatal were treated with mitomycin-C to prevent replication then used to immunize syngeneic mice (A/J). Tumor antigens are known to be weak immunogens in syngeneic hosts but some reports have appeared in which "helper" determinants attached to tumor cells enhanced the response to tumor antigens (90-92). H-2 identical allogenic mice (B10·A) possess differences from A/J in minor histocompatibility antigens which could serve as "helper" determinants for the anti-tumor response. These mice were immunized with L1117 cells in a manner identical to that used for A/J mice to determine what effect these differences might have on the anti-tumor antibody response.

The first objective was to determine whether or not the mice were capable of recognizing the tumor antigens and producing an antibody response. The presence of anti-tumor antibodies and the specificity of the response were shown by complement-mediated cytotoxicity tests, by absorption of cytotoxic activity with cells and by immunofluorescence.

The second objective was to determine the kinetics of the antibody response. Preliminary work had indicated that prolonged immunization was necessary to produce anti-TA antiserum. If this period was much greater than the survival time of tumor-bearing mice it would suggest that little if any antibody was available during tumor development and any hypothetical role for anti-TA antibody would have to take this into consideration.

Having established at this point that the mice were capable of responding to tumor antigens in a highly specific manner and that antibody was detectable, although in very small amounts, early enough to be relevant to the tumor-bearing model, the third objective was the determination of antibody classes produced during the response. Since different classes of antibody are involved in complement-mediated lysis, antibody-induced cellular cytotoxicity, and antibody-mediated tumor enhancement, the production of a single class of anti-tumor antibody would limit the possible roles for such antibodies. In addition, a knowledge of which immunoglobulin classes are produced provides information about the basic recognition processes by the host animal. An interesting example is that of the so-called T-independent antigens which produce a prolonged IgM response but little or no IgG antibody (93-96). This contrasts strikingly with

the normal T-dependent response to alloantigens which is characterized by a transient IgM response and a prolonged, elevated IgG response. Therefore analysis of the antibody classes produced could also provide information about the type of immunorecognition systems involved.

In summary then, this study was undertaken with the general aim of clarifying the role of anti-tumor antibodies in the tumor-bearing host through the use of a model system. Three aspects of the problem were examined: the ability of the mice to produce specific antibodies; the kinetics of the antibody response; and the classes of immunoglobulin produced during the response.

# CHAPTER IV

# MATERIALS AND METHODS

1. Animals: Mice of inbred strains A/J, B10·A/SgSn, C57Bl/6J and C3H/HeJ were purchased from Jackson Laboratory, Bar Harbour, Maine. Rabbits used for production of heterologous antisera were New Zealand White rabbits purchased from North American Laboratory Supply, Gunton, Manitoba.

2. Characteristics of tumors: The lymphoma L1117, discovered in this laboratory, has been shown to be derived from thymus cells of A/J mice. It has been maintained by i.p. transfer into 3- to 5-month-old A/J mice in vivo and by culture in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) (8).

   The sarcoma 1509a induced by methylcholanthrene in A/J mice was donated by Dr. P. Wright of the National Institutes of Health, Bethesda, Md. For propagation of the tumor, $10^5$ sarcoma cells grown in the peritoneum of tumor-bearing donors and harvested in the ascitic fluid were transfered i.p. into 3-month-old mice or were cultured in RPMI 1640 medium (Grand Island Biological Co., Long Island, N.Y.) supplemented with 5% FCS. After 3 subcultures the cells could be successfully transfered in vivo, a minimum dose of $10^4$ cells being necessary for subcutaneous transfer and killing the host within 2 months.

3. Cell Culture: L1117 lymphoma cells obtained from lymph nodes of tumor-bearing mice were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (RPMI c 5% FCS) and have been subcultured 11 times to date.

4. B10·A anti-L1117 antiserum: B10·A mice were immunized bi-weekly with a minimum of $10^6$ mitomycin-C treated L1117 cells per mouse. The first injection was into the footpads with an emulsion in

complete Freund's adjuvant (CFA), the second was of cells suspended in Hank's solution injected subcutaneously (s.c.), and all subsequent injections were i.p. with cells suspended in Hank's solution. Serum samples were collected bi-weekly by tail bleeding prior to immunization then stored at -30°C.

5. A/J anti-L1117 antiserum: A/J mice were immunized and bled according to the same protocol used for B10·A mice, sera were stored at -30°C.

6. Alloantisera: Antiserum to A/Jax histocompatibility antigens was prepared by immunization of C3H/HeJ mice by 7 weekly injections of $10^6$ A/J spleen cells. Serum was collected, pooled, and stored at -30°C. A/J anti-C57Bl/6 antiserum was prepared in the same manner.

7. Antisera to mouse immunoglobulins: Specific rabbit anti-mouse IgG1 and rabbit anti-mouse IgG2a were gifts of Dr. B. Carter of this department.

Anti-mouse IgG2 antiserum was prepared by immunizing a rabbit with 3 biweekly injections of 120 µg protein of mouse IgG2 emulsified in CFA; mouse IgG2 had been obtained by preparative agar block electrophoresis.

Anti-mouse µ-chain antiserum was prepared by immunizing a rabbit with 3 biweekly injections of 90 µg protein of mouse IgM emulsified in CFA. Antiserum obtained 10 days after the last injection was absorbed with an immunosorbent consisting of Sepharose 4B-coupled mouse 7S immunoglobulins to remove anti-light chain activity. Mouse IgM used for immunization was obtained as follows: normal mouse serum was precipitated 3

times with 50% saturated ammonium sulfate then concentrated by ultracentrifugation at 100,000 x G for 4 hours in a Beckman L2-65B preparative ultra centrifuge. The pellet was resuspended in borate-buffered saline pH 8.0 (BBS) then applied to a Sephadex G200 column to isolate the macroglobulin fraction. This fraction was further purified by preparative agar block electrophoresis.

8. Antiserum to mouse $F(ab')_2$ fragments: This antiserum was prepared by immunizing a rabbit with $F(ab')_2$ fragments prepared by pepsin digestion of a mouse IgG2 sample as described by Fujimoto et al. (8).

9. Gel filtration: Sera were fractionated by gel filtration using a column of Sephadex G200 (2.5 x 100 cm, Pharmacia, Uppsala, Sweden). Elution was carried out with BBS at an upward flow rate of 18 ml/hr. Three fractions, corresponding to serum proteins with sedimentation coefficients of approximately 19S, 7S, and 4S, were collected and concentrated separately by negative pressure dialysis.

10. Agar block electrophoresis: Serum components were fractionated by preparative agar block electrophoresis in barbital buffer (pH 8.6, $\mu$=0.05). Samples were applied to the center of a 1.5 x 8 x 30 cm agar block (0.85%) and run at 100 mA for 26 hours.

11. Immunosorbents: Immunoglobulins were coupled to Sepharose 4B using the method of Cuatrecases (83). Sepharose 4B was washed with 0.1 M carbonate buffer (pH 9.0) (CB) and glycine-HCl buffer (pH 2.8) (GHB) and resuspended in CB. CNBr solution was added (100 mg CNBr/ml packed Sepharose) and the pH was adjusted to

11.0 with 4N NaOH and held there for 10 minutes. The activated Sepharose was washed with cold CB (100x vol. of Sepharose) on a Buchner funnel, then added to the immunoglobulin solution (2 mg protein/ml Sepharose in CB equal to volume of packed Sepharose) and incubated 18 hours at 4°C on a rotator. This coupled immunosorbent was washed with PBS, then once with GHB, then again with PBS until O.D. 280 nm $\leq$ 0.01. Each sample was added, incubated 18 hours at 4°C with rotating, then washed with PBS until O.D. 280 nm $\leq$ 0.01. Dissociation was accomplished by elution with GHB at 4°C. Eluates were neutralized immediately, dialyzed against PBS, then concentrated to their original volume by negative pressure dialysis.

12. $^{51}$Cr-release cytotoxicity test:

(a) Standard assay: This was performed by a modification of the method of Wigzell (80). 2x $10^7$ target cells in 1 ml of Veronal buffer containing 10% FCS were incubated for 30 minutes at 37°C with 100 µCi of $^{51}$Cr as sodium chromate (Atomic Energy of Canada Ltd., Ottawa). The cells were washed 5 times then resuspended in Veronal/10% FCS to a concentration of 1 x $10^6$ cells/ml. Serial dilutions of 0.1 ml volumes of the test antisera were prepared in tissue culture plates (Microtest II, Falcon Plastics, Oxnard, Calif.) and 0.1 ml of a suspension of labeled cells ($10^5$ cells) added to each well. As a complement source, 0.1 ml of guinea pig serum (fresh or stored at -70°C), suitably diluted with Veronal/10% FCS was added to each well. The plates were covered with an adhesive sealer, mixed well, and incubated at 37°C for 40 minutes. The plates were then

centrifuged at 200 x G for 8 minutes and 0.1 ml. samples of the supernatant from each well were counted in a well-type scintillation counter (model 4230, Nuclear Chicago Corp., DesPlaines, Ill.) to measure the radioactivity released into the medium.

The percentage of target cells lysed by the antiserum was calculated as 100 x (E - C)/(T - C), where: E = counts per minute (cpm) in the supernatant of the experimental sample; C = cpm in the highest control ( cells $\bar{c}$ buffer, Ab or C); and T=cpm released by a strongly cytotoxic antiserum (approximately 80% of that released by repeated freezing and thawing).

(b) Preincubation assay: Serial dilutions of 0.1 ml volumes of antisera were prepared and 0.1 ml of labeled cells added as in the standard assay. The plate was then covered, mixed and incubated for 45 minutes at $4^{o}$C. The plate was centrifuged 200 x G for 8 minutes, the supernatant removed, and the cells washed once with Veronal/10% FCS. 0.2 ml of suitably diluted guinea pig serum was added as a source of complement, the plates again covered with an adhesive sealer, then incubated, centrifuged and counted as in the standard assay.

13. Indirect membrane immunofluorescence: Immunofluorescent staining was done according to the following procedure. Initially $5 \times 10^{7}$ test cells (L1117 cells, normal A/Jax thymus cells or lymph node cells, or 1509a sarcoma cells) were suspended in 1.0 ml of staining buffer (SB) (0.01 M phosphate buffered saline pH 7.2 containing 5% FCS) then washed twice by discontinuous gradient centrifugation (100%, 75%, 50% FCS in SB) at 600 x G for 5 minutes and once in SB. With each cell type $5 \times 10^{6}$ cells

were transfered to a small test tube, 0.25 ml of antiserum added (B10·A anti-L1117 antiserum diluted 1:3 with SB; A/J anti-L1117 antiserum 1:1; normal B10·A or A/J sera 1:3 and 1:1 respectively) and incubated at 4°C for 1 hour with constant rotating. The cells were washed twice through the gradients and once with SB, then 0.25 ml of FITC-conjugated rabbit anti-mouse $F(ab')_2$ was added. The cells were again incubated at 4°C with rotating for 30 minutes, washed twice through the gradient, once with SB, then resuspended in 0.25 ml SB. One drop was placed on a slide, mounted with a cover glass and examined under a fluorescence microscope.

14. Preparation of fluorescein-conjugated antibodies: Rabbit anti-mouse $F(ab')_2$ was prepared as in section 8 then diluted to make a 1% solution in 0.5 M carbonate buffer pH 9.1. While stirring gently at 4°C fluorescein isothiocyanate was added (1 mg FITC/100 mg protein) and the reaction allowed to continue for 6 hours. Uncoupled FITC was removed by gel filtration through Sephadex G25 with 0.01 M phosphate buffer pH 8.0, then the sample was concentrated, dialyzed against the same buffer, and applied to a DEAE cellulose column. Stepwise elution with 0.01 M, 0.05 M, 0.10 M phosphate buffers pH 8.0, and 0.01 M, phosphate buffer/1 M NaCl produced 4 fractions which were concentrated separately and dialyzed against PBS. Fraction III was used for cell membrane staining.

15. Absorption of antiserum with cells: To determine antibody specificity samples of B10·A anti-L1117 and A/J anti-L1117 antisera were incubated with living cells then residual cytotoxic

activity was measured by the two-step cytotoxicity test. Preliminary experiments were done to determine the number of L1117 cells necessary to remove specific activity from each antiserum, then an excess of each cell type based on an estimate of cell surface area was used for each absorption. Antiserum was diluted 1:3 with Veronal buffer then added to a pellet of washed cells. The cells were resuspended then placed on a rotating test tube rack at 4°C for 1 hour. After centrifugation (300 x G, 8') the supernatant serum was removed and tested for residual cytotoxic activity.

16. Preparation of radioiodinated anti-mouse Ig antibodies: Rabbit anti-mouse Ig antisera were prepared as described in section 7. Labeling was done using the method of McConahey and Dixon (84) as modified by Campbell et al. (85). Working at 4°C with continous stirring 500 µCi of $^{125}I$ in 0.2 ml of 0.05 M phosphate buffer pH 7.0 were added slowly to 1 mg of protein solution in 0.5 ml of buffer. Chloramine-T (200 µg in 0.2 ml buffer) was added rapidly, 5 minutes later sodium metabisulfite ( 200 µg in 0.2 ml buffer) was added to stop the reaction. Each sample was dialyzed extensively against PBS. Labeled anti-mouse IgG1 , IgG2 , IgG2a , and anti-mouse IgM were adsorbed to immunosorbents of mouse 7S and 19S globulin fractions respectively, then eluted with GHB, neutralized and dialyzed against PBS.

17. Determination of antibody class: To determine the class of antibodies in which specific anti-L1117 activity resided, $1 \times 10^7$ washed, cultured L1117 cells were incubated with 1.0 ml of anti-L1117 antiserum or normal mouse serum (diluted 1:3 with PBS) for

2 hours at 4°C with constant sample rotation. These cells were washed 3 times with PBS to remove unbound protein, then resuspended in 1.0 ml of PBS containing 1% normal rabbit serum. 0.1 ml of cell suspension ($1 \times 10^6$ cells) was added to 0.1 ml of $^{125}$I-labeled anti-mouse immunoglobulin and incubated 40 minutes at 4°C with intermittent agitation. These cells were washed 3 times with PBS/1% NRS then counted in a well-type scintillation counter (Nuclear Chicago model 4230).

18. Mercaptoethanol treatment: Samples of each antiserum were separated on Sephadex G200 columns as described above, then the first and second elution peaks were pooled separately and concentrated to their original volume. An aliquot of each fraction was tested for cytotoxic activity by the $^{51}$Cr-release test, and a second aliquot was treated with an equal volume of 2-mercaptoethanol (0.2 M in tris-HCl buffer 0.5 M, pH 8.2) for 1 hour at room temperature. The treated samples were then cooled in ice and used immediately in the 2-step $^{51}$Cr-release cytotoxicity test.

# CHAPTER V

# RESULTS

## 1. SPECIFICITY OF THE ANTIBODY RESPONSE

Some tumor-bearing animals possess circulating anti-tumor antibodies (39, 46) but in other cases these cannot be detected and in fact free antigen has been found (8). Also it has been possible in a number of cases to produce anti-tumor immunity by immunizing syngeneic animals with irradiated or mitomycin-treated cells (97). The results however are not uniformly successful and consequently cytotoxic antisera are routinely prepared in allogeneic or xenogeneic systems. These variations may reflect fundamental differences in the recognition of tumor antigens by different hosts which in turn may determine the type of response and the ultimate fate of the neoplastic growth.

The murine lymphoma L1117 was found to be capable of inducing cytotoxic antisera in two strains of mice, one syngeneic (A/J) and the other allogeneic but H-2 identical (B10·A), upon repeated immunizations with mitomycin-treated cells. As a result this system was chosen to study the antibody response to tumor antigens with the aim of providing some insight into the processes which govern the recognition and subsequent response to tumor antigens. The response was considered in terms of its specificity, rate of antibody production, class(es) of antibody produced, and compared with the ability of each strain to produce a cell-mediated anti-tumor response.

A. SPECIFICITY FOR TUMOR ANTIGENS

Both A/J mice and B10·A mice produce cytotoxic antisera when immunized with L1117 cells according to the procedure in the section on Materials and Methods. The specificity of these antisera for TA was tested by direct cytotoxicity assays and by loss of cytotoxic activity upon absorption with cells. Confirmation by indirect immunofluorescence studies also ruled out the presence of non-cytotoxic antibodies directed to other antigens.

Titration of the two antisera produced significant lysis of L1117 cells, but not of A/J thymus or lymph node cells nor of cells from an unrelated tumor, the fibrosarcoma 1509a (Fig. 1). This high degree of specificity indicated for the response of both strains of mice was confirmed by an absorption study. Incubation of aliquots of antiserum from each strain with L1117 cells for 1 hour at 4°C removed virtually all cytotoxic activity toward L1117 target cells, however comparable numbers of normal A/J thymus or lymph node cells or 1509a fibrosarcoma cells produced no reduction in the titre of A/J anti-serum and only a slight decrease for B10·A anti-serum (Fig. 2).

As both of these tests detect only complement-fixing antibodies, indirect immunofluorescence was used to confirm the specificity of the responses. Incubation of L1117 cells with B10·A anti-L1117 or A/J anti-L1117 antiserum, then with fluorescein-labeled rabbit anti-mouse $F(ab')_2$ antibodies produced the specific membrane fluorescence shown in Figure 3a. In contrast, Figure 3b illustrates the diffuse, pale staining obtained when L1117 cells were incubated with normal mouse serum during the

Figure 1. Specificity of cytotoxic activity: Lysis of L1117 lymphoma cells (o), normal lymph node cells (▽), normal thymus cells (△) and 1509a fibrosarcoma cells (□) of A/J mice by B10·A anti-L1117 antiserum (1a) and A/J anti-L1117 antiserum (1b) as measured by the two-step $^{51}Cr$-release microcytotoxicity test.

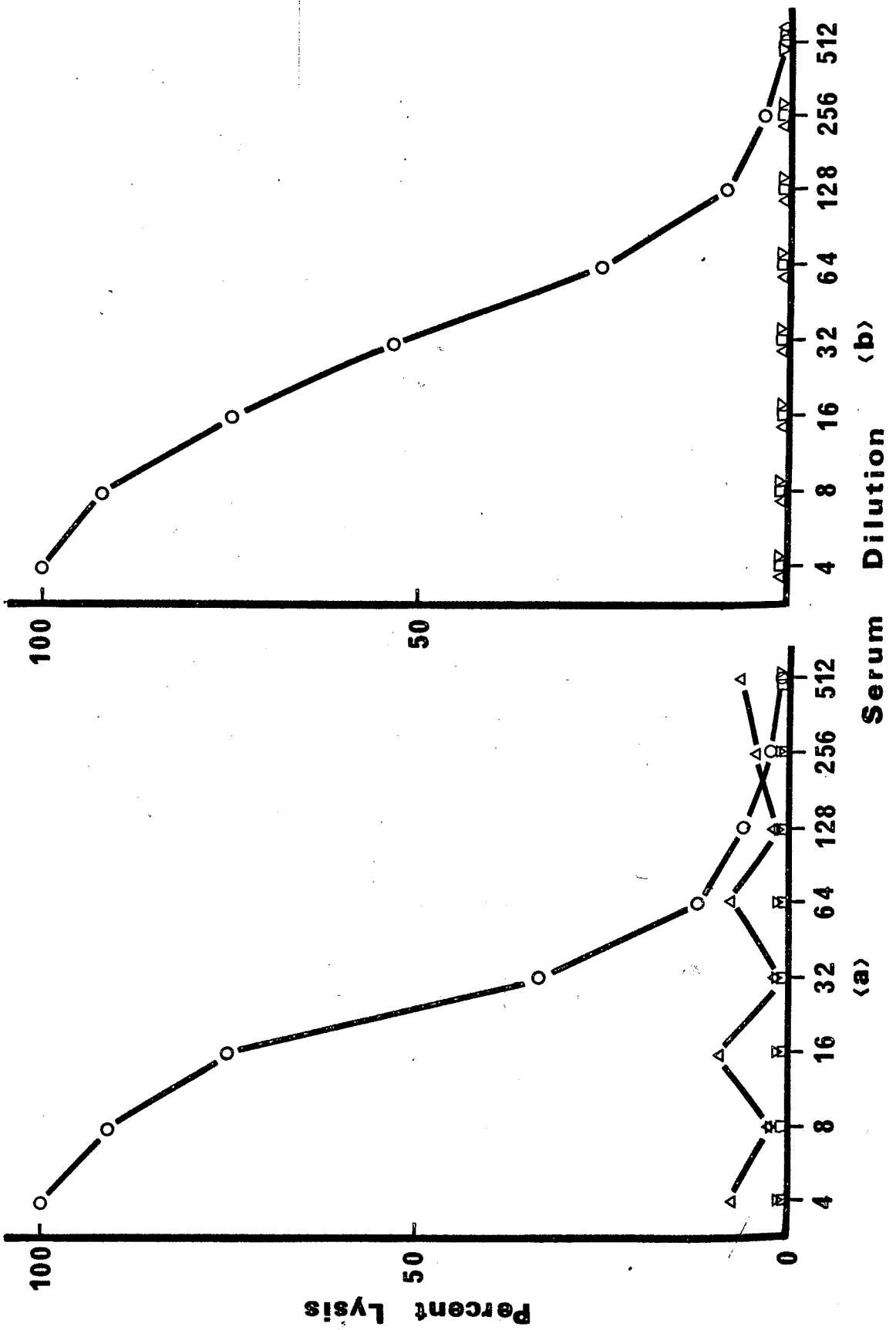

Percent Lysis
100
50
0
4
8
16
32
64
128
256
512
Serum Dilution
(a)
(b)

Figure 2. Absorption of cytotoxic activity by cells: Residual cytotoxic activity for L1117 cells following incubation of antisera with cells. Figure 2a: 0.45 ml of B10·A anti-L1117 antiserum (diluted 1:3) absorbed with 6 x $10^7$ L1117 cells (o), 1.2 x $10^8$ normal thymus cells (▽), 2.4 x $10^8$ normal lymph node cells (△), or 2 x $10^7$ 1509a fibrosarcoma cells (□) of A/J mice. Figure 2b: 0.45 ml of A/J anti-L1117 antiserum (diluted 1:3) absorbed with 6 x $10^7$ L1117 cells (o), 1 x $10^8$ normal thymus cells (▽), 3 x $10^7$ normal lymph node cells (△) or 1 x $10^7$ 1509a fibrosarcoma cells (□) of A/J mice. The non-absorbed antisera are shown by the solid circles (⊗).

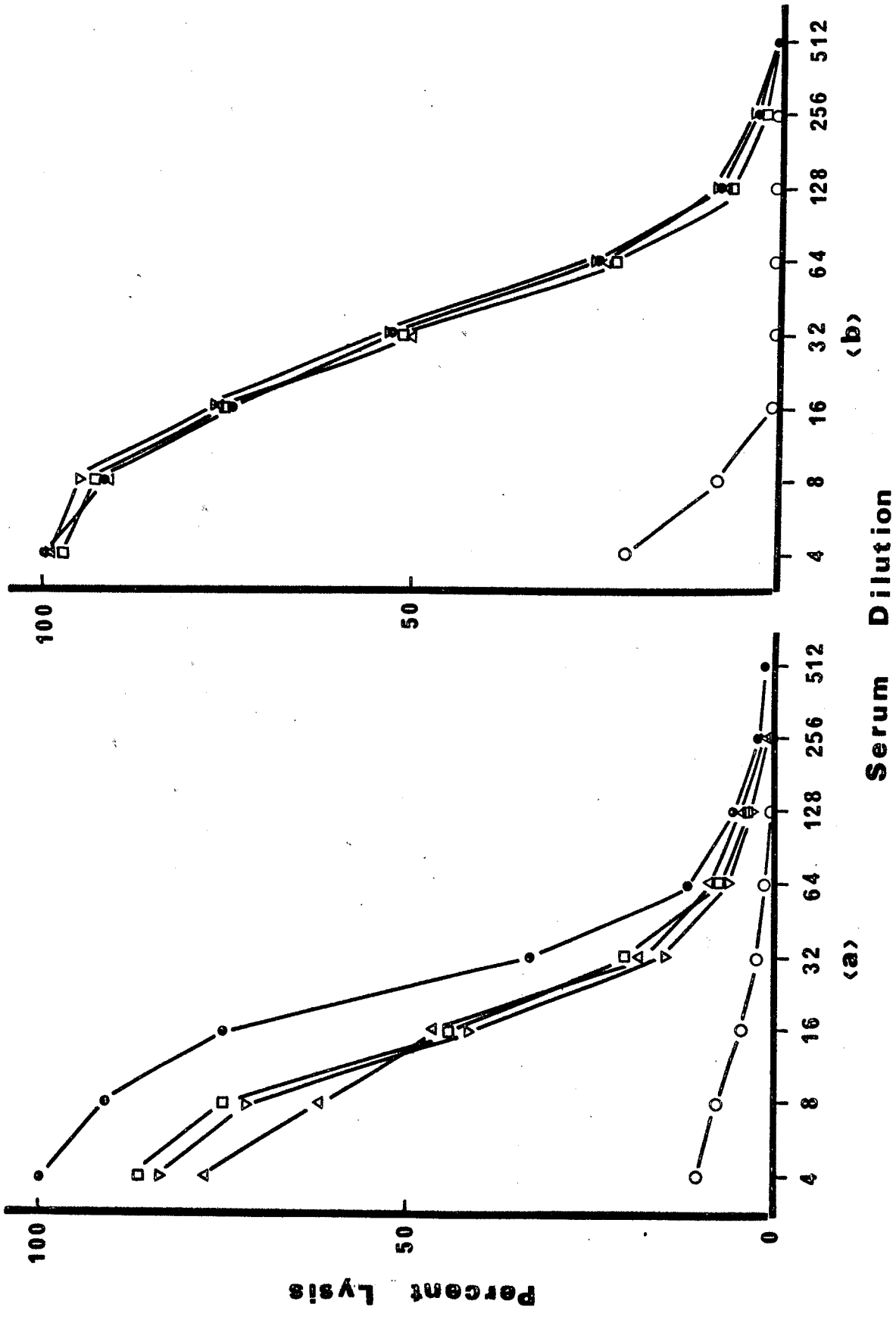
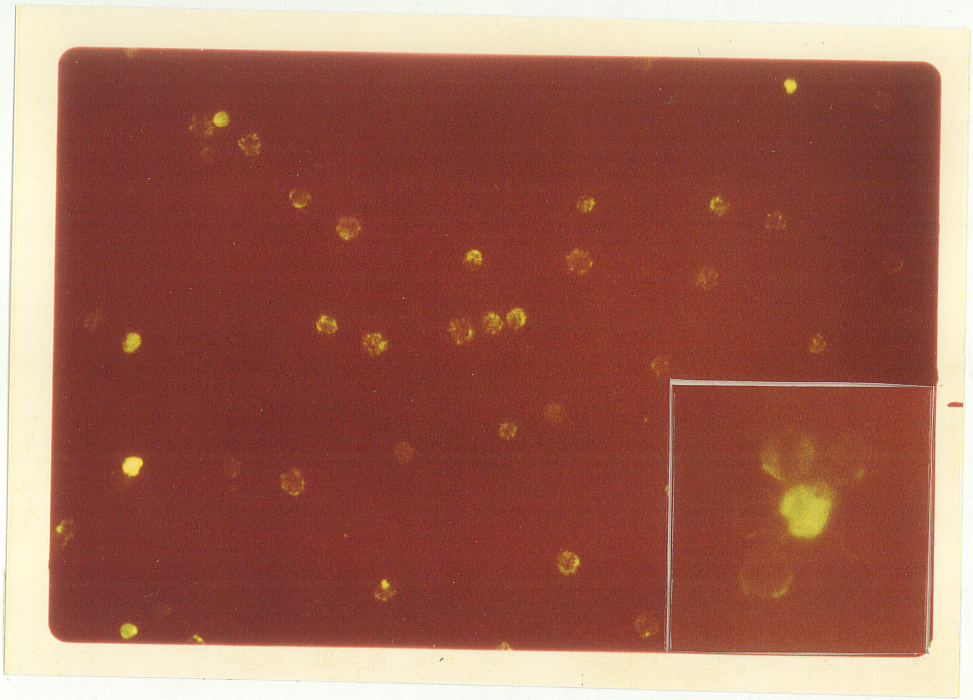
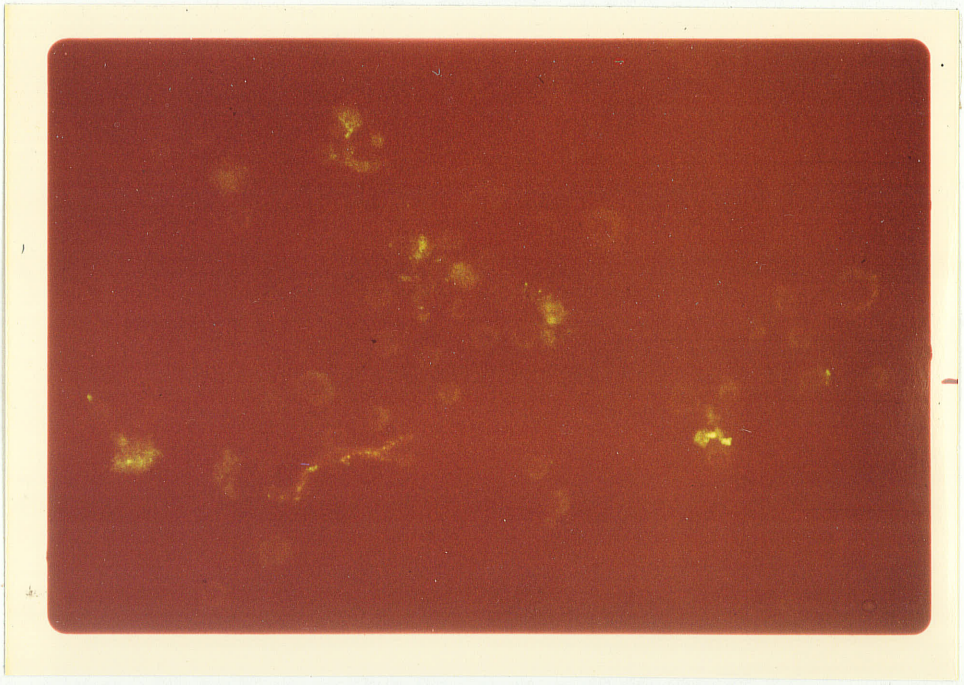

Percent Lysis
100
50
0
4
8
16
32
64
128
256
512
(a)
(b)
Serum Dilution

Figure 3. Specificity of immunofluorescent staining: The specific binding of antibodies from B10.A anti-L1117 antiserum to L1117 lymphoma cells shown by positive indirect immunofluorescence ( 3a, magnification X160, inset X640 ). Figure 3b shows the pale, non-specific staining observed with 1509a fibrosarcoma cells incubated with anti-L1117 antiserum ( X160 ); similar negative results were obtained for normal A/J lymph node and thymus cells.



(a)



(b)

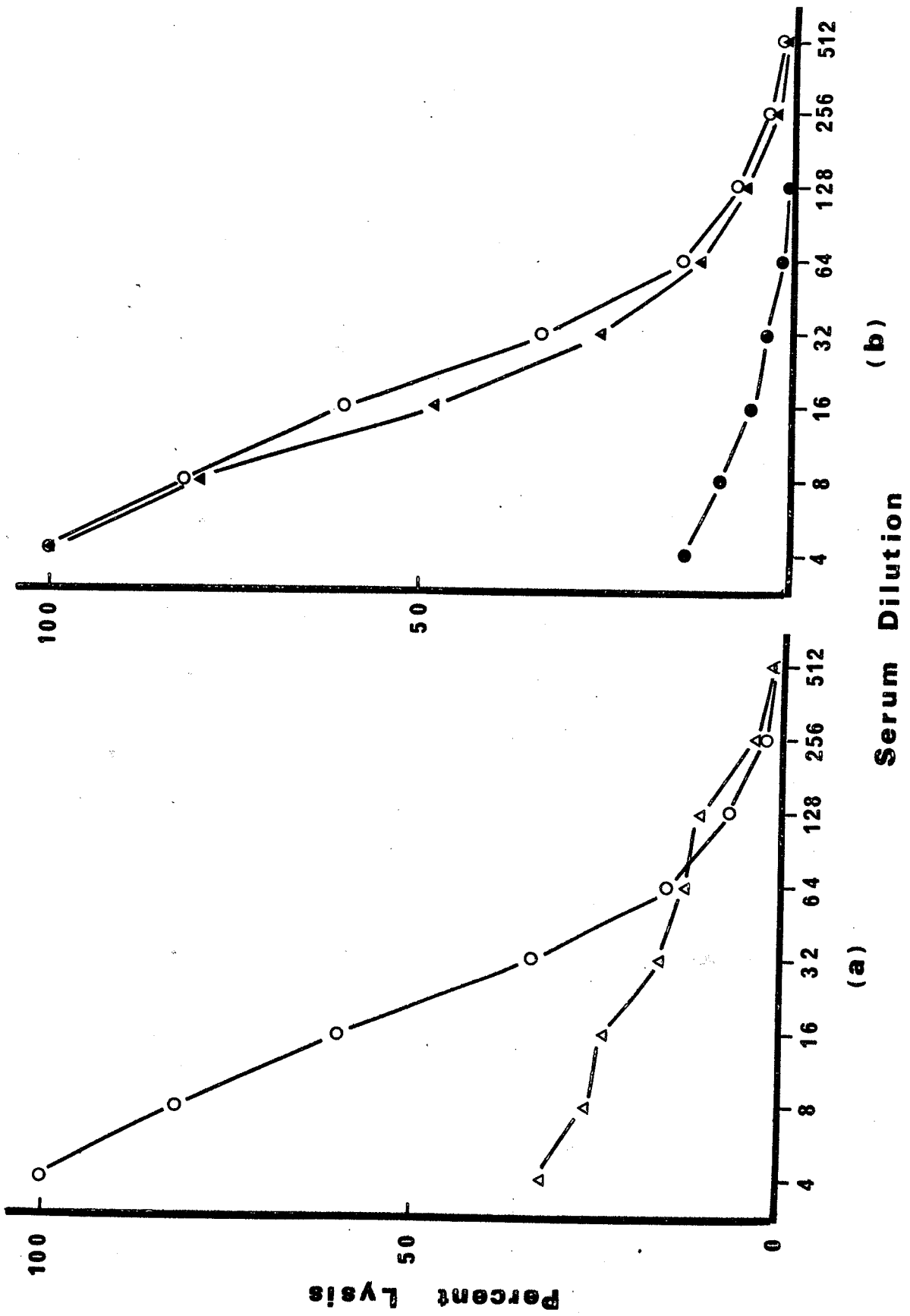
first step, or when normal A/J lymph node cells or thymus cells or 1509a fibrosarcoma cells were incubated with anti-L1117 antiserum then stained.

It can be concluded from these results that under the conditions of immunization used in this work A/J mice respond in a highly specific manner to the tumor antigens of L1117 cells. The results of the cytotoxicity tests and the immunofluorescence study suggests that the B10.A response is also highly specific, however the slight loss of activity upon absorption with normal cells may indicate a weak response to the different minor histocompatibility antigens of the A/J strain.

B. CROSS-REACTIVITY WITH FETAL ANTIGENS

It has been reported that antisera prepared against fetal cells will also react with a membrane antigen present on tumor cells, and in some of these cases the reverse was also true, that is an antiserum to tumor cells was cytotoxic for fetal cells (86). A heterologous antiserum to unfertilized mouse eggs. for example, was cytotoxic for SV40 transformed mouse cells, however the serum from mice immunized with syngeneic SV40 transformed cells was not cytotoxic to mouse eggs (88). These results, and others of a similar nature, have led to the hypothesis that malignant transformation may in some cases cause "derepression" of silent genes resulting in the reappearance of antigenic moieties normally present only on fetal cells in addition to the specific tumor antigens found only on transformed cells. To determine whether the responses in the two strains of mice under study were directed against a fetal antigen or a specific tumor antigen, or possibly both, cell suspensions were prepared from mouse fetuses during the first trimester of gestation and tested for susceptibility to lysis by anti-L1117 antiserum and for ability to absorb cytotoxic activity upon incubation with antiserum at $4^{o}C$.

Results of the cytotoxicity tests with B10·A anti-L1117 antiserum are shown in Figure 4a. Some lysis of fetal cells was observed, but this was substantially less than that for L1117 cells. In addition, the serial dilution of antiserum did not produce the sigmoid curve normally associated with an antiserum titration suggesting that other factors may be responsible for this low level lysis of fetal cells.


Percent Lysis
100
50
0
4
8
16
32
64
128
256
512
(a)
(b)
Serum Dilution

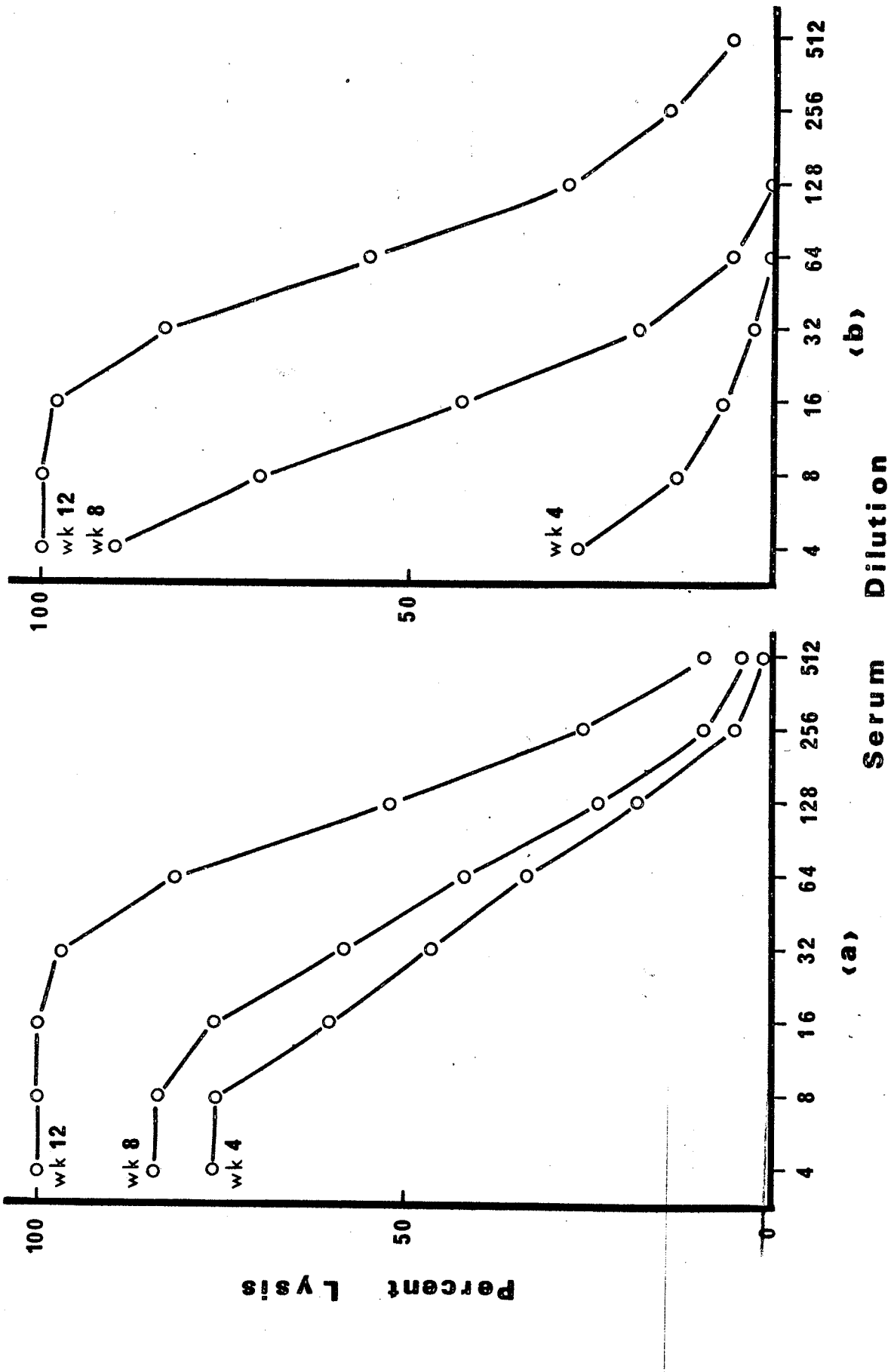
Incubation of B10·A anti-L1117 antiserum with L1117 cells for 2 hours at 4°C reduced cytotoxic activity against L1117 cells to minimal levels while incubation with a large excess of fetal cells produced no decrease in titre.

The low level lysis of fetal cells may indicate that B10·A mice recognize two antigens on the L1117 cells, one of which is a fetal antigen. However the relative levels of tumor cell and fetal cell lysis, and the inability of fetal cells to absorb anti-L1117 activity would suggest that the response is primarily directed toward L1117 tumor antigens.

## 2. KINETICS OF THE ANTIBODY RESPONSE

The response to immunization with L1117 lymphoma cells was followed in syngeneic and allogeneic mice by means of serum samples collected by tail bleeding immediately prior to each biweekly injection. Cytotoxic activity, as measured by the two-step $^{51}Cr$-release microcytotoxicity test, was detected in the first test bleeding from B10·A mice at week 2 and continued to rise until week 10 (Fig. 5a). Continued immunization maintained this level until week 20, at which time the mice were sacrificed, but produced no appreciable increase in titre.

A/J mice responded rather more slowly. Cytotoxicity was again detected after 2 weeks, but levels remained below those for B10·A mice throughout the period of immunization (Fig. 5b). The titre continued to rise until week 14 however, eventually reaching levels comparable to those obtained with B10·A mice. While the results reported in the previous section indicate that the response in both strains is directed specifically to a tumor antigen it may be that the minor histocompatibility differences between this strain and strain A, from which the tumor is derived, are responsible for producing the higher response in B10·A mice. This possibility will be considered further in a later section.


Percent Lysis
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wk 12
wk 8
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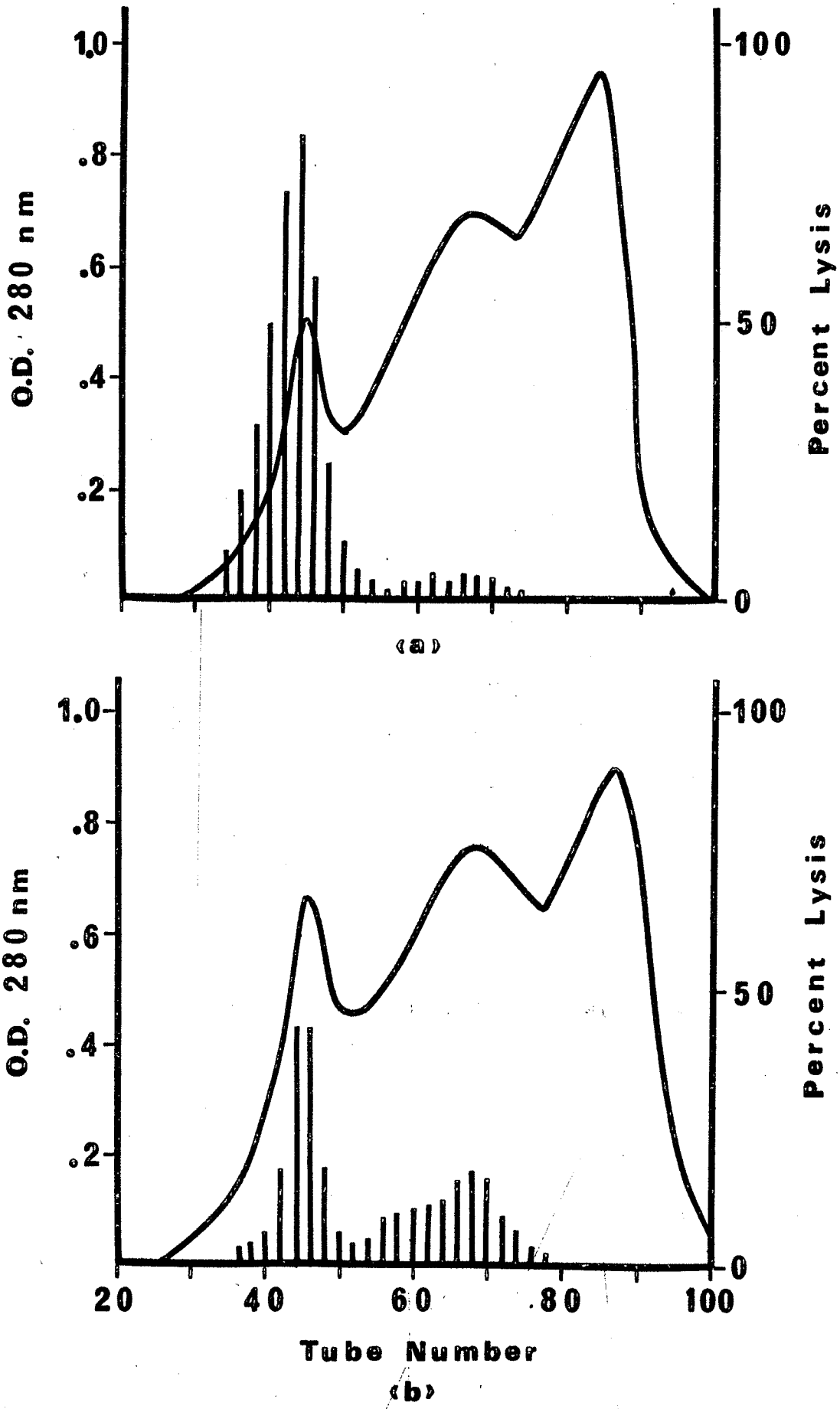
(a)
Serum Dilution
(b)

## 3. ANTIBODY CHARACTERIZATION

Both B10·A and A/J mice produce cytotoxic antibodies in response to immunization with L1117 cells, yet the kinetics of the responses are quite different. In order to examine the nature of the response further antisera from both strains were tested to determine which class(es) of antibodies were responsible for the observed cytotoxic properties.

Gel filtration on Sephadex G200 was used to assign an approximate molecular size to the anti-L1117 antibodies. A sample of B10·A anti-L1117 was applied to the column and eluted with an upward flow of borate-buffered saline. The protein content of each 4 ml. sample of eluate was estimated by measuring the optical density at 280 nm, producing the elution pattern shown in Figure 6a. The three peaks are characteristic of mouse serum and correspond to proteins with sedimentation coefficients of approximately 19S, 7S, and 4S, with elution in that order. Samples (0.1 ml) taken from every second tube were tested, undiluted, for their ability to lyse L1117 cells in the 2-step microcytotoxicity test. The results are shown by the vertical bars indicating the percent of $^{51}Cr$ released through cell lysis compared to the maximum releasable by a strongly cytotoxic alloantiserum.

Cytotoxic antibodies present in B10·A antiserum after 16-20 weeks of biweekly immunization are predominantly localized in the first elution peak, the 19S immunoglobulins (Fig. 6a). A very low level of lysis, less than 5%, appears in the 7S region. In itself this level of cytotoxicity is not significant, however in a


1.0
.8
.6
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.2
O.D. 280 nm
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0
Percent Lysis
(a)
1.0
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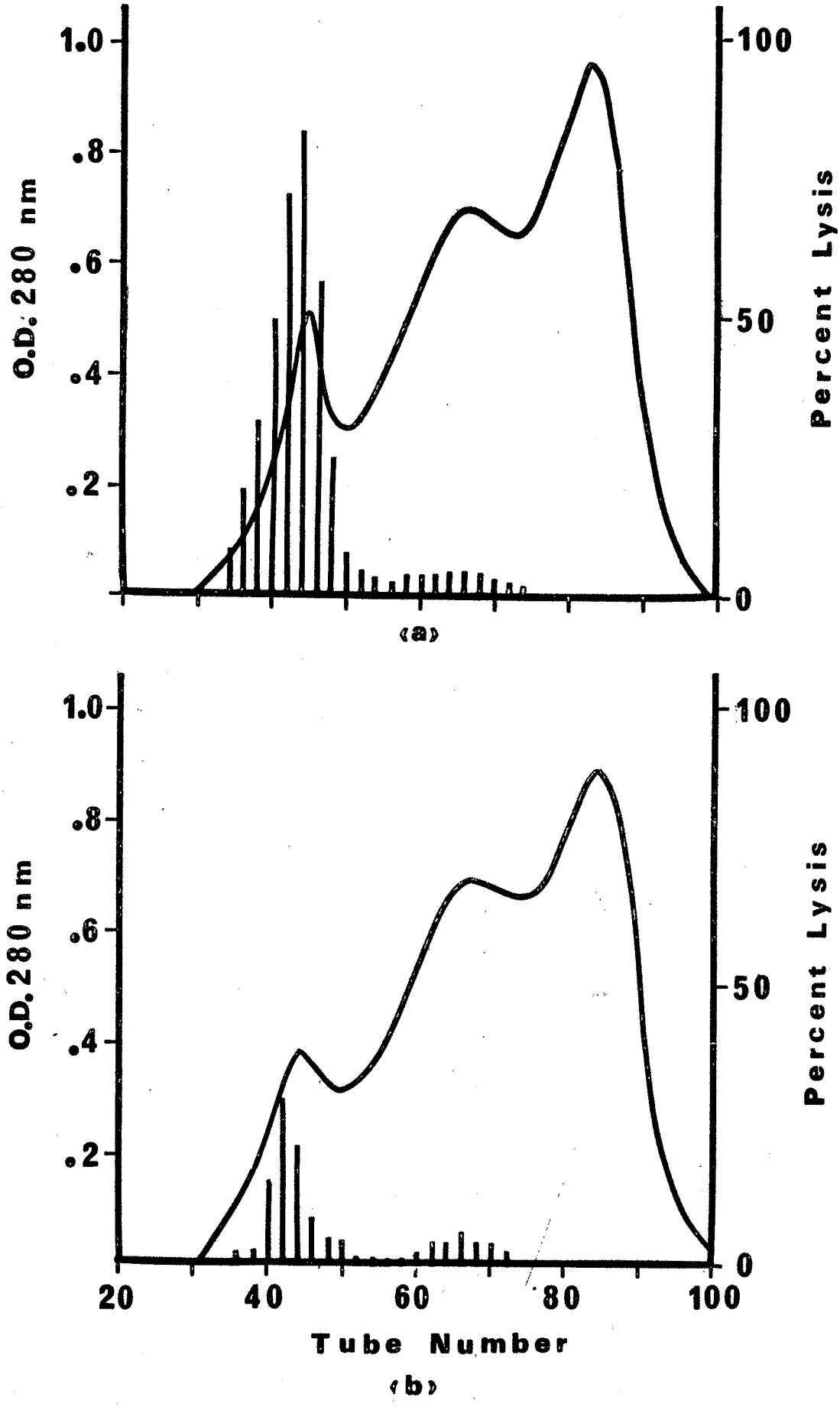
.6
.4
.2
O.D. 280 nm
100
50
0
Percent Lysis
20
40
60
80
100
Tube Number
(b)

Figure 7. Characterization of cytotoxic antibody in A/J antiserum: Figure 7b shows the distribution of cytotoxic activity in the serum of A/J mice after 19 weeks of biweekly immunization with L1117 cells. Elution from Sephadex G200 and measurement of cytotoxic activity in each sample was performed in the same manner as used for the B10·A antiserum. The distribution of cytotoxic activity in B10·A antiserum after 20 weeks is shown in Figure 7a for comparison.


1.0
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O.D. 280 nm
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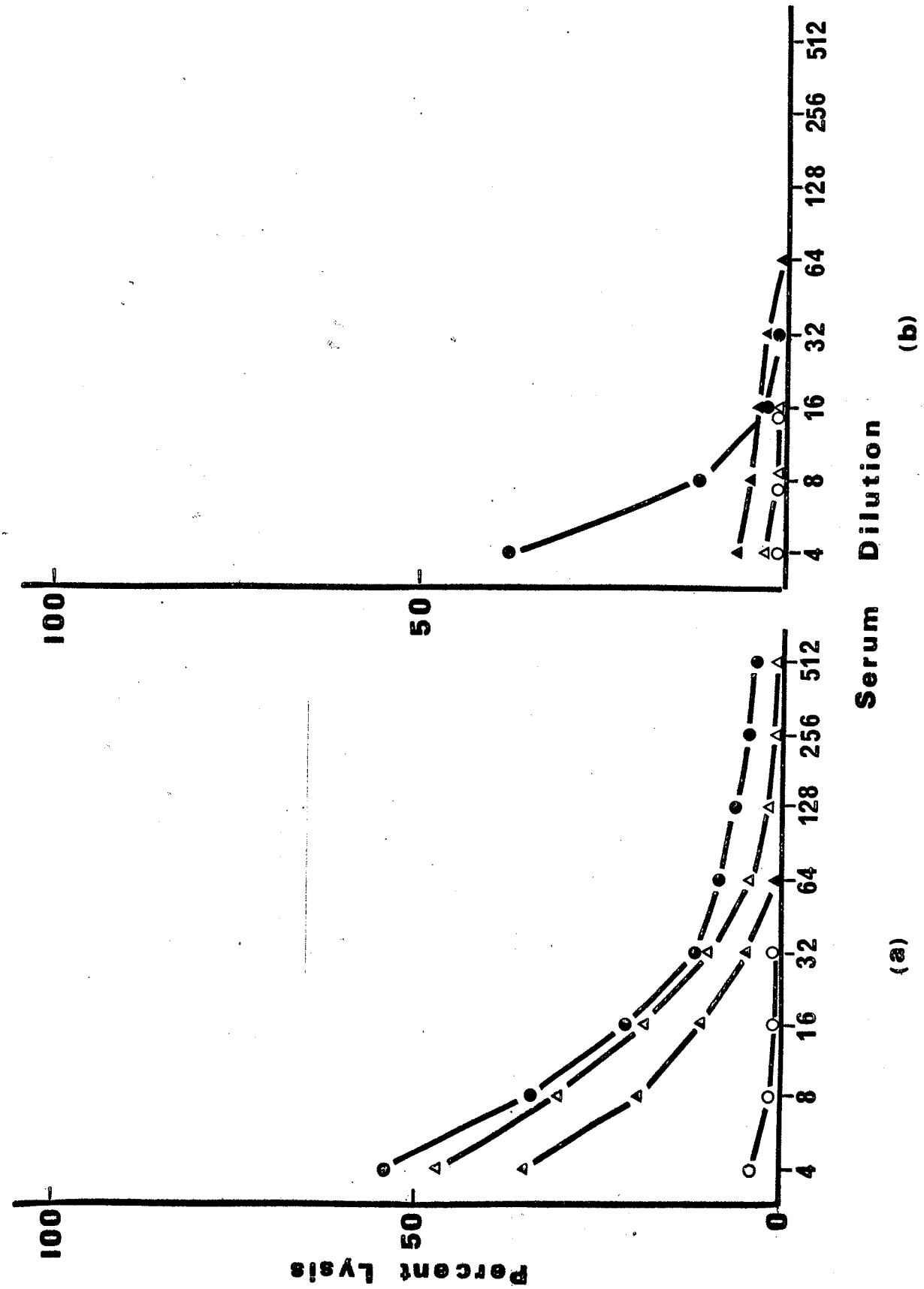
0
Percent Lysis
(a)
1.0
.8
.6
.4
.2
O.D. 280 nm
100
50
0
Percent Lysis
20
40
60
80
100
Tube Number
(b)

preliminary experiment a group of B10·A mice had been immunized for 40 weeks and tests with this antiserum did indicate significant levels of cytotoxic 7S antibodies (Fig. 6b). However even in this antiserum, after 10 months of immunization with L1117 cells, 19S antibodies still account for much of the cytotoxic activity.

It would appear then that B10·A mice respond to repeated immunization with L1117 cells by a prolonged production of cytolytic 19S antibodies and a very late, low level production of 7S antibodies.

A sample of A/J anti-L1117 antiserum was separated on a Sephadex G200 column in a similar manner. Figure 7 compares the elution pattern of cytotoxic activity in B10·A antiserum (Fig. 7a) with that obtained for A/J antiserum (Fig. 7b). The titre of the unfractionated A/J antiserum was slightly lower than that of the B10·A antiserum and this is reflected in the maximum level of lysis obtained, however activity is again localized in the 19S fraction.

It is difficult to evaluate the significance of the very low levels of 7S antibody detected by these tests, especially when IgM is known to be much more efficient in complement-mediated target cell lysis. It is quite possible that the cytotoxic activity of the 7S fractions represents an appreciable IgG response. To study this problem further, the samples within each peak were pooled separately and concentrated to the original sample volume. Aliquots were tested for cytotoxic activity toward L1117 cells before and after treatment with 2-mercaptoethanol for 1 hour at room


Percent Lysis
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Serum Dilution
(a)
(b)

temperature, a procedure known to destroy the ability of IgM to bind complement without affecting IgG (96). B10·A anti-L1117 antiserum showed cytotoxic activity in both the 19S and 7S fractions (Fig. 8a) before treatment, but only in the 7S peak after treatment, thus confirming the presence of both IgM and IgG anti-L1117 antibodies in the serum at 20 weeks. The results for A/J anti-L1117 fractions were parallel, but in this case the level of lysis effected by IgG antibodies is much lower (Fig. 8b) relative to that due to IgM.

It seems clear from these results that the antibodies produced by B10·A mice in response to tumor cells are of both IgM and IgG classes. While A/J mice definitely produce IgM antibodies, the evidence for the production of IgG antibodies by A/J mice is somewhat weaker. A radioimmunoassay was used to confirm the presence of IgG anti-tumor antibodies. This method is more sensitive than the cytotoxicity test, especially for IgG antibodies which are much less efficient than IgM at complement-dependent cell lysis. It is also capable of detecting non-complement-fixing antibodies such as those of the IgG1 class (97). L1117 cells were incubated with antiserum from B10·A or A/J mice for 2 hours at 4°C, washed, then incubated with $^{125}$I-labeled anti-$IgG_1$ or anti-IgG2. Both anti-immunoglobulins bound to coated cells at levels several times those obtained with controls using normal A/J serum or phosphate buffered saline for the first incubation (Table I ), thus confirming the presence of IgG antibodies in A/J anti-L1117 antiserum as well as in B10·A anti-L1117 antiserum. Preliminary experiments which utilized anti-IgG2a and anti-IgM as

Table 1

Determination of antibody classes with $^{125}$I-labeled anti-immunoglobulins.[a]

| Sample | $^{125}$I-Anti-M IgG1 | $^{125}$I-Anti-M IgG2 |
|---|---|---|
| B10·A anti-L1117 wk 40 | 1748 ± 138 | 1552 ± 57 |
| B10·A anti-L1117 wk 20 | 1886 ± 62 | 1660 ± 31 |
| A/J anti-L1117 wk 19 | 1732 ± 53 | 1104 ± 45 |
| normal A/J serum | 537 ± 36 | 237 ± 16 |
| PBS | 592 ± 50 | 171 ± 1.8 |

[a]Counts per minute bound to L1117 cells incubated with antiserum samples (column 1) for 2 hours at 4°C, then washed and incubated with radiolabeled anti-Ig.

well as anti-IgG1 and anti-IgG2 showed binding by all four antisera to anti-L1117-coated cells, results which agree with those presented here.

It can be concluded from these results that both B10·A and A/J strains of mice respond to immunization with L1117 lymphoma cells by a prolonged production of IgM anti-L1117 antibodies, weak IgG antibody response. Even after 10 biweekly injections of mitomycin-C treated L1117 cells, IgM antibodies account for most of the cytotoxic activity toward L1117 cells in in vitro tests, IgG anti-L1117 antibody levels being just sufficient for detection by the cytotoxicity test. In contrast, cytotoxic activity of an antiserum raised in C57B1/6 mice by immunization with normal A/J lymphoid cells was restricted entirely to the 7S peak upon gel filtration after only 7 biweekly immunizations in a control experiment.

## 4. TUMOR GROWTH IN VIVO

It has been recognized that tumor rejection is mediated primarily by cytotoxic T cells (88), consequently the cellular response to the L1117 lymphoma was studied in both strains of mice using rejection of growing tumors as an assay system. Groups of mice were challenged with graded doses of viable, cultured L1117 cells injected s.c. into the dorsum of the animal and the number of successful takes as well as the final outcome recorded for each subject. From results shown in Table 1 it can be seen that there is a marked difference in the responses of the two strains of mice. At the 3 doses tested there were successful takes in all A/J mice followed by progressive tumor growth culminating in the death of the host approximately one month later. In contrast only 50% of the B10·A mice receiving the highest tumor cell dose had detectable tumors and none were observed for any of the lower doses. In both takes the growing tumor was more localized than those in A/J mice. One tumor was rejected within 19 days following challenge, after reaching its maximum size at about day 10; the second reached its maximum size about day 35 then became necrotic and eventually disappeared by day 50.

These results parallel those obtained with the antibody response although the difference is far more dramatic here. The cellular response in A/J mice must be so weak that it exerts little or no inhibitory effect on a growing tumor. Even with the lowest challenge dose, where growth inhibition should be most obvious, tumor growth was so rapid death occured only a few days later than

Table II

The cell-mediated response to tumor antigens.[a]

Strain A/J

| Challenge (No. L1117 cells) | Number of Takes | Animal Survival Time (Mean) |
|---|---|---|
| $5 \times 10^7$ | 4/4 | 31 ± 1d |
| $5 \times 10^6$ | 4/4 | 33 ± 2d |
| $5 \times 10^5$ | 4/4 | 35 ± 2d |

Strain B10·A

| Challenge (No. L1117 cells) | Number of Takes | Tumor Fate |
|---|---|---|
| $5 \times 10^7$ | 2/4 | rejected: 19d, 45d |
| $5 \times 10^6$ | 0/4 | --- |
| $5 \times 10^5$ | 0/4 | --- |

[a]Measured by the ability to reject graded doses of tumor cells. Challenge doses of living L1117 cells were injected s.c. into the dorsum of each mouse. Tumor size could be estimated both visibly and palpably and each mouse was followed until death or complete rejection of the tumor.

in the case of mice receiving a dose of cells 100 times as large.

B10·A mice mount a much more vigorous cellular response, tumor growth occuring in only 2 mice at the highest dose and even these later being rejected. The possibility that histocompatibility differences play a role in this rejection cannot be eliminated as the specificity of the rejection reaction was not established.

# CHAPTER VI

# DISCUSSION

## 1. SPECIFICITY OF THE ANTIBODY RESPONSE

### A. SPECIFICITY FOR TUMOR ANTIGENS

The antibody response to syngeneic tumor cells should be directed only toward tumor antigens, those antigens which appeared as a result of the neoplastic transformation and consequently did not exist on the tissue from which the tumor arose. The L1117 lymphoma is probably derived from A/J thymus cells as it exhibits a lymphoid morphology and carries the θ antigen (8). As expected, A/J anti-L1117 antiserum exhibited no specificity for normal A/J thymus cells or lymph node cells even at concentrations producing 100% lysis of lymphoma cells. Studies by Möller (66) show that absorption tests may detect much lower levels of antigen than assays using complement-mediated cytotoxicity, as fixation of complement by IgG molecules requires a minimum concentration of antigenic sites on the cell surface. However incubation of syngeneic antiserum with large numbers of normal A/J thymus or lymph node cells produced no reduction in titre, confirming the specificity of the syngeneic response for tumor antigens.

Immunization of allogeneic mice introduces the possibility of a polyspecific response producing antibodies directed toward alloantigens as well as tumor antigens. The B10·A response appears to be primarily directed toward the tumor antigens as cytotoxicity tests and indirect immunofluorescence both failed to detect any antibodies binding to normal A/J thymus or lymph node cells. However absorption with a large number of A/J cells, either thymus, lymph node, or 1509a fibrosarcoma cells, produced a slight decrease in anti-L1117 activity

gen, and they can be immunized with allogeneic (strain A) TL+ leukemia cells to produce an antiserum cytotoxic in vitro for all TL+ cells including TL+ leukemias of C57Bl/6. Unfortunately a direct comparison with the present system cannot be made because C57Bl/6. and A strain mice differ at the H-2 locus. Also, TL- mice cannot be immunized with syngeneic TL+ leukemia cells because loss of TL antigen through antigenic modulation allows progressive tumor growth resulting in the death of the animal. It would be of interest to know whether immunization with cells treated to prevent modulation, could produce an anti-TL antiserum. The present results indicate that such syngeneic antisera would be highly specific for the TL antigen.

While these results show that both strains of mice respond specifically to a tumor antigen, the exact nature of this determinant on L1117 cells is not known. Several possibilities exist including fetal antigens, viral antigens, tumor specific antigens, and antigens similar to those of the TL system which are a product of a gene normally silent but expressed in the neoplastic cell.

Tests for cross-reactivity with other murine tumor lines were all negative. No other A/J lymphoma lines were available during this study, however an unrelated strain A tumor, the 1509a fibrosarcoma, was used as a routine control in cytotoxicity tests, absorption experiments, and indirect immunofluorescence studies, and showed no reactivity with either the syngeneic or allogeneic anti-L1117 antiserum. Preliminary screening also included a mye-

which was not observed with A/J anti-L1117 antiserum. This may indicate that B10·A mice respond to an antigen of normal A/J cells present on the L1117 tumor cells, however to account for the failure of cytotoxcity tests and immunofluorescence to detect binding of B10·A antibodies to A/J cells other than L1117 lymphoma cells it is necessary to postulate that the antigen in question is present in extremely low concentration on A/J cells, or is possessed by only a very small subpopulation of normal cells. Although the L1117 lymphoma and normal B10·A cells share the same major histocompatibility locus (H-2) they differ in some of their minor histocompatibility antigens. It is not possible however to determine from the data presented here whether the minor histocompatibility antigens could account for these observations.

A monospecific response was also observed by Reif and Allen (87) in a very similar system during their studies of the θ antigen, or Thy-1b antigen as it is presently designated. When AKR strain mouse thymus cells were injected into C3HeB/Fe mice, the antiserum produced was highly cytotoxic for cells bearing the θ-AKR antigen. These two strains of mice carry the same H-2 allele but presumably differ in other histocompatibility factors, yet the C3H antiserum was specific for AKR thymocytes and no lytic activity could be detected against other AKR lymphocytes or marrow cells, which do not carry the θ antigen.

The work of Old, Boyse and their coworkers on the TL antigen (72-74) is also of interest. C57Bl/6J mice are TL-, that is their thymus cells do not normally express the thymus leukemia (TL) anti-

locytic leukemia of strain A mice designated YAAC1 and again no cross-reactivity was observed.

The L1117 lymphoma can be transfered with ultrafiltrates of tumor cells, indicating a viral etiology (8), however the virus has not been isolated to date, excluding the possibility of studying cross-reactivity with other tumors caused by the same virus, and at the same time making it impossible to determine whether the antigen recognized by the anti-L1117 antisera is present on the virion or unique to the tumor cell.

B. CROSS-REACTIVITY WITH FETAL ANTIGENS

A number of TA have been shown to cross-react with antigens present on embryonal tissue during the first trimester of gestation (88), for example the carcino-embryonic antigen (CEA) described by Gold and Freedman (89). These are antigenic determinants which can be detected on embryonal tissue early in development but disappear upon further tissue differentiation, being absent from normal adult tissues. Their reappearance during oncogenesis, accompanied by loss of differentiation and loss of contact inhibition suggests a process of reversion or loss of differentiation.

In the present study, cell suspensions prepared from normal A/J fetuses removed at 6.5 to 7 days gestation did not remove anti-L1117 activity from B10·A anti-L1117 antiserum during absorption at 4°C for 1 hour, however in the cytotoxicity tests these fetal cells were lysed although to a much lesser extent than were L1117 cells. The titration curve is somewhat different than the usual sigmoid curve, possibly indicating that the lysis is due to some non-specific factor rather than anti-L1117 antibody. Alternatively, L1117 cells may express fetal antigens which are recognized by the B10·A mice. The inability of fetal cells to reduce the anti-L1117 activity during absorption would mean that any antibodies directed to fetal antigens account for very little of the cytotoxic activity of B10·A antiserum for L1117 cells.

From these results it can be concluded that both syngeneic A/J mice and allogeneic B10·A mice respond to the tumor antigen of A/J lymphoma L1117 in a highly specific manner. While related lymphoma

lines were not available for testing and cannot be completely excluded, no specificity for normal A/J lymphocytes or unrelated A/J tumor cells could be detected in A/J anti-L1117 antiserum. B10·A mice may produce antibodies which react with normal A/J cells and/or A/J fetal cells, but these form a very small part of the response which is directed predominantly to the L1117 tumor antigen.

## 2. NATURE OF THE ANTIBODY RESPONSE

### A. KINETICS OF THE ANTIBODY RESPONSE

The response to immunization with protein immunogens is usually characterized by a primary response consisting mainly of 19S IgM production at a relatively low level, then a secondary response, upon rechallenge, consisting mainly of 7S IgG production at levels many times those of the primary response. The immunological memory responsible for the secondary response is thought to be a property of long-lived recirculating T cells, it is specific, and may be demonstrated repeatedly by subsequent challenges with the same antigen.

The results observed with the present system differ from this pattern at a number of important points. Experimental mice were first bled two weeks after the initial injection of L1117 cells at which time cytotoxic antibodies were detected in both strains of mice at relatively low levels. However a second injection immediately after the test bleeding produced only a small increase in antibody levels during the next 2 weeks, especially in A/J mice. Continued biweekly injections produced a gradual rise in cytolytic antibody levels in both strains, however B10·A levels were consistently higher, reaching a maximum at 10 weeks while A/J mice took 14 weeks to reach the same point. This maximum level remained constant or even declined slightly upon further immunization.

Tumor antigens are known to be weak immunogens in syngeneic hosts and attempts have been made to overcome this difficulty by attaching new antigenic determinants to tumor cells in the hope

of specifically enhancing the anti-TA response. Several attempts have been made with some significant successes reported. Martin et al. (91) compared the immunogenicity of EL-4 mouse lymphoid leukemia cells in syngeneic C57B1/6 mice after coating the cells with concanavalin A (Con A) or 2,4-dinitropheynylaminocaproate (DnpC). Using a $^{51}Cr$-release cytotoxicity test they found that spleen cells from mice immunized with coated EL4 cells were more effective at lysing EL-4 target cells than spleen cells from mice immunized with EL-4 cells or chemical alone. Moreover this activity was specific for the EL-4 cell antigens as no significant lysis of DnpC-coated unrelated tumor cells was observed.

Kurth and Bauer (92) utilized a similar procedure to enhance the humoral response to tumor antigens. Inbred STU mice were primed with ovalbumin (OA) to induce immediate (IH) or delayed hypersensitivity (DH), or both, then injected with syngeneic D4 tumor cells alone or coupled to OA. Serum from mice which had demonstrated a strong reaction to OA and were subsequently immunized with OA-coupled D4 cells showed substantial anti-D4 activity in a microcytotoxicity assay. Sera from unprimed mice or mice primed with OA then given only D4 cells were significantly less cytotoxic.

Mitchison (90), one of the early proponents of this idea, describes it in terms of a "helper" determinant not unlike the carrier-hapten model and perhaps utilizing a similar mechanism. There are also parallels in the T cell-B cell cooperation observed with thymus-dependent antigens. While these speculations have not yet been verified, it is certain that in some cases at least, added antigenic determinants can enhance the immune response to TA. This

may account for the difference observed between the response of the two strains of mice used in the present experiment with minor histocompatibility differences in B10·A mice acting as helper determinants.

The enhanced response to tumor antigens observed in these experiments when a "carrier" determinant was provided suggests that in such a case tumor antigens alone may not be capable of inducing effector and/or helper T cells in the syngeneic host. Tyan (93) explored such a possibility in the case of several murine tumors. Mice immunized with irradiated or mitomycin-C treated tumor cells produced anti-TA antisera, however this afforded minimal protection against transplantation of viable cells. Thymectomized, irradiated, bone marrow reconstituted mice showed no decrease in their antibody response to TA when immunized in the same way, nor to lipopolysaccaride (LPS) and DNP-poly-L-lysine (DNP-PLL) two thymus independent antigens. A similar comparison with DNP-bovine IgG (DNP-BGG) or DNP-keyhole limpet hemocyanin (DNP-KLH), 2 T-dependent antigens, in normal and T-cell depleted mice showed significantly impaired responses in the latter.

A report by Iverson and Lindenmann (94) into the response to F-antigen proposed a similar model. F-antigen is a water soluble extract of murine liver. Anti-F antibody prepared by immunizing mice with extract of allogeneic liver will precipitate F-antigen from all strains of mice, including that in which the antiserum was prepared, however F-antigen will neither induce nor boost an anti-F response in syngeneic mice. The authors proposed that the

F-antigen acted as a hapten which required an alloantigen "carrier" to produce a response. They proceeded to show that the anti-F response was indeed thymus dependent and could be abrogated by tolerizing the mice to the carrier alloantigen prior to immunization; such tolerant mice could respond however to F-antigen from a third strain. Further experiments indicated that the H-2 antigen was not responsible, and they conclude that another alloantigen, possibly a minor histocompatibility antigen, serves as the carrier.

Comparison with the present study reveals some interesting parallels to these hapten-like tumor antigens and liver antigens. The response to L1117 cells in syngeneic mice was very weak, despite the use of complete Freund's adjuvant (CFA) for the initial injection, while B10·A mice produced equivalent antibody levels in approximately two-thirds the length of time. If TA are incapable of stimulating T cells this would explain why no memory effect was observed upon second antigenic challenge.

B. SIGNIFICANCE OF THE 19S RESPONSE

Several reports have appeared of antigens which are generally designated as "T-independent". Antibody production upon challenge with these antigens has the characteristics of a primary response: primarily IgM antibody with little or no detectable IgG antibody. They also produce no immunological memory, the response to a second challenge of the same antigen being essentially another "primary" type response. Experiments in vivo and in vitro with T-cell depleted systems have shown that T cells are not required for this response suggesting that these antigens may be capable of directly stimulating B cells to respond. Within this general class of antigens there appear to be two sub-groups, one of which is exemplified by DNP-Ficoll (82,98) which cannot be shown to activate either helper or suppressor T cells and so will be designated T-independent, and the other which includes Type III pneumococcal polysaccaride (S-III) (95,96), bacterial lipopolysaccarides (LPS) (96), polyvinylpyrrolidone (PVP) (96), and polymerized flagellin (POL) (96), which appear to preferentially activate suppressor T cells. This latter group thus exhibits a response similar to the former, as only the directly stiulated B cells can produce antibody, however if the suppressor T cells can be eliminated a normal primary and secondary response is observed. While these have been referred to as T-independent antigens in the literature it now appears that this term may be inaccurate so for this discussion they are designated "T-independent" to distinguish them from the former group.

Two recent reports of studies into the antibody response to SIII by several strains of mice have suggested a possible explanation

for the unusual properties of these antigens. The first of these, by Baker et al. (95), reports that treatment with antithymocyte (ATS) or antilymphocyte serum (ALS) following immunization with SIII produced an increase in antibody response. This treatment causes a depletion of T cells, which would not be expected to have any effect on the response to a T-independent antigen. Moreover, congenitally athymic "nude", mice, which respond to SIII as normal mice do, showed no enhancement of the anti-SIII response following similar treatment with ATS or ALS. The authors suggest that the response to SIII, and presumably other "T-independent" antigens, is in fact regulated by the thymus. They hypothesize that two functionally distinct types of T cells, suppressor and amplifier cells, regulate the response to SIII. In normal mice SIII would preferentially stimulate suppressor T cells, exerting a negative influence on antibody production by B cells so that only the "T-indepdent" IgM response is observed. Treatment with ALS or ATS at the time of immunization with SIII would remove or reduce the suppressor cell population, producing the enhanced antibody response observed experimentally. Similar treatment of athymic "nude" mice should have no effect on their antibody response as they lack a T cell population, and this is exactly what was observed.

Support for this hypothesis also came from a study by Braley-Mullen (96). Using IgG production and immunlogical memory as evidence for T-cell cooperation she examined the effect of several manipulations on the antibody response to SIII. Mice immunized twice with SIII coupled to sheep erythrocytes (SIII-SRBC) produce IgG antibody specific for SIII and also develop SIII-specific

immunological memory. Priming by SIII-SRBC was evidenced by IgG production upon subsequent challenge with SIII alone. IgG antibody to SIII was also produced if mice were immunized with SIII then treated with ALS, confirming the results obtained by Baker.

It would appear from these results that "T-independent" antigens preferentially activate suppressor T cells, consequently the only observed response is the primary type IgM antibody production which apparently does not require T cell cooperation. The existance of these specific suppressor cells prevents IgG antibody production, eliminating the possibility of a secondary response. Treatment with ATS to destroy suppressor cells, or administration of a T-dependent form of the antigen results in the familiar anamnestic IgG production and in the presence of immunological memory.

Although depletion of T cells had no effect on the response to DNP-Ficoll it is not clear whether this reflects a fundamental difference between the two types of antigens or whether it is a matter of degree. The authors are careful to note (98) that a response induced by DNP-Ficoll and tolerance induced by DNP-D-GL (DNP coupled to a linear copolymer of D-glutamic acid and D-lysine) occur in the absence of <u>detectable</u> T cells.

The responses observed in the present study resemble those observed for "T-independent" antigens both in the production of predominately IgM antibody and in the absence of an immune memory. While the data presented here is insufficient to definitely identify TA as belonging to either type "T-independent" antigen it leads to some interesting speculations. Graft rejection, and presumably tumor rejection also, are considered to be mediated by cytotoxic T

cells. If tumor antigens are incapable of stimulating T cells, or preferentially activate suppressor T cells, this might account for the ability of some tumors to grow in an immunocompetent host. In terms of the present data, the primary-type response of A/J mice observed upon repeated challenge with L1117 cells suggests that this may be an example of a "T-independent" antigen system, while B10·A mice, for which minor histocompatibility antigens may serve as "helper" determinants produce a more rapid response. It is not clear at this time whether the mechanism of suppression operating in the case of the antibody response is the same as that of suppression of cell mediated immunity, consequently it is difficult to make any direct comparisons between the tumor growth in vivo and the observed antibody responses.

While both strains of mice are capable of producing an IgM response to L1117 tumor antigens, it should be noted that this is unlikely to play a major role in the fate of the lymphoma in vivo. Comparison of the kinetics of antibody production with the fate of mice with growing tumors shows that A/J mice have already died, and B10·A mice are rejecting their tumors at the time anti-L1117 antibody titres are just beginning to rise in the immunized mice. A/J mice immunized with L1117 cells for 16 weeks were also unable to reject a challenge of $1 \times 10^6$ viable L1117 cells, this dose killing 15 out of 15 mice. This strain is known to be deficient

in complement (99), and mouse complement is generally ineffective for antibody-mediated cell lysis *in vitro* (100), suggesting that even if titres were much higher during tumor development they would provide little protection for the host.

# 3. CONCLUSIONS

It has been shown that B10.A and A/J mice respond in a highly specific manner to tumor antigens of the A/J lymphoma L1117. The response in both strains exhibits a very slow rise in antibody levels, reaching a maximum only after 3 months of biweekly immunizations. It is also unusual in that the antibody produced is primarily IgM even after 20 weeks of immunization. The similarity between these characteristics and those reported for "T - independent" antigens suggests that TA may be unable to stimulate T cells or may preferentially stimulate suppressor T cells.

If these observations represent a general phenomenon, the fate of neoplastic cells may depend upon the ability of their particular TA to stimulate effector and/or helper T cells rather than suppressor T cells, or in some cases their inability to trigger any T cell response. Considerable work remains, both in this system and in others, to establish the validity of this proposal but the initial results are promising. It will be important in future studies to establish more precisely the role of regulatory T cells in controlling the antibody response.

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# APPENDIX I

## THE TWO-STEP CYTOTOXICITY TEST

Alloantisera are routinely tested in this laboratory using the $^{51}Cr$-release cytotoxicity test of Wigzell (80) modified as described in the chapter Materials and Methods. Isotope-labeled target cells are incubated with antiserum plus a complement source, usually guinea pig serum, for 35 minutes at 37°C then an aliquot of the supernatant medium tested for $^{51}Cr$ released by cell death. The method is simple, rapid, permits assay of a large number of samples simultaneously, requires no subjective judgements, and is highly reproduceable. These advantages made it the method of choice to test routine bleedings from immunized B10·A and A/J mice, however the initial results were unsatisfactory. An example of the type of titration curve obtained for B10·A antiserum with this assay is shown in Figure 8. It shows a marked prozone followed by a plateau, which appeared between 55 and 65 percent upon repeated trials, then begins to drop off at high dilutions. Tests with another alloantiserum, A/J anti-C57B1/6J, and the corresponding target cells, C57B1/6J lymph node cells, indicated the presence of a non-specific inhibitory factor possibly acting at the complement level. The situation was complicated by the fact that preparation of the 7S antibody fraction of immune B10·A serum by precipitation with 50 percent ammonium sulfate solution followed by Sephadex G200 gel filtration had not removed the inhibitory factor. It must be concluded that hyperimmune B10·A anti-L1117 antiserum contains a factor which elutes with the 7S proteins during gel filtration and which inhibits the action of guinea pig complement in the one-step microcytotoxicity test. Utilization of a similar immunization procedure for the production

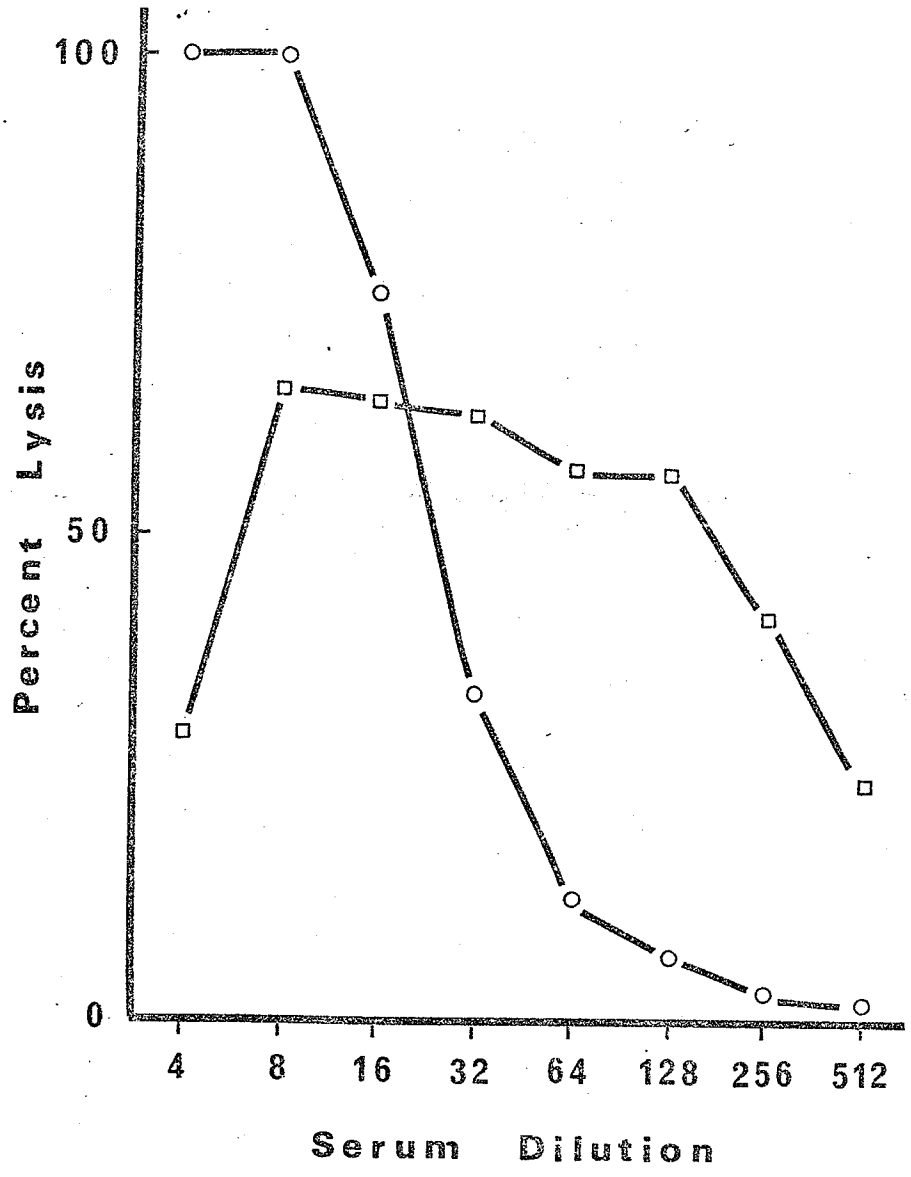
of specific anti-TA antiserum must take into account the possibility of inducing a similar factor.

To overcome this problem a two-step assay was used in which cells were first incubated with the antiserum dilutions alone at 4°C for 45 minutes in the microtitre plate, washed once with 0.1 ml of Veronal buffer with 10% FCS, then 0.2 ml of suitably diluted complement was added to each well for the final incubation of 35 minutes at 37°C. The plates were then centrifuged and 0.1 ml of supernatant removed for measurement of released $^{51}Cr$.

This procedure produced the same type of sigmoidal curve observed for alloantiserum titrations with the conventional test, and as shown in Figure 8 the maximum lysis is now 100 percent instead of 65 percent. It appears that the assay is less sensitive however, at high dilutions the two-step test has dropped to zero while the one-step test shows significant lysis. Presumably at this point the inhibitory factor has been diluted out while antibody concentrations are still high enough to be lytic. The sensitivity of the two-step procedure could not be improved by increasing incubation times for cells and antiserum to 2 hours, suggesting that the decrease may be due to loss of lightly bound antibodies which are washed off the target cells following preincubation but which effect some lysis in the one-step assay.

The decreased sensitivity associated with the use of this assay produced no problems in the present study as comparisons were always made between results obtained with the same procedure, comparisons which are very difficult with the type of curve produced by the one-step assay. The lower sensitivity should be noted however,

Figure 8. Comparison of cytotoxicity tests: A sample of B10·A anti-L1117 antiserum was divided into two aliquots and titrated for cytotoxic activity toward L1117 cells using either a one step assay (□) in which cells, antiserum and complement are added together then incubated 35 minutes at 37°C, or a two-step assay (o) in which cells and antiserum are first incubated 45 minutes at 4°C then washed and complement added for a further 35 minutes at 37°C.


100
50
0
Percent Lysis
4
8
16
32
64
128
256
512
Serum Dilution

especially for comparisons with results published by other authors and for future use where comparison with results obtained by other procedures may be anticipated.

# APPENDIX II

## A STATISTICAL EVALUATION OF THE CYTOTOXICITY TEST DATA

A statistical evaluation of the data from the two - step cytotoxicity test used for many of the experiments in this thesis is particularly difficult. Part of the data used to produce figure 2a is shown in Table III as an example. The values used for 100% lysis and background lysis are based on triplicate samples, while experimental values are based on duplicate samples. As can be seen from this table, the standard deviation calculated from these values is so small that it would fall within the size of the symbols used to plot the points in Figure 2. In contrast to this high degree of precision, comparison of data from tests made on separate days reveals much larger differences if the values for any one dilution are compared, even when parameters such as complement source, $^{51}Cr$ sample, and target cell conditon are unchanged. However the relative positions of the titration curves tends to remain the same. As data from different runs cannot be compared in terms of actual values, but only relative to the other curves, only two values are available for the calculation of each point. To rigorously determine the significance of a difference between two curves it would be necessary to transform them to straight lines then use the standard error of the means as a basis for statistical tests of significance. As an alternative method, the one used in this study is empirical in nature but makes some allowance for uncontrolled variables which are always present when dealing with biological samples and living cells. Each experiment was repeated a number of times keeping as many factors as possible constant. Although the absolute values changed, the relative positons of the titration curves proved

TABLE III

Lysis of L1117 cells by anti - L1117 antiserum[a]

| Sample | Antiserum Dilution | | | | |
|---|---|---|---|---|---|
| | 1/8 | 1/16 | 1/32 | 1/64 | 1/128 |
| B10·A anti - L1117 | 93.0 ± 0.3 | 76.0 ± 0.6 | 31.7 ± 4.0 | 11.6 ± 0.1 | 5.7 ± 4.2 |
| B10·A anti - L1117 absorbed with L1117 cells | 8.1 ± 1.7 | 4.7 ± 0.7 | 2.8 ± 0.1 | 1.6 ± 0.2 | 0.6 ± 0.7 |
| B10·A anti - L1117 absorbed with thymus cells | 71.9 ± 0.9 | 42.1 ± 2.0 | 14.9 ± 0.5 | 6.4 ± 0.2 | 3.8 ± 0.6 |
| B10·A anti - L1117 absorbed with LNC | 67.4 ± 0.7 | 45.7 ± 5.5 | 19.0 ± 0.5 | 8.1 ± 0.7 | 4.8 ± 1.1 |
| B10·A anti - L1117 absorbed with 1509a cells | 75.1 ± 0.8 | 44.7 ± 2.2 | 20.2 ± 2.9 | 7.9 ± 1.4 | 3.5 ± 0.5 |

[a]Percent lysis calculated for duplicate samples according to the formula in section 12 of Materials and Methods, ± the standard deviation.

highly reproduceable. The curves shown in Figure 2a are illustrative of several runs in which the non - absorbed curve was the highest; the three absorbed sera produced curves which were always somewhat lower but within the group the three showed no fixed order; and the serum absorbed with L1117 cells was always less than 10%. This would indicate that there is a small but reproducible loss of activity upon incubation with normal cells or 1509a cells, and an almost complete lack of activity in serum absorbed with L1117 cells. This method of evaluating the results is obviously less rigorous than a statistical evaluation, but it is probably a more realistic approach when the inherent variability of the assay is taken into account.