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THE ROLE OF THE ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY MECHANISM IN THE MURINE P815 MASTOCYTOMA TUMOR SYSTEM

by

LEROY KONDO

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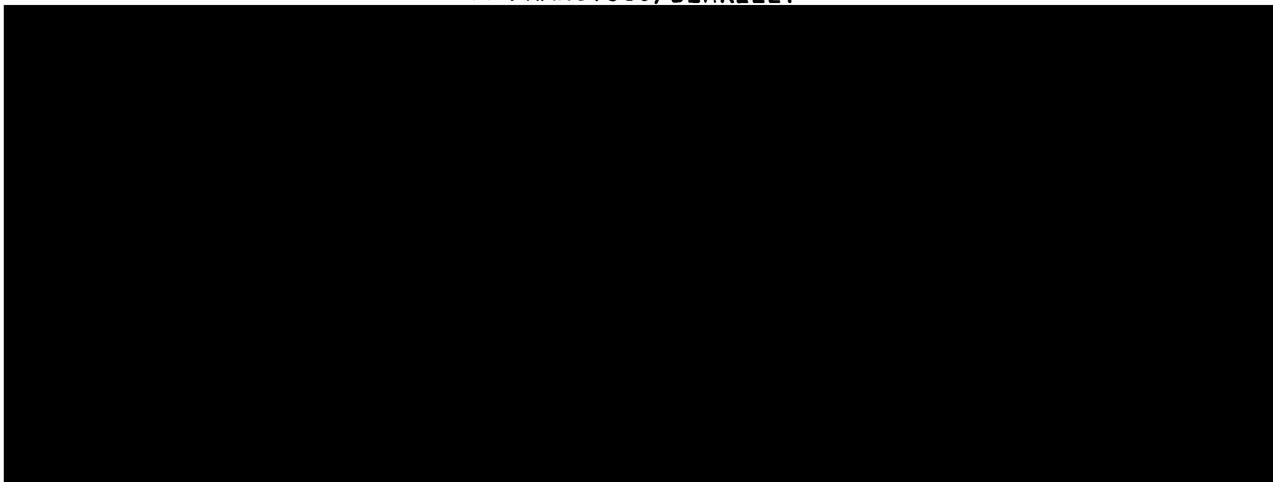

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THE ROLE OF THE ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY MECHANISM IN THE MURINE P815 MASTOCYTOMA TUMOR SYSTEM

by

LeRoy L. Kondo

ABSTRACT

The antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism was examined for its ability to effect killing of the murine P815 mastocytoma tumor *in vitro* and *in vivo*. Xenogeneic rabbit anti-P815 antisera, but neither allogeneic C57Bl/6 nor syngeneic DBA/2 anti-P815 antisera, were able to mediate ADCC against chromium-51 labelled P815 tumor targets *in vitro*. While C57Bl/6 anti-P815 antisera were incapable of mediating *in vitro* ADCC of P815 targets, these sera were able to mediate complement-dependent lysis of P815 targets and also were able to mediate both ADCC and complement-dependent lysis of L1210 tumor, an $H\text{-}2^d$ tumor syngeneic to P815 and to the murine DBA/2 strain. The possible mechanisms of resistance of P815 tumor targets to cytolysis by means of the ADCC pathway are discussed.

Several effector cell populations were tested for their abilities to mediate ADCC against P815 in the presence of xenogeneic rabbit anti-P815 antisera. Only human peripheral blood lymphocytes and rat spleen cell effectors were able to induce lysis of antibody-coated P815 targets. Syngeneic adult DBA/2 spleen cells were unable to lyse antibody-sensitized P815 targets, although these same effector cells were able to lyse antibody-sensitized chicken red blood cell (CRBC) targets. Alloactivated murine spleen cells lysed unsensitized P815 targets; this lysis was blocked by the addition of xenogeneic anti-P815 antiserum.

Rabbit anti-P815 antisera were absorbed either *in vitro* with DBA/2 spleen, lymph node, thymus, blood, liver and bone marrow cells at various temperatures (4°, 20°, 37° C) or *in vivo* by passage through DBA/2 mice. These sera were then tested for their specificity in the complement-mediated lysis and ADCC assays against P815 tumor targets, L1210 tumor targets and normal spleen cell targets. The absorption process nonselectively removed antibody activity directed against both the specific P815 target and the nonspecific L1210 and normal spleen cell targets.

The *in vivo* P815 dose response experiments indicated the P815 mastocytoma to be a highly lethal tumor with a TD_{50} and an LD_{50} of 80 cells per mouse and 83 cells per mouse respectively. Regression of tumor growth was rare. Results of the *in vivo* ADCC protection experiments using xenogeneic rabbit anti-P815 antisera and either human PBL or mouse spleen cell effectors were inconclusive. Although protection appeared to occur in initial sporadic instances, ADCC protection in the P815 system was not confirmed in subsequent controlled experiments.

I met a seer.
He held in his hands
The book of wisdom.
"Sir," I addressed him,
"Let me read."
"Child—" he began.
"Sir," I said,
"Think not that I am a child,
For already I know much
Of that which you hold;
Aye, much."

He smiled.
Then he opened the book
And held it before me.
Strange that I should have grown
So suddenly blind.

— Stephen Crane,
"The Book of Wisdom"

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List of Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity

ads: adsorbed serum

C': complement

CFA: complete Freund's adjuvant

CRBC: chicken red blood cell

E:T: effector cell to target cell

HBSS: Hanks Balanced Salt Solution

HIFCS: heat-inactivated fetal calf serum

i.d.: intradermal

Ig: immunoglobulin

i.p.: intraperitoneal

i.v.: intravenous

K cell: "killer" cell of ADCC

MEM: minimum essential medium

MEM-complete: 187 ml MEM, 10 ml HIFCS, 1 ml 200mM glutamine 2 ml antibiotic-antimycotic solution

NK: natural killer cell

NMS: normal mouse serum

NRS: normal rabbit serum

PBL: peripheral blood lymphocyte

PBS-CMF: phosphate-buffered saline, calcium and magnesium free

PMN: polymorphonuclear leukocyte (neutrophil)

SAP: saponin detergent lysis

sc: spleen cells

SR: spontaneous release

SRBC: sheep red blood cell

List of Abbreviations
(Continued)

T: total counts control

TDHP: ten donor human plasma

Test medium: 100 ml Dulbecco's modified Eagle (High Glucose) medium (GIBCO #196G) plus 2.5 ml 1 M Hepes (GIBCO) plus 5 ml of heat-inactivated fetal calf serum

TH: 10X Tris buffer, 175 ml HBSS, and 5 ml HIFCS

unads: unadsorbed serum

Z.C.: complement-opsonized zymosan particles

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I. INTRODUCTION

The role played by effector cells in tumor immunity and rejection is highly controversial. A wide variety of cell types mediate specific and non-specific tumor cell killing through a number of postulated mechanisms. Among the anti-tumor phenomena which have been explored by recent investigations are cytotoxicity and cytostasis mediated by cells (T cells, macrophages, natural killers, antibody-dependent cells) and by soluble factors (complement, lymphokines).

The purpose of this introduction is to focus on one of these anti-tumor phenomena, that of antibody-dependent cell-mediated cytotoxicity (ADCC), and to provide evidence suggesting the role of ADCC in tumor immunity. Section A will deal with tumor systems in which _in vitro_ ADCC has been shown. Section B will present _in vivo_ evidence indicating the association between tumor regression and the appearance of ADCC activity. Section C will provide support for the hypothesis that passive transfer of anti-tumor antibody and cells protects hosts from tumor challenge. Section D outlines factors governing the selection of the P815 mastocytoma system for the study of the ADCC mechanism _in vitro_ and _in vivo_.

Antibody-dependent cell-mediated cytotoxicity describes an _in vitro_ phenomenon, first observed by Moller (113) in 1965, in which antibody-coated target cells are lysed by unsensitized leukocytes. The heterogeneous effector cells which kill antibody-coated targets _in vitro_ fulfill two basic criteria: 1) they bear membrane receptors for the Fc region of immunoglobulin, and 2) they have the inherent capacity to kill target cells. Although ADCC relies on antibody for its specificity (like the complement-dependent pathway), the ADCC mechanism is a separate complement-independent lytic pathway.

A. Tumor Cell Systems In Which In Vitro ADCC Has Been Shown

The first clear demonstrations of ADCC in a tumor system were those of Holm and Perlmann (140) and MacLennan et al. (106) who reported that human polyploid Chang liver cells were lysed in the presence of xenogeneic anti-Chang antiserum and unsensitized allogeneic or xenogeneic lymphoid cells. Since this initial pioneering work, a large number of mouse, rat, and human tumors have been successfully established as targets in in vitro ADCC assays. Among the murine tumors which have been examined in ADCC studies are the EL-4 leukemia (186),the YAC tumor (165), the SL-2 lymphoma (52), the MNB neuroblastoma (21), the 6C3HED lymphoma (53), and the P815 mastocytoma (102). The murine Moloney sarcoma virus (MSV)-induced tumor yielded particularly interesting results. A high percentage of Balb/c mice injected with MSV developed tumors which spontaneously regressed. Regressors possessed both anti-tumor antibody and cell populations (T and non-T) which interacted synergistically in vitro to kill and/or inhibit growth of target cells* bearing Moloney leukemia virus (MLV)-determined antigens (90).

Although most studies of the role of ADCC in tumor immunity were performed in mice, some have also been done in rats and humans. For example, the extensively studied rat tumor, (C58NT)D lymphoma, has been used to study the antibody-coded specificity of ADCC (133). In man, several tumors have been examined, including acute myeloid leukemia (119), colon carcinoma, urinary bladder carcinoma (135), osteogenic sarcoma (183), and Epstein-Barr virus-induced lymphoblastoid lines (73).

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*Ambiguities in the interpretation of microcytotoxicity assay results, such as those obtained in the MSV tumor system, will be discussed in Section II.B.

B. Appearance of ADCC Activity Associated with Tumor Regression

1. Involvement of serum antibody

The demonstration of an association between tumor regression and the appearance of lymphocyte-dependent antibody was among the first evidence providing indirect support for the hypothesis that ADCC may function in tumor immunity. Shortly after Moloney sarcoma virus (MSV) inoculation (10-14 days), sera from tumor-bearing mice mediated *in vitro* ADCC (89). While sera from regressor mice possessed very pronounced antibody activity (90), sera from mice with progressively growing tumors (40-50 days post-inoculation, were generally ineffective in mediating cell-dependent microcytotoxicity. It was hypothesized that the early appearance of cell-dependent anti-tumor antibody 15 days post-virus inoculation may represent the critical host response determining tumor regression or progression.

Further evidence for the role of lymphocyte-dependent antibody in tumor immunity was found in the syngeneic (C58NT)D rat tumor system. As in the MSV system, rejection of the (C58NT)D tumor in regressors was closely associated with the appearance of serum ADCC activity (133). Furthermore, tumor-immunized rats which reject tumors upon challenge possessed sera with marked ADCC activity but lacked T cells with detectable direct cytolytic ability.

Fractionation of the regressor and immune sera revealed that the arming activity was localized to the 7S peak, indicating that the active component of serum was associated with the fraction containing 200,000 dalton molecular weight IgG immunoglobulin (133). Since minute amounts of rabbit anti-rat IgG serum completely blocked the serum arming activity, it was suggested that IgG antibody was the specific arming factor (134).

2. <u>Modulation of non-T effector cell populations during tumor growth</u>

If regressor and immunized animals possess serum antibodies which sensitize tumor targets *in vitro* and activate the cell-mediated cytolysis mechanism, then antibody-armed cytolytic effector cells might also be isolated from such animals. However, since the methodology for the detection of antibody-armed effectors does not yet exist, alternative approaches must be considered. One promising approach has been to measure non-T cell-mediated tumor-specific cytolytic activity, the rationale being that specific non-T cell mediated cytolysis may be identical to ADCC. The following describes experiments which correlate the appearance of non-T cell mediated anti-tumor activity with tumor regression and immunity.

In the Moloney sarcoma virus (MSV) system, Lamon and collaborators (90) showed that the anti-tumor activity of immune lymphocytes from regressor mice was contained in the theta negative, Ig positive fraction of cells. The activity of immune lymphocytes was highly specific for MLV antigens, since MLV-negative control target cells were not killed. Although these experiments do not rule out the possibility that B lymphocytes participate in ADCC, perhaps by secretion of tumor-specific antibody, experiments in other systems (56,57), particularly those demonstrating normal ADCC effector cell activity from patients with severe hypogammaglobulinemia (180), strongly suggest that the differentiated B lymphocyte lacks the ADCC capability. A more probable explanation of Lamon's data is that non-T, non-B, Fc receptor-positive cells are the killer cells, and that their surface immunoglobulin was passively adsorbed to the cells rather than synthesized by them. In support of this

explanation is evidence by Greenberg et al. (49) that the effector cell involved in ADCC against murine tumor targets is a non-T, non-B, non-phagocytic cell, perhaps in the monocyte lineage.

Evidence for the role of non-T cells in tumor immunity in the murine mammary tumor virus system was found by Blair et al. (14) who reported that the non-T cell-mediated cytolytic activity present in the spleens of multiparous female Balb/cfC3H mice disappeared as mammary tumors progressed in these hosts to large size. In contrast, the splenic cytolytic T cell activity remained unchanged throughout tumor growth. These investigators speculated that the loss of non-T cell activity in large tumor bearers may have contributed to the development of the tumor in these hosts. Furthermore, since the presence of a T cell response was insufficient to destroy tumor in vivo, Blair et al. suggested that perhaps non-T cells were functionally more important than T cells in delaying the onset of mammary tumor development.

Preliminary evidence for the participation of non-T cells in defense against tumors also exists in humans. Peripheral blood leukocytes isolated from patients with bladder carcinoma were specifically cytotoxic to bladder carcinoma cells in vitro (17, 135). The predominant or total cytolytic activity was present in the non-T lymphocyte fraction. Thus, cells passed over an anti-Ig column were devoid of cytolytic activity (as measured by both a microcytotoxicity assay and a lytic Cr^{51} release assay)

in comparison to controls.*

C. Protection Against Tumor Growth by Antibody-dependent Mechanisms

1. Protection effected by administration of passive antibody

Treatment of tumors by passive administration of serum antibody was attempted by many investigators. As early as 1895, Hericourt and Richet (69, 70) treated sarcoma patients with xenosera. Since these initial studies, numerous reports appeared (reviewed in Motta) in which antitumor antisera were used to treat tumors in man and in experimental animals.

Perhaps some of the most extensive and informative studies were performed using the murine EL-4 leukemia tumor. In 1956, Gorer and Amos (46) described substantial protection against EL-4 lymphoma challenge when recipient mice were given allogeneic antibody prior to tumor injection. Antibody given two days after tumor injection also afforded a smaller degree of protection. More recently, Zighelboim (185) confirmed and extended Gorer and Amos' initial findings by demonstrating that passive administration of antiserum containing ADCC activity into tumor bearers resulted in tumor rejection.

Passive antibody induced tumor regression in the EL-4 system was

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*Although this evidence is consistent with the hypothesis that Ig-bearing non-T cells mediate ADCC, there is another interpretation of the data which needs to be evaluated; that is, since the anti-Ig antibody which was conjugated to the column in these experiments possessed intact Fc portions, it is possible that Fc receptor bearing T cells attached to the column through the chemically activated Fc regions. To rule out this possibility, it is necessary to select antibody for these experiments which is incapable of binding to cellular Fc receptors, ideally F(ab) or $F(ab')_2$ fragments of anti-Ig antibody which lack the Fc region, or perhaps alternatively goat IgG anti-Ig antibody which has been reported to be incapable of binding to human cellular Fc receptors (179).

shown to depend on host cells, particularly macrophages and platelets (Shin, *et al*. 161, 162); and tumor suppression did not require complement (160). Further investigation revealed that host accessory cells (macrophages) rather than lymphocytes were the primary cell type involved in antibody-dependent suppression of tumor growth (161, 162, 164).

The hypothesis that macrophage-mediated cytotoxicity was responsible for antibody-induced protection against the EL-4 tumor was presented when it was found that transferred macrophages suppressed *in vivo* lymphoma growth. Since killed macrophages were as effective as live ones, it was postulated that cytotoxicity was mediated by macrophage membranes. This hypothesis was supported by unpublished observations by Zacharchuck and Shin (184) which showed that isolated macrophage membrane vesicles were themselves cytotoxic *in vitro*. An alternative explanation for the phenomenon of macrophage-mediated protection is macrophage-mediated cytostasis or growth inhibition (137, 138). Further investigation is necessary to distinguish between cytotoxic and cytostatic mechanisms.

In addition to macrophages, platelets also play a role in antibody-induced EL-4 tumor regression [Shin, *et al*. (161, 162)]. The finding by Shin that platelets were active in *in vivo* ADCC was confirmed *in vitro* by Lovchik and Hong (101, 102). The mechanism of platelet-induced tumor cytolysis is not known; however, it may be similar to that bacteriolytic process which is mediated by means of a soluble platelet factor (30).

Less extensive examinations into passive immunotherapy of tumors using serum antibody have been reported in other tumor systems. For example, in the murine Moloney sarcoma virus system, passive antibody from regressor mice was shown to 1) protect unimmunized hosts against tumor challenge (139), and 2) cause established growing tumors to regress

(165, 65, 39). The mechanism of antibody-mediated protection remains to be elucidated. Similarly, xenogeneic rabbit antibody, raised against purified ovarian mouse tumor cell components, was able to prolong the survival of tumor bearing mice (125). Treatment of mice with silica, an inhibitor of macrophage function, reduced the efficacy of antibody, suggesting a role for antibody-dependent macrophage-mediated tumor suppression.

Passive administration of anti-P815 serum alone delayed the onset of growth of the P815 mastocytoma in C5 deficient DBA/2 mice (12). This result suggested that the complement-mediated lytic pathway was not involved in protection. Instead, anti-tumor antibody may have activated host cellular elements to mount a cytolytic response against the mastocytoma. Compatible with this explanation is evidence that aged New Zealand mice possess a spontaneously arising cytotoxic lymphoid cell which specifically killed P815 (50). The cytotoxic cell was not a T cell nor a phagocyte. The speculation was that the cytotoxic effector was a K cell armed by circulating anti-self antibody-antigen complexes known to exist in NZB mice.

Antibody-dependent protection has also been shown in rat tumor models. Bansal and Sjogren (5, 72) reported antibody-induced tumor regression in the polyoma virus-induced tumor. Extensively adsorbed xenogeneic rabbit sera and syngeneic rat sera both were capable of prolonging survival of rats given syngeneic polyoma tumors. In an unrelated rat lymphoma system, heterologous rabbit antisera were found by Hersey (72) to be capable of inhibiting the onset of tumor growth when antibody treatment was given up to five days after tumor injection. Interestingly, this xenogeneic antibody contained high levels of ADCC activity, whereas allogeneic rat

antibody against the same tumor lacked lymphocyte-dependent antibody and also lacked in vivo protective capacity.

While studies of passively transferred antibody have yielded significant information concerning antibody-mediated anti-tumor mechanisms, the interpretation of results from these studies is necessarily complex. If the complexity of host contributions to immunity (e.g., complement, T cells) could be reduced, interpretation of results would be simplified. A simpler model might be one in which both anti-tumor antibody and ADCC effector cells were provided to a host. This would insure that the necessary components of the ADCC mechanism were available. If these components were injected into an immunodeficient host (e.g., T cell deficient nude mouse, complement-deficient mouse), the chances of studying ADCC in isolating would be enhanced.

Few studies to date have employed this approach to examine anti-tumor ADCC in vivo. However, one such study was that of Byfield and co-workers (21). They reported protection from growth of antibody-coated murine neuroblastoma tumors when mice receiving antibody-sensitized tumor cells were simultaneously given rat spleen cells. Both specific antibody and effector cells were required for tumor growth inhibition to occur. These results indicated that the ADCC effectors prevented growth of antibody-coated tumors.

Is it possible to halt growth of established malignancies by injecting ADCC effector cells into the host? This question may be technically difficult to answer. If anti-tumor antibody and effector cells are injected into a solid tumor-bearing host, ADCC-mediated growth inhibition may be expected to be minimal. This result may be explained by the steps involved in activation. In order to activate the ADCC effector cells, antibody

must first bind to the tumor cells, then antibody must bind to the effector cells. Killing of tumor cell targets by effectors may then occur. The efficiency of activation may be enhanced by injecting antibody-armed effector cells into the host. One step in the activation process, that of binding of antibody to effector cells, is eliminated. The efficiency of tumor cell killing is increased and depends upon the kinetics of binding of antibody-armed effector cells to targets.

One study utilizing armed effector cells has demonstrated an anti-tumor effect. Van Loveren and Den Otter (173) showed that macrophages armed *in vitro* by incubation with culture supernatants from spleen cell and SL-2 tumor cell mixtures were capable of prolonging survival of syngeneic mice injected intraperitoneally with the macrophages two hours after tumor injection. The "arming" factor responsible for conferring specificity to the macrophage may have been cytophilic antibody. In support of this is evidence that after putative antibody was stripped from the cell membranes of "armed" macrophages (trypsin treatment or incubation at 56°C for 30 minutes), the macrophages could be "re-armed" by immune serum containing anti-tumor antibody (145).

D. Factors Governing the Selection of the P815 Mastocytoma System for the Study of the ADCC Mechanism In Vitro and In Vivo

The P815 mast cell neoplasm arose as a methylcholanthrene-induced solid tumor in a male mouse of the DBA/2 strain (32). The solid tumor was easily adapted to ascites growth as a nonhemorrhagic, highly concentrated neoplasm. The tumor was subsequently cultivated *in vitro* in Spinner culture, allowing the production of large quantities of purified, uniform tumor cells devoid of host cell and host factor contamination. There are

several advantages in using the P815 tumor system in ADCC studies: 1) The P815 tumor cell is easily labelled with the Cr^{51} isotope and exhibits low spontaneous release which makes it ideal for in vitro cytotoxicity studies. This tumor has classically been used as a target in direct T cell-mediated cytotoxicity experiments (2, 22) and recently in indirect ADCC experiments (12, 33, 34, 102). 2) The tumor is easily maintained both in vivo (as a solid and as an ascites tumor) and in vitro in Spinner culture. 3) The mouse tumor has high metastasizing potential, which makes it similar to many human tumors. 4) The P815 tumor is syngeneic to the DBA/2 mouse, a strain which lacks the fifth component of complement and therefore has an aborted lytic pathway (25, 121, 152). Since complement-mediated lysis of tumors has been implicated as an effective anti-tumor mechanism in some tumor systems examined (e.g., leukemias) (46, 47, 98), and since both complement-mediated lysis and ADCC mechanisms depend on serum antibody to confer specificity to immune attack against tumor targets, these two mechanisms have been difficult to distinguish in vivo. The complement-deficient DBA/2 mouse, whose C' dependent lytic pathway has been aborted, offered a unique model in which one could study the ADCC mechanism in vivo in relative isolation. 5) The tumor has been studied extensively as a model of allograft rejection (22), although immunity to syngeneic P815 tumor has not been well characterized. 6) The P815 tumor has been studied in the laboratory of Dr. John Harris at UCSF. There are also disadvantages to this system: a) The P815 tumor is a mastocytoma, a tumor type which is relatively uncommon among mouse and human tumor histological types, b) P815 tumor has a rapid proliferation rate which makes it dissimilar from more slowly growing arising neoplasms, and c) finally, unknown to us prior to beginning the experiments, P815 mastocytoma targets are resistant to lysis

by means of the ADCC mechanism. The first two disadvantages are relatively minor ones; however, the last disadvantage, which was discovered during the course of experimentation with P815, made interpretation of ADCC results in the P815 tumor system difficult. This topic will be discussed in greater detail in the discussion section.

II. MATERIALS AND METHODS

A. Tumors

The tumor cell lines used in these experiments were the P815 mastocytoma, the P388 leukemia, and the L1210 lymphocytic leukemia, all syngeneic to H-2d DBA/2 mice. The P815 and P388 lines were generously donated by Dr. John Harris, Laboratory of Radiobiology, U.C.S.F. L1210 was obtained from Dr. David Martin, Department of Biochemistry, U.C.S.F. All cell lines were maintained by the U.C.S.F. Cell Culture Facility in Corning T-75 (75 cm^3) tissue culture flasks in 30 ml of media. The P388 and L1210 lines were grown in Dulbecco's medium with 10% added fetal calf serum. P815 was grown in Swims 77 with 10% calf serum. Flasks were placed in a 37^oC, 5% CO_2 incubator; medium was replenished daily to maintain maximum cell viability (assessed by trypan blue exclusion).

B. Rationale for the Use of the ADCC Assay

1. The microcytotoxicity assay

There are several *in vitro* methods available for assessing cell-mediated cytotoxicity. One of the first methods to be developed was the microcytotoxicity test of Takasugi and Klein (170). In this assay, tumor cells are allowed to adhere to culture wells, then incubated with effector cells and antibody, in the case of ADCC, for 24-72 hours. After the appropriate incubation period has elapsed, effector cells and dead or dying target cells detach and are washed from the plate, leaving viable, adherent target cells in each well. Plates are stained, and cells in each well are enumerated. Cytotoxicity is evaluated by comparing remaining target cells in experimental versus control wells.

There are several disadvantages associated with the microcytotoxicity assay: 1) Since living, dividing tumor cells are used as targets in this relatively long-term assay, this method measures cytostatic as well as cytotoxic effects. That is, any factor which inhibits the growth of the target cells will cause a reduction in cell number in the experimental groups when compared with controls. 2) For target cells to be enumerated at the assay's conclusion, they must not only be viable but also attached to the well bottoms. Thus, any factor which dislodges target cells from wells without killing them will also be mistakenly measured as exhibiting "cytotoxicity" in this assay. 3) Finally, cell counting is subjective and extremely tedious.

2. The chromium-51 radioisotope release assay

Faced with these disadvantages, investigators developed radioisotope pre-labelling release assays for accurate, objective, and more rapid assessments of cell-mediated cytotoxicity effects. Bean et al. (8) described several criteria for evaluating an isotopic label for its usefulness in cell-mediated cytolysis assays:

> "(a) the amount of isotope incorporated into target cells should be sufficient to allow adequate labeling of relatively small numbers of cells without toxicity; (b) the isotope should be evenly distributed among the individual target cells, irrespective of their metabolic activity or cell cycle stage; (c) the incorporated isotope should be released in the supernatant fluid only when irreversible damage of the target cells has occurred; (d) release should be rapid and complete; and (e) once released, the isotope should not be reutilized by target cells or lymphocytes."

Among the radioisotopic markers now available for use in the cell-mediated cytotoxicity assays are chromium-51-labelled sodium chromate, iodine-125-labelled deoxyuridine ($I^{125}UdR$), tritiated proline, Rb^{86}, and

C^{14}-nicotinamide. The isotopic label which has been used in the majority of cellular cytotoxicity studies is chromium-51.

The chromium release assay meets most of the criteria established by Bean et al. for the evaluation of isotope release assays. First, the radioactive chromate ion ($^{51}CrO_4^{2-}$) readily and rapidly diffuses into target cells where it binds to cytoplasmic macromolecules with a molecular weight ranging from 2000-4000 daltons (113). Relatively small numbers of cells may be labelled adequately with minimal cytotoxicity. Secondly, the efficiency of the radiolabelling process is independent of the cell cycle stage and of the cells' metabolic activities. Presumably, the isotope is evenly distributed among the target cell population; however, this hypothesis needs to be critically evaluated, possibly using limiting dilution experiments. Thirdly, the chromium-51 method primarily measures advanced, irreversible cell damage associated with cell death. Chromium release from target cells correlates well with other indicators of cell death such as loss of cloning ability and permeability to vital stains (i.e., trypan blue) (178, 169, 16). Radioactive chromate is released from cells subsequent to the efflux of electrolytes, and simultaneously with cytoplasmic protein molecules. Martz speculated that the chromate ion ($^{51}CrO_4^{2-}$) enters the cell and is reduced to Cr^{3+} form which binds to protein (113). If this hypothesis is correct, then the chromium release assay may measure the irreversible efflux of macromolecular proteins immediately preceding the cell lysis event. Finally, once chromium-51 is released from the cell, it is not reutilized and incorporated into other target cells. The chromium release assay therefore fulfills most of the necessary criteria for an ideal isotopic label.

However, there are disadvantages which restrict the applicability of

the chromium release assay to certain tumors and the interpretation of experimental results. First, the rate of spontaneous release for most primary tumors and many *in vitro* established tumor lines is prohibitively high. Thus, only tumor lines which retain an acceptable level of the chromate label (e.g., P815 and L1210) for 6-8 hours may be studied using this method. Since all known cell-mediated cytolytic mechanisms (ADCC, T cell-mediated cytolysis, natural killer cell-mediated cytolysis, macrophage-mediated cytolysis) are demonstrable in short term assays, the chromium release assay is suitable for studies of these mechanisms. Second, even with target cells which retain label, the rate of release of chromium-51 label from damaged cells may vary from cell to cell. Lysis may reach completion within a few minutes or a few hours. Thus, this assay may not be appropriate for studies of the kinetics of cell lysis. Third, less than 100% of cell-incorporated label is released upon cytolysis. Furthermore, Burakoff (18) has found that various methods used to lyse cells differ in their efficiency of label extraction. Detergent or distilled water solubilizes the greatest amount of label; then, in descending order of efficiency are free-thawing, T cell-mediated lysis, and complement-mediated lysis. In our experiments with the P815 tumor, saponin detergent lysis is used as a "maximum lysis" standard. Lysis of P815 targets by means of the ADCC pathway always released less than 100% of the saponin control counts.

C. ADCC Effector Cell Sources

1. Human peripheral blood lymphocytes (PBLs)

Human peripheral blood lymphocyte preparations depleted of phagocytes were isolated from heparinized normal donor blood (10 units

Liquaemin ® heparin/ml blood) by 1) iron filing depletion and 2) density centrifugation over Ficoll-Hypaque (ρ = 1.077). To each 10 ml of heparinized blood was added 32 ml of Technicon lymphocyte separator reagent (Technicon Instruments Corporation, Tarrytown, New York 10591) in a sterile centrifuge tube. Tubes were rotated for 30 minutes at 37°C, and phagocytes ingesting iron were removed with a magnet. Then 10 ml of Ficoll-Hypaque was added to the bottom of the tube using a cannula-fitted syringe. Tubes were centrifuged at 400g for 40 minutes at 18-20°C. The cloudy bands containing lymphocytes were harvested and washed three times in PBS-CMF. PBS-CMF was decanted, and the cell pellet was resuspended in "test" medium consisting of 100 ml Dulbecco's modified Eagle (High Glucose) medium (GIBCO #196G), 2.5 ml 1M Hepes (GIBCO) buffer, and 5 ml HIFCS.

2. DBA/2 mouse spleen cells

Single cell suspensions (3×10^6/ml) from pooled DBA/2 spleens were layered over Ficoll-Hypaque (ρ = 1.09) in sterile centrifuge tubes, and the tubes were spun at 400g at 20°C for 20 minutes. Bands containing lymphocytes were harvested and washed 3 times in "test" medium.

3. Rat spleen cells

Individual rat (Fisher 344 or Lewis) spleens were minced, and single cell suspensions separated over Ficoll-Hypaque (ρ = 1.09) as above. Cells were washed three times in HBSS, then suspended in "test" medium for assay.

4. Mouse peritoneal macrophages

To stimulate the induction of peritoneal macrophages, 1.5 ml of thioglycollate broth was injected into each of 10 male DBA/2 mice (18-20g);

then, peritoneal cells were harvested five days later to be used in ADCC assays in vitro.

5. Alloactivated mouse splenic cell effectors

Two male C57Bl/6 mice were injected with 2.8×10^7 P815 cells i.p. each, according to the procedure established by Brunner et al. (16) for activation of cytolytic T cells. Ten days later, spleen cells were purified and assayed for direct cell-mediated cytolysis against radio-labelled P815 as outlined in Section II.E.1.

Since Kimura et al. (84) reported the ability of alloactivated murine T cells to mediate ADCC, two allogeneic systems were examined for ADCC activity: 1) DBA/2 mice each were sensitized with 7.2×10^7 DBA spleen cells, and 2) C57Bl/6 mice each were sensitized with 1.2×10^8 DBA/2 spleen cells. Five days later, spleens from alloactivated DBA/2 and C57Bl/6 mice were excised, cell suspensions were made, then splenic lymphocytes were isolated by Ficoll-Hypaque density centrifugation and washed.

D. Antiserum Directed Against P815

1. Xenogeneic rabbit anti-P815 antiserum

a. Immunization schedule

Four New Zealand White rabbits (#8, 9, 10, 11) each were immunized subcutaneously with 2×10^8 P815 cells in complete Freund's adjuvant (CFA), then boosted monthly for four months with the same P815-CFA mixture. Animals were bled sequentially 7-18 days after each boost to determine the day of peak anti-P815 antibody activity, at which time the rabbits were anesthetized and bled of 100-150 ml blood.

b. In vitro adsorption procedure

Prior to adsorption, rabbit sera were heat-inactivated (56°C, 1 hour). Single cell suspensions from the following DBA/2 mouse organs were made: blood, spleen, liver, lymph node, thymus, and bone marrow. Suspensions were washed twice, pooled, and centrifuged. The supernatant was decanted, and to the cell pellet was added heat-inactivated rabbit serum at 1:1 serum to packed cell volume ratio, and the serum-cell mixture was rotated for 2-18 hours. Evaluations of sera adsorbed up to three times at different temperatures (4°C, 20°C, 37°C) were made. Based on these tests, sera adsorbed both at 4°C and 20°C (twice adsorbed) were chosen for subsequent in vivo ADCC experiments.

c. In vivo adsorption procedure

DBA/2 mice were injected i.p. or i.v. with 1-2 ml of rabbit anti-P815 antiserum. After 4-12 hours, animals were bled, and sera was tested in vitro for anti-P815 and anti-H-2 activity.

2. Allogeneic C57Bl/6 anti-P815 serum

C57Bl/6 anti-P815 allosera were donated by Dr. Una Chan and Dr. Robert Mishell, University of California, Berkeley.

3. Syngeneic DBA/2 anti-P815 serum

DBA/2 mice each were injected i.p. or i.v. with $4x10^7$ cesium-137(4540R) irradiated P815 cells, then boosted two weeks later. Mice were bled 7 days after the boost, and sera were tested for anti-P815 activity.

A second source of syngeneic DBA/2 anti-P815 serum was 1-2 week ascites tumor-bearer serum obtained from Dr. Una Chan and Dr. Robert Mishell, University of California, Berkeley.

E. Antibody-Dependent Cell-Mediated Cytolysis (ADCC) Assay

1. Radiolabelling of cell targets

a. Mammalian cell targets

$2x10^6$ mammalian cell targets (P815, L1210, P388, or normal spleen cells) were placed in a centrifuge tube and spun at 1500 rpm for 5 minutes. The supernatant was decanted, and to the pellet were added 200λ test medium and 200λ radioactive sodium chromate in sterile isotonic saline (1mCi/ml chromium-51, Amersham Corporation). The mixture was incubated at 37°C for 45 minutes. Then 10 ml of test medium were added, and the cell suspension centrifuged. The radioactive supernatant was decanted, ten ml fresh test medium were added and mixed, then the suspension was reincubated at 37°C for 30 minutes. The cell suspension was centrifuged, and the pellet was washed three times before suspension in 3 ml test medium for counting.

b. Chicken red blood cell targets

0.5 ml of blood was drawn from a White Leghorn chicken into heparinized Tris-Hanks (TH) buffer (175 ml HBSS, 5 ml HIFCS, 20 ml 10x Tris). Cells were washed three times in TH buffer, then suspended to a concentration of $1.5x10^8$ cells/ml. To 200λ of cell suspension were added 200λ of TH buffer and 200λ of sodium chromate (1mCi/ml chromium-51). The mixture was incubated at 37°C for 60 minutes, then washed three times in TH buffer. Cells were resuspended in 3 ml of MEM-complete (187 ml MEM, 10 ml HIFCS, 1 ml 200 mM glutamine, 2 ml antibiotic-antimycotic solution) and counted.

2. Effector cell preparation

Effector cells were prepared as indicated in Section II.C. above.

Washed cells were then suspended in either 1) test medium for ADCC assay against nonerythrocyte targets, or 2) MEM-complete for ADCC against CRBC targets.

3. Preparation of antibody-coated target cell mixtures

Radiolabelled target cells were suspended as follows: Cell concentrations were selected on the basis of minimum spontaneous lysis data obtained with varied target cell concentrations. Final target cell concentrations were $5x10^4$ cells/ml for P815, $2.5x10^5$ cells/ml for P388, $2.5x10^5$ cells/ml for L1210, $8x10^5$ cells/ml for CRBC, and $5x10^5$ cells/ml for murine spleen cell targets.

Target cells were coated with anti-target cell antiserum, normal serum, or medium by the following procedure: Tubes containing two parts of radiolabelled target cells and one part of serum or medium were vortexed, for a final 3/4 dilution.

4. Microtiter plate arrangement

A representative microtiter plate arrangement for ADCC experiments is diagrammed in Figure 1. The P815 target was used as an example; ADCC experiments with other targets were arranged in a similar manner.

a. Spontaneous release (SR), Total (T), and Saponin (SAP) controls

To wells A 1-8 of Linbro 96 well, round bottom plates (#76-014-05) were added P815 target cells (100λ /well) and medium (50λ /well). Wells A 1-4 served as the spontaneous release (SR) control measuring the amount of chromium-51 radioisotope released spontaneously from cells over the specified assay time period (e.g., 8 hours for P815 targets). Wells A 5-8 served as the "total counts" control; after the

elapsed assay period, an aliquot of cells and medium from each well was deposited into a corresponding labelled tube for counting. Thus, the "total counts" control represents a measure of the amount of chromium-51 present in each well. To wells A 9-12 were added 100λ of target cells and 50λ of saponin (SAP) (10g/100 ml), a detergent which lyses cells maximally.

b. Effector cells alone control

To wells B 1-4 were added 100λ of target cells and 50λ of effector cells at the maximum effector to target (E:T) cell ratio used in the experiment. This control would detect cytotoxic effects of effector cells alone against targets.

c. Serum alone control

To quadruplicate wells were added target cells coated with either anti-P815 antiserum (D 1-12) or normal serum (C 1-12) dilutions (100λ /well) and medium (50λ /well). This control screens potential cytotoxic effects of sera alone against radiolabelled targets.

d. Normal serum plus effector cells control

To quadruplicate wells (row E) were added normal serum-coated target cells (100λ /well) and effector cells (50λ /well), at each designated E:T ratio and serum dilution tested.

e. Experimental: anti-target cell antiserum plus effector cells

To quadruplicate wells (row F) were added anti-P815 serum-coated target cells (100λ /well) and effectors (50λ /well) at the chosen E:T ratios and antiserum dilutions tested.

Figure I: Microtiter plate arrangement

	1 2 3 4	5 6 7 8	9 10 11 12
A	P815 spontaneous release (SR)	P815 total counts (T)	P815 saponinlysis (SAP)
B	P815 + peripheral blood lympho- cytes (PBL)		
C	P815 + normal rabbit serum (NRS) (1:10)	P815 + NRS (1:100)	P815 + NRS (1:1000)
D	P815 + rabbit anti-P815 serum (1:10)	P815 + rabbit anti-P815 (1:100)	P815 + rabbit anti-P815 (1:1000)
E	P815 + NRS (1:10) + PBL	P815 + NRS (1:100) + PBL	P815 + NRS (1:1000) + PBL
F	P815 + rabbit anti-P815 (1:10) + PBL	P815 + rabbit anti-P815 (1:100) + PBL	P815 + rabbit anti-P815 (1:1000) + PBL

5. Plate incubation, supernatant harvesting, and radioactivity counting

Microtiter plates were incubated in a 37°C, 5% CO_2 incubator for 8 hours, in the case of mammalian cell targets, or for 4 hours in the case of chicken erythrocyte targets. During this time, 12x75 mm glass tubes were labelled to correspond to the microtiter plate wells. After the appropriate incubation period had elapsed, 75λ of well suspension from each of the wells labelled A 5-8 were deposited into each tube with the corresponding marking; these tubes represented the "total counts" control. The microtiter plates were then centrifuged for 5 minutes at 1000 rpm. After centrifugation, 75λ of each well's supernatant was deposited into its corresponding glass tube. Tubes were counted for 10 minutes each in a Packard Auto-gamma Scintillation Spectrometer on a channel with a window setting from 300 to 335 Kev, a setting appropriate for chromium-51.

6. Percent specific lysis computation

The percent specific lysis was calculated by using the following formula:

$$\% \text{ specific lysis} = \frac{E - C}{M - C} \times 100\%, \text{ where}$$

E = counts released in the experimental group

C = counts released in the control group

M = maximum lysis control

In ADCC experiments, the experimental group was that set of wells receiving anti-target serum-coated target cells and effector cells; the control group was generally normal serum-coated targets and effector cells, although in early experiments, targets and effector cells served as the control; the maximum lysis was the saponin detergent lysis control. In complement-mediated lysis experiments, the experimental group was that set of wells receiving anti-target serum-coated target cells and guinea pig complement; the control group was generally anti-target serum-coated targets alone, and maximum lysis was the saponin lysis control.

F. Complement-mediated Target Cell Lysis

1. Complement titration

Guinea pig and rabbit complement (GIBCO) were tested for lytic activity against sheep red blood cells (SRBC). One ml of sheep blood in Alsevier's solution was washed three times in PBS, then twice in HBSS plus sodium bicarbonate. To 2 ml of SRBCs (1×10^9/ml) was slowly added 2 ml of a 1:100 dilution of anti-sheep hemolysin (GIBCO). The mixture was incubated at 37°C for 10 minutes.

To each of 12x75 mm glass tubes was added 200λ of sensitized SRBC, 200λ of a complement dilution, and 1.1 ml of HBSS. Tubes were incubated

at 37°C for 1 hour, and centrifuged for 5 minutes in an Adams serofuge. The endpoint of total hemolysis was determined visually. The endpoint for titration was the highest dilution of complement giving complete hemolysis (CH_{100}).

Once rabbit anti-P815 antisera was tested and proven to contain complement-mediated lytic antibody, both guinea pig and rabbit complement were retitrated on chromium-51 labelled P815.

2. Tumor target cell lysis

P815 tumor cells were labelled with chromium-51 as described in Section II.E.1. Initially, complement-mediated plate assay was established. In the tube method, to 12x75 mm tubes each were added 200λ radiolabelled P815 (5×10^4/ml), 100λ of antiserum dilution, and 100λ of complement (1:10). Tubes were incubated at 37°C for 1 hour; then 500λ of cold PBS was added to each tube to halt lysis. Tubes were centrifuged at 2500 rpm for 5 minutes. 250λ of each supernatant was deposited in corresponding labelled glass tubes containing 1 ml of distilled H_2O. Tubes were counted from 4 to 10 minutes each in a Packard gamma counter.

In the microtiter plate method, target cells were labelled and coated with antiserum as described in Section II.E.3. 100λ of the antiserum-coated target cells and 50λ of complement (1:5) were added to each well. Plates were incubated for 2-8 hours, then supernatants were harvested and counted as described in Section II.E.5.

G. In vivo ADCC Protocol

1. P815 dose response curve determination

In vitro grown P815 cells with viability greater than 95% were

washed 3 times in test medium and counted. In the first dose response experiment, dilutions of P815 cells were prepared for injection into 5 groups of mice (10 mice per group) to receive 10^6, 10^5, 10^4, 10^3, and 10^2 cells per mouse in a 50λ volume. Mice were anesthetized with 50-100λ of a 1:10 dilution of Innovar-Vet (Pitman-Moore, Inc., Washington Crossing, New Jersey) i.p. in PBS. Mice were shaved of hair over the thorax with a scalpel. 50λ of cells was injected intradermally over the sternum of each mouse using a tuberculin syringe with a 25-27 gauge needle. Groups were examined for date of tumor appearance, tumor growth, and date of mouse death.

Since greater than 80% of mice developed lethal tumors in each of the groups of the first experiment, the tumor cell dose range was reduced to 5, 10, 20, 50, 100, and 1000 cells per group (5 mice per group) in the second experiment. The third experiment was designed to more closely define the dose response curve obtained in experiment #2. In the third experiment, mouse groups (15 mice per group) were injected with 10, 30, 40, 50, 60, 70, 90, and 110 cells respectively. Dates of death were noted. The TD_{50} (50% tumor, take dose) was calculated to be 83 cells per mouse, and the LD_{50} (50% mouse killing dose) was calculated to be 80 cells per mouse. Subsequent ADCC protection experiments employed P815 at 83 cells per mouse, the TD_{50} tumor dose.

2. <u>ADCC protection protocol</u>

P815 cells were washed and counted as previously described in Section II.G.1. Immediately prior to injection into DBA/2 mice, P815 cells were diluted and mixed with an appropriate serum and phagocyte-depleted effector cell dilution. Into each mouse was injected 50λ of the cell mixture intradermally. A representative experimental design

is tabulated in Table I.

Table I: ADCC protection protocol

Mouse Group	P815 $4x10^4$ cells/ml	Sera (1:2 dilution)		Human PBL $3x10^6$/ml	Test Medium
		Rabbit anti-P815 serum	Normal rabbit serum (NRS)		
I. P815 + PBL	600λ	--	--	800λ	200λ
II. P815 alone	600λ	--	--	--	1000λ
III. P815 + rabbit anti-P815 serum	600λ	200λ	--	--	800λ
IV. P815 + normal rabbit serum (NRS)	600λ	--	200	--	800λ
V. P815 + rabbit anti-P815 serum + PBL	600λ	200λ	--	800λ	--
VI. P815 + NRS + PBL	600λ	--	200	800λ	--

III. RESULTS

A. Testing of Anti-P815 Antisera Against the P815 Tumor Target in a Complement-dependent Lysis Assay

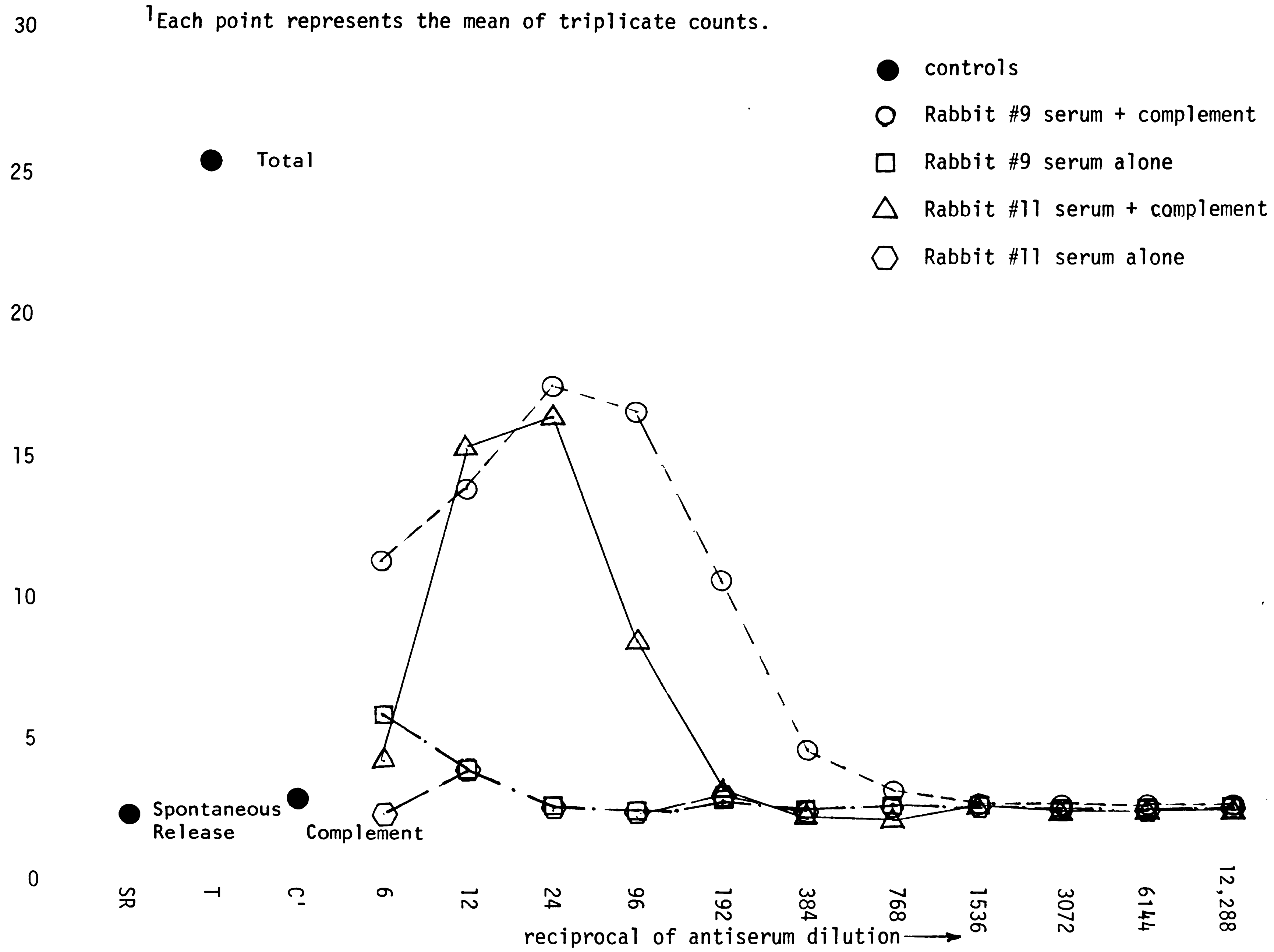
Anti-P815 antisera were raised by immunization of either New Zealand White rabbits or C57Bl/6 or DBA/2 mice. Sera were then examined for the presence of anti-P815 antibodies by assay of complement-mediated lysis of chromium-51 labelled P815 targets. Once high titer anti-P815 antisera were obtained, they were tested for their ability to support ADCC.

1. Rabbit anti-P815 antisera testing

Rabbits were immunized and boosted with P815 in CFA (see Section II.D.1). At various times after boosting, serum samples were obtained and tested in a complement-mediated lysis assay against P815 targets. Prior to immunization, the rabbit sera contained no naturally occurring complement-dependent cytolytic activity against P815. After immunization and boosting, serum complement-dependent cytolytic activity appeared. Anti-P815 activity was detectable in serum as early as five days post-boost. Serum antibody activity rose with time, until peak cytolytic activity was observed between 10 and 13 days post-boost. By 16-18 days post-boost, cytolytic activity diminished. Rabbit #9 and #11 sera taken 13 days after the fourth booster injection were titrated and found to contain high titers of anti-P815 complement-dependent cytolytic antibodies (Figure 2). Large quantities of these sera were aliquoted and stored at 20°C for evaluation in the ADCC assay.

Figure 2. Titration of rabbit #9 and #11 sera taken 13 days post-boost in a complement-dependent microtiter assay[1]

[1]Each point represents the mean of triplicate counts.



2. Mouse anti-P815 antisera testing

Two sources of mouse anti-P815 sera were obtained from Dr. Una Chan and Dr. Robert Mishell: 1) C57Bl/6 anti-P815 sera #35 and #40, and 2) DBA/2 tumor-bearer sera #28, #29 and #30. Only allosera #35 and #40 contained complement-mediated anti-P815 lytic activity; syngeneic tumor bearer sera, normal rabbit serum, and normal mouse serum lacked complement-dependent lytic activity ($p < .05$) (Table II).

Syngeneic anti-P815 sera, raised by immunization of DBA/2 mice with irradiated P815 tumor cells (Section II.D.3.), lacked detectable complement-dependent antibodies.

B. Cytolytic Potential of Effector Cell Populations in the ADCC Assay

Various syngeneic, allogeneic, and xenogeneic cell populations were tested for their ability to lyse rabbit anti-P815 antibody-sensitized radiolabelled P815 targets *in vitro*. Only xenogeneic effector cells (rat spleen cells and human PBLs) were able to lyse the sensitized targets.

1. Murine effector cells

Ficoll-Hypaque purified syngeneic DBA/2 spleen cells were first examined for their ability to mediate ADCC against rabbit anti-P815 antibody-coated P815 targets. Although the rabbit #11 anti-P815 antiserum supported high levels of complement-mediated lysis (Table III), the antiserum failed to support spleen cell-mediated lysis of P815 targets at the 371:1 and 185:1 effector to target cell ratios employed ($p > .05$). Even at a 1000:1 effector to target ratio, no ADCC activity was demonstrable.

Table II: Testing of syngeneic, allogeneic, and xenogeneic anti-P815 antisera in a complement-dependent microtiter assay against radiolabelled P815

	Without complement		With complement	
	%	Counts	%	Counts
Spontaneous release (SR)	20.6	1272	--	--
Saponin (SAP)	100	6178	--	--
Complement (C')	--	--	19.5	1202
Normal mouse serum (NMS)	18.3	1130	18.6	1147
Normal rabbit serum (NRS)	18.1	1117	18.0	1109
Syngeneic DBA/2 tumor bearer #28	17.3	1071	18.6	1147
Syngeneic DBA/2 tumor bearer #29	18.0	1109	17.8	1099
Syngeneic DBA/2 tumor bearer #30	17.8	1097	18.0	1114
Allogeneic C57Bl/6 #35	19.6	1212	69.5*	4296*
Allogeneic C57Bl/6 #40	18.6	1152	75.5*	4662*
Xenogeneic rabbit #11	39.1	2417	119*	7343*

*Significantly greater than serum alone controls using the unpaired t test ($p < .0005$)

Table III: Mouse spleen cell ADCC against rabbit #11 anti-P815 serum-coated P815 targets

	#11 Serum	Complement	Spleen cells		No serum		Rabbit #11 anti-P815 antiserum		
			371:1 E:T	185:1 E:T	%	Counts	1:6	1:60	1:600
Spontaneous release (SR)	--	--	--	--	13.8	2258	--	--	--
Saponin (SAP)	--	--	--	--	100	16,353	--	--	--
Complement (C')	--	+	--	--	21.6	3535	--	--	--
Anti-P815 alone	+	--	--	--	--	--	31.3	12.9	15.5
Anti-P815 + C'	+	+	--	--	--	--	82.0	82.4	26.0
Anti-P815 + S.C.	+	--	+	--	--	--	17.7	14.3	16.4
Anti-P815 + S.C.	+	--	--	+	--	--	23.0	14.5	15.8

While Ficoll-Hypaque purified DBA/2 splenic effector cells were unable to lyse antibody-sensitized P815 cells, they were able to lyse antibody-coated chicken red blood cell targets (Figure 3).

Next, syngeneic DBA/2 peritoneal macrophages were examined for their ability to mediate ADCC. At a 250:1 effector to target ratio, no significant lysis of antibody-coated P815 targets occurred (Table IV).

Since unsensitized syngeneic effector cells lacked ADCC activity against P815 targets, allosensitized murine spleen cells were tested. Alloactivated mouse T lymphocytes have been reported to be capable of expressing Fc receptors and ADCC function (Kimura, et al., 84). Based on this report, two alloactivated mouse spleen cell populations were examined: 1) DBA/2 spleens from mice injected with CBA/J mouse spleen cells, and 2) C57Bl/6 spleens from mice injected with DBA/2 spleen cells. Five days after receiving donor spleen cells, recipient mice were sacrificed, and their splenic cells were separated over Ficoll-Hypaque (ρ = 1.09) and tested in ADCC. Alloactivated spleen cells from C57Bl/6 and DBA/2 mice produced significantly higher levels of lysis from anti-P815 serum-coated targets when compared to normal rabbit serum-coated targets, suggesting spleen cell-mediated ADCC occurred; however, no difference was observed in lysis between uncoated and anti-P815 serum-coated targets (Table V). The high spontaneous release observed in this experiment makes results difficult to interpret. Thus, the alternative hypothesis that no ADCC occurred and that normal rabbit serum, but not anti-P815 serum protected P815 targets from spontaneous lysis must be considered.

Further data providing support for the hypothesis that alloactivated spleen cells fail to mediate ADCC were provided in the following experiment.

Figure 3: Percent specific lysis[1] plot of mouse spleen cell ADCC against CRBC targets

$$^{1}\text{Percent specific lysis} = \frac{(\text{anti-CRBC targets} + \text{S.C.}) - (\text{NRS} + \text{S.C.})}{\text{SAP} - (\text{NRS} + \text{S.C.})}$$

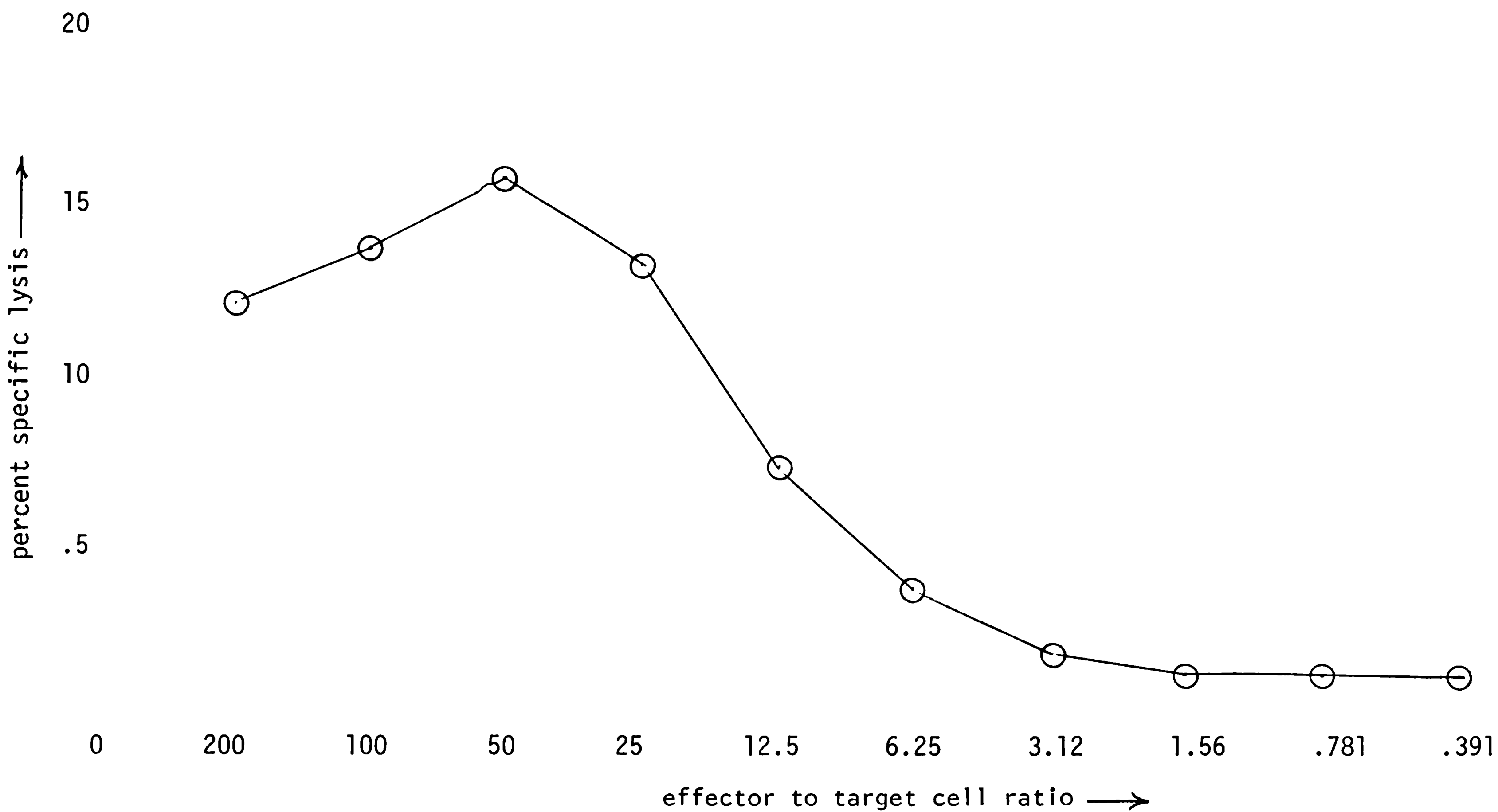


Table IV: Mouse peritoneal exudate cell ADCC against radiolabelled P815 targets

	Complement	Effector cells	No serum		Rabbit #11 anti-P815 serum		
			%[1]	Counts	1:6	1:60	1:600
Spontaneous release (SR)	--	--	34.4	2825	--	--	--
Saponin (SAP)	--	--	100	8218	--	--	--
Complement (C')	+	--	37.6	3089	--	--	--
Anti-P815 serum	--	--	--	--	81.6	27.2	31.5
Anti-P815 serum + C'	+	--	--	--	90.5*	83.3*	40.0*
DBA/2 mouse peritoneal exudate cells (250:1, E:T)	--	+	24.8	2041	46.6	28.6	26.5

[1] percent lysis = $\frac{E}{SAP} \times 100\%$

* Significantly greater than serum alone controls using the unpaired t test ($p < .0005$).

Table V: Mouse alloactivated spleen cell ADCC against P815 targets[1]

	Complement	Spleen cells	Serum (1:60 dilution)					
			No serum		Normal rabbit		Anti-P815 serum	
			%	Counts	%	Counts	%	Counts
Spontaneous release (SR)	--	--	75.9	5450	57.6	4133	75.6*	5425
Saponin (SAP)	--	--	100	7180	--	--	--	--
Complement (C')	+	--	62.9	4516	--	--	91.9	6596
Alloactivated C57B1/6 spleen cells (31:1, E:T)	--	+	69.6	4998	59.3	4258	72.0*	5170*
Alloactivated DBA/2 spleen cells (15:1, E:T)	--	+	60.8	4363	50.3	3609	69.7*	5002*

[1] percent lysis = $\frac{E}{SAP} \times 100\%$

* Significantly greater than the normal rabbit serum controls using the unpaired t test ($p < .005$) but not significantly greater than the no serum controls or the anti-P815 alone controls ($p > .05$).

C57Bl/6 mice were immunized with P815 cells. Ten days later, allo-sensitized spleen cells were separated over Ficoll-Hypaque (see Section II.C.5) and tested for cytolytic activity against radiolabelled P815 targets. Alloactivated spleen cells lysed P815 targets directly (Figure 4). In the presence of rabbit anti-P815 antiserum, this direct cytolytic activity was blocked rather than enhanced ($p < .001$).

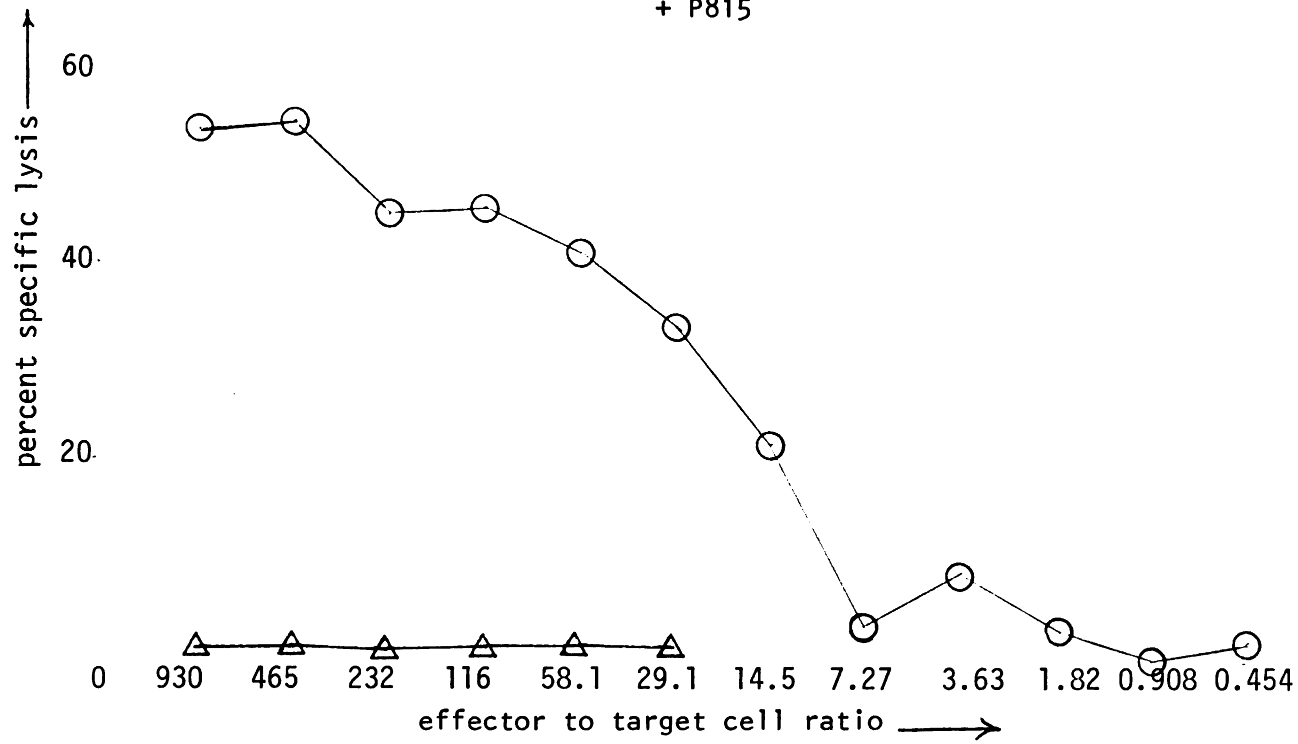
2. Rat spleen cell effectors

Since murine syngeneic and allogeneic effector cell populations were incapable of lysing antibody-sensitized P815 targets *in vitro*, xenogeneic rat spleen cells were tested. Fisher 344 rat spleen cells were isolated by the method described in Section II.C.3. Although high background lysis occurred at 1:6 serum dilutions, both 1:60 and 1:600 dilutions of anti-P815 serum exhibited sufficiently low background lysis levels by which to evaluate possible ADCC effects (Table VI). Rat spleen cells induced significant ADCC of antibody-coated P815 targets. Note that ADCC mediated by rat spleen cells was observed at a 1:600 serum dilution, one which fails to activate the complement-lytic pathway (see Figure 2). In addition, the amount of target cell lysis correlated directly with the E:T ratio; that is, for a given antiserum concentration, greater numbers of effector cells induced greater amounts of target cell lysis.

Similar results were obtained using Lewis rat spleen effector cells (Table VII). ADCC of P815 targets by rat spleen cells was highly significant ($p < .0025$) at all antiserum dilutions tested. Percent specific lysis values are for these splenic effector cells and depicted graphically in Figure 5.

Figure 4: Percent specific lysis plot of alloactivated C57Bl/6 spleen cell cytotoxicity against P815[1]

[1]Percent specific lysis = $\frac{\text{experimental counts-SR*}}{\text{SAP - SR}} \times 100\%$



*SR = 56.8% in this 8.5 hour assay

Table VI: Rat spleen cell ADCC against radiolabelled P815 targets

	Complement	Effector cells	No serum		Rabbit #11 anti-P815 serum		
			%[1]	Counts	1:6	1:60	1:600
Spontaneous release (SR)	--	--	34.4	2825	--	--	--
Saponin (SAP)	--	--	100	8218	--	--	--
Complement (C')	+	--	37.6	3089	--	--	--
Anti-P815 serum	--	--	--	--	81.6	27.2	31.5
Anti-P815 serum + C'	+	--	--	--	90.5*	83.3*	40.0*
Fisher rat spleen cells (500:1, E:T)	--	+	30.3	2490	71.8	57.2*	48.6*
Fisher rat spleen cells (250:1, E:T)	--	+	27.9	2291	61.9	46.7*	41.7*

[1] percent lysis = $\frac{E}{SAP} \times 100\%$

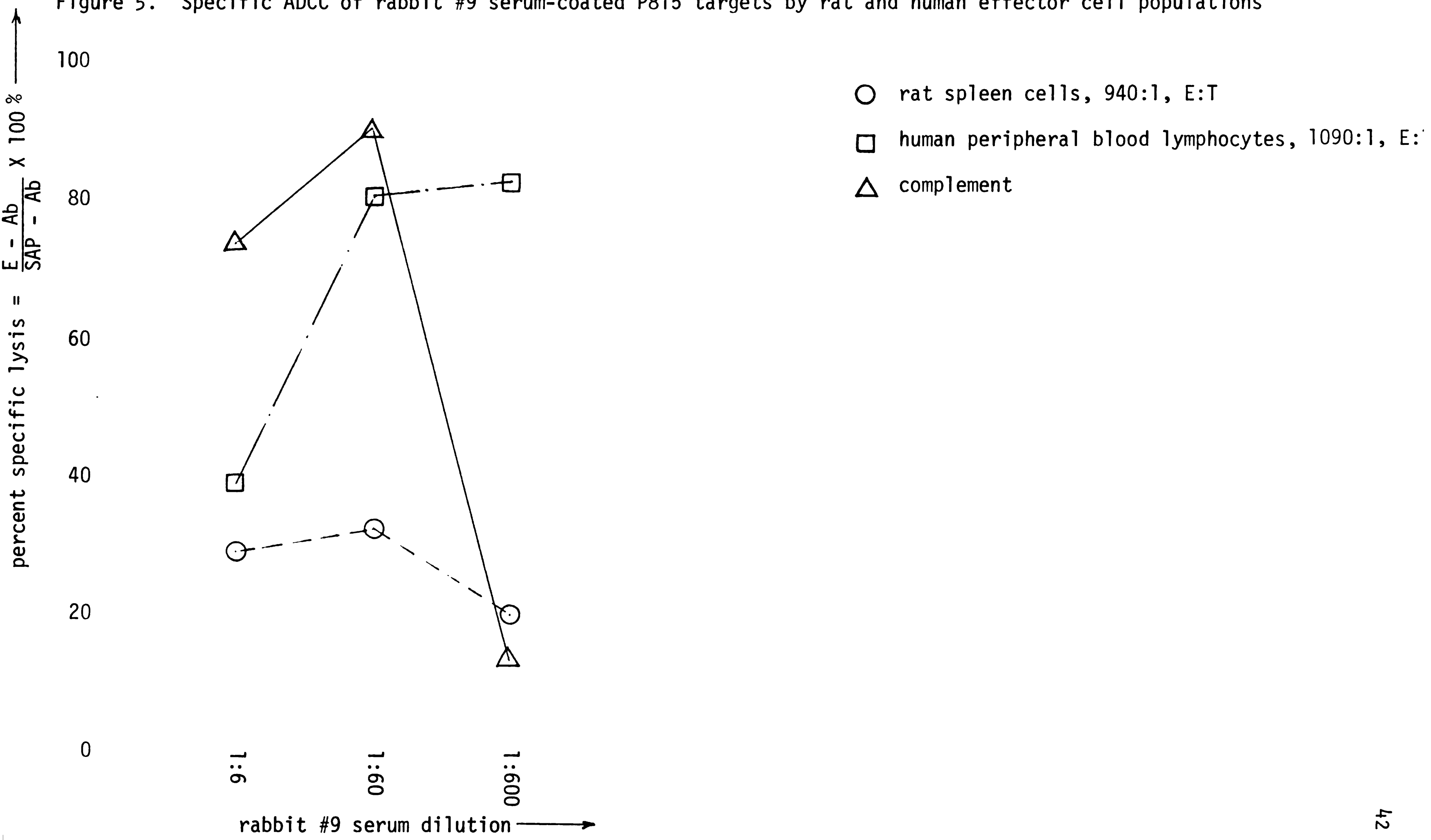
* Significantly greater than serum alone controls using the unpaired t test (p .0005).

Table VII: Rat spleen cell and human PBL ADCC against P815 targets

	Complement	Effector cells	No serum		Rabbit #9 anti-P815 antiserum		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	49.3	3952	--	--	--
saponin (SAP)	--	--	100	8010	--	--	--
complement (C')	+	--	40.6	3251	--	--	--
Anti-P815 serum alone	--	--	--	--	67.1	33.4	35.6
Anti-P815 serum + C'	+	--	--	--	91.2*	94.3*	43.9*
Rat spleen cells (940:1, E:T)	--	+	--	2513	76.4*	55.0*	47.6*
Human PBLs (1090:1, E:T)	--	+	--	2739	79.7*	86.6*	88.2*

* Significantly greater than corresponding antiserum alone control ($p < .0025$) using the one-tailed unpaired t test.

Figure 5. Specific ADCC of rabbit #9 serum-coated P815 targets by rat and human effector cell populations



3. Human peripheral blood lymphocytes (PBLs)

Human peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density centrifugation as discussed in Section II.C.1. ADCC against rabbit #9 coated P815 targets was carried out at a 1090:1 effector to target cell ratio. Significant ($p < .0025$) ADCC of P815 targets was observed using human PBL effector cells at each of the antiserum dilutions tested (Table VI and Figure 5). A comparison of rat and human effectors may be visualized in the plot of percent specific lysis* presented in Figure 5. Note that human PBL effectors possessed greater lytic activity than rat splenic effectors at all antiserum dilutions tested.

Human PBL effectors were used for further in vitro and in vivo ADCC experiments for the following reasons: 1) murine effector cells failed to lyse rabbit anti-P815 coated P815 target cells in vitro, 2) of the two xenogeneic species (rat and human) tested, human PBLs possessed the highest lytic activity, 3) the ADCC effector cell present in human peripheral blood and cytolytic for tumor targets had been well characterized as a non-T, non-B, nonphagocytic cell, perhaps in the monocyte series (49), 4) human PBL sources were relatively easily obtained, 5) methods for human PBL separation and characterization were available.

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*Plots of percent specific lysis are useful since they permit the exclusion of nonspecific cytolytic effects observed with serum alone controls. However, these plots are subject to artifacts which must be evaluated. For example, the 1:6 dilution points are spuriously low due to high background lysis levels. In other experiments with lower backgrounds, these points would be higher.

C. Titration of Human PBL Effector Cells and of Rabbit Anti-P815 Antisera in the ADCC Assay

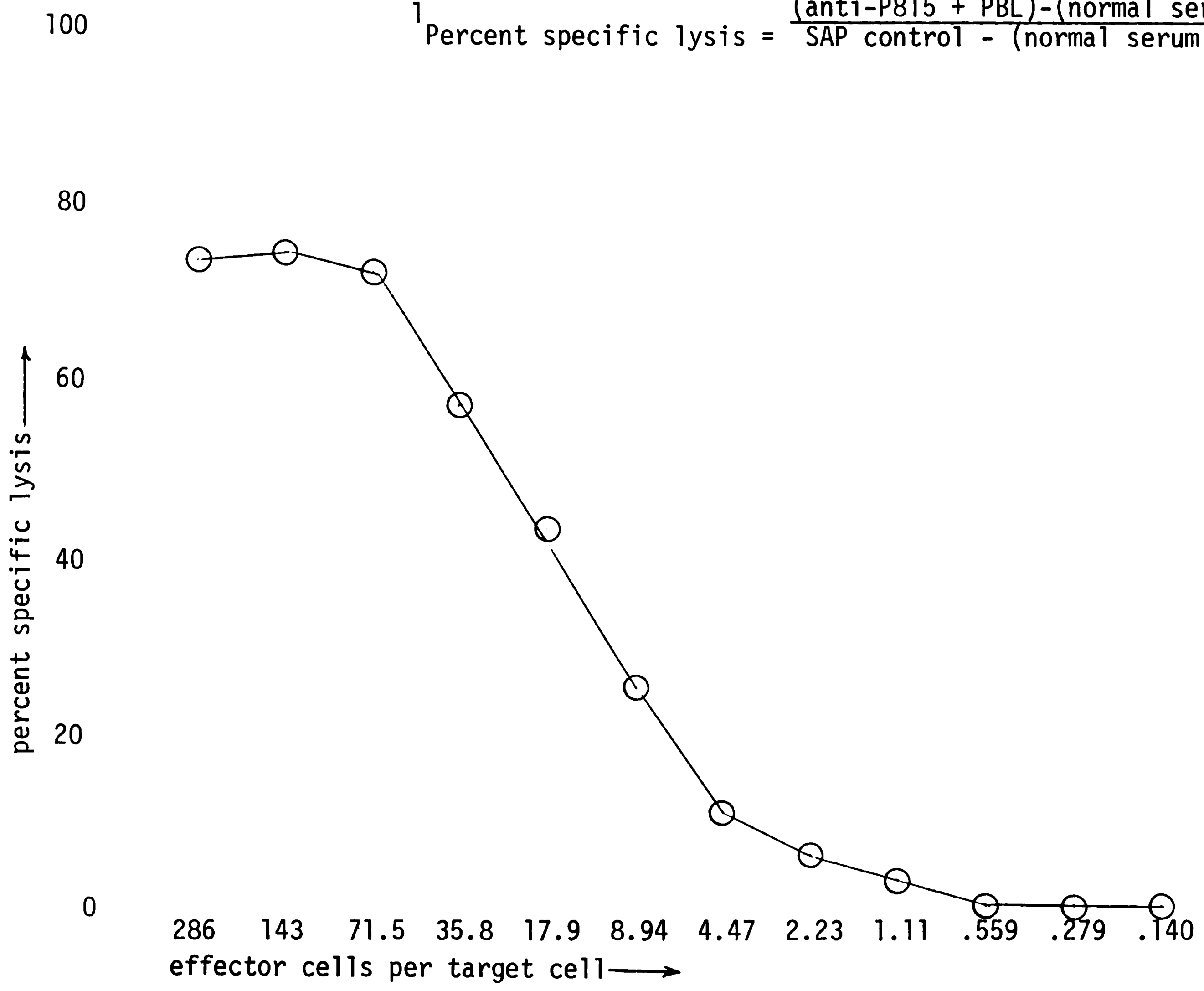
The purpose of these experiments was to determine the minimum effector to target cell ratio and anti-P815 antiserum concentration which supported complete lysis of P815 *in vitro*. Results from these experiments formed the basis for choosing the E:T ratios and serum dilutions for the *in vivo* ADCC protection experiments which follow. A representative experiment (Figure 6) shows peak lysis occurred at a 71.5:1 effector to target ratio.

In conclusion, human peripheral blood lymphocytes were found to lyse antibody-sensitized chromium-51 labelled P815 targets. Peak lysis of target cells occurred at a 71.5:1 effector to target cell ratio.

1. Rabbit anti-P815 antisera titration

The anti-P815 antibody requirements of both ADCC and complement-mediated lysis pathways were assessed to evaluate whether the two pathways were distinguishable. Results indicate the pathways are distinct (Figure 7). The PBL-mediated lytic mechanism required less antibody than the complement-mediated cytolytic mechanism to effect target cell lysis. Observe that the percent specific lysis value for a 1:3072 antiserum dilution on the ADCC curve remained near plateau level, whereas the specific lysis value for the same serum dilution on the complement curve fell to background levels (Figure 7). This datum is consistent with the hypothesis that ADCC against the P815 target mediated by PBLs occurs by means of a complement-independent cytolytic pathway.

Figure 6: Percent specific lysis plot of human PBL titration[1]



[1] $\text{Percent specific lysis} = \frac{(\text{anti-P815 + PBL}) - (\text{normal serum + PBL})}{\text{SAP control} - (\text{normal serum + PBL})}$

Figure 7: Percent lysis[1] plot of titration of rabbit #9 serum in the presence of complement or human PBLs[2]

[1]Percent lysis = $\frac{E}{SAP}$ x 100%

[2]Each point represents the mean of triplicate or quadruplicate counts

D. Adsorption and Specificity Testing of Rabbit Anti-P815 Antisera

Xenogeneic rabbit anti-P815 antisera were elicited by immunization of rabbits with P815 mastocytoma cells. In addition to antibodies directed against tumor-associated antigens, these antisera also contain antibodies directed against normal murine surface antigens, particularly H-2 antigens. The goal of the adsorption protocol was to remove these "anti-normal tissue" antibodies from the serum, leaving antibodies specific for P815 TAAs. In order to evaluate the completeness of adsorption, adsorbed sera were tested for their ability to mediate cytotoxicity against the specific P815 target and also against a nonspecific target, either the normal spleen cell or the L1210 leukemia cell, which shares normal antigens ($H-2^d$ and others) with the P815 cell.

1. Specificity testing against normal murine spleen cell targets

Rabbit #11 serum was adsorbed once with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells at 4^oC for 18 hours. Then it was tested for its specificity in the complement-mediated lysis and ADCC assays against P815 tumor and DBA/2 normal spleen cell targets. The adsorption of anti-P815 serum with normal tissue resulted in the nonspecific reduction of complement-mediated lytic activity against both normal spleen (Tables VIII, IX, $p < .005$) and P815 tumor (Tables X, XI, $p < .0005$) targets.

Results of serum specificity testing in ADCC were inconclusive. While ADCC of P815 targets was demonstrable, neither unadsorbed nor adsorbed sera were capable of mediating ADCC of normal spleen cell targets; therefore, the efficiency of serum adsorption against normal cell antigens could not be evaluated.

Table VIII: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against normal spleen cell targets

	Complement	PBL 46:1, E:T	No serum		Serum dilution		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	63.9	11,710	--	--	--
saponin (SAP)	--	--	100	18,312	--	--	--
complement (C')	+	--	65.0	11,910	--	--	--
normal rabbit serum alone	--	--	--	--	50.5	50.4	75.6
normal rabbit serum + PBL	--	+	--	--	34.3	--	--
unadsorbed #11 serum alone	--	--	--	--	47.5	48.1	55.3
unadsorbed #11 serum + C'	+	--	--	--	100*	95.2*	67.2*
unadsorbed #11 serum + PBL	--	+	--	--	51.8*	50.1	51.8
adsorbed #11 serum alone	--	--	--	--	49.6	52.2	56.0
adsorbed #11 serum + C'	+	--	--	--	53.0*	58.9*	61.0*
adsorbed #11 serum + PBL	--	--	--	--	42.2	48.5	46.1

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver and bone marrow cells at $4^{o}C$, 18 hours.

* Significantly greater lysis than in the corresponding antiserum alone controls ($p < .05$).

Table IX: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against normal spleen cell targets

	Complement	PBL 46:1, E:T	No serum		Serum dilution		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	63.9	11,710	--	--	--
saponin (SAP)	--	--	100	18,312	--	--	--
complement (C')	+	--	65.0	11,910	--	--	--
unadsorbed #11 serum + C'	+	--	--	--	100	95.2	67.2
adsorbed #11 serum + C'	+	--	--	--	53.0	58.9	61.0
unadsorbed #11 serum + PBL	--	+	--	--	51.8	50.1	51.8
adsorbed #11 serum + PBL	--	--	--	--	42.2	48.5	46.1

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver and bone marrow cells at 4°C, 18 hours.

Table X: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against P815 targets

	Complement	PBL 75:1, E:T	No serum		Serum dilution		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	21.3	6333	--	--	--
saponin (SAP)	--	--	100	29,717	--	--	--
complement (C')	+	--	18.3	5425	--	--	--
unadsorbed #11 serum	--	--	--	--	18.5	20.7	20.8
unadsorbed #11 serum + C'	+	--	--	--	93.1*	88.5*	19.5
unadsorbed #11 serum + PBL	--	+	--	--	59.2	--	--
adsorbed #11 serum	--	--	--	--	18.7	19.6	20.1
adsorbed #11 serum + C'	+	--	--	--	18.4	18.6	17.9
adsorbed #11 serum + PBL	--	+	--	--	56.3	--	--

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells at 4°C, 18 hours.

* Significantly greater lysis than in the corresponding antiserum alone controls ($p < .0005$).

Table XI: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against P815 targets

	Complement	PBL 75:1, E:T	No serum		Serum dilution		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	21.3	6333	--	--	--
saponin (SAP)	--	--	100	29,717	--	--	--
complement (C')	+	--	18.2	5425	--	--	--
unadsorbed #11 serum + C'	+	--	--	--	93.1	88.5	19.5
adsorbed #11 serum + C'	+	--	--	--	18.4	18.6	17.9
unadsorbed #11 serum + PBL	--	+	--	--	59.2	--	--
adsorbed #11 serum + PBL	--	+	--	--	56.3	--	--

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells at 4°C, 18 hours.

The effector to target cell ratio in the normal spleen cell target system was raised from 46:1 to 115:1 in an attempt to promote ADCC lysis of normal spleen cell targets. Tables XII and XIII show that ADCC against the normal spleen cell target was insignificant ($p \ngtr .05$) at a 115:1 effector to target cell ratio. In contrast to the lack of ADCC activity observed, complement-mediated cytolysis against normal spleen cell targets was pronounced ($p < .001$) in the presence of unadsorbed serum (1:6 and 1:60 dilutions). Thus, it was concluded that normal spleen cell targets were resistant to ADCC, although they were sensitive to complement-mediated lysis. For this reason, another target, the L1210 tumor cell, was chosen for further serum specificity experiments.

2. Specificity testing against L1210 tumor targets

The L1210 lymphocytic leukemia is syngeneic to DBA/2 mice and should therefore share $H\text{-}2^d$ surface antigens with P815 mastocytoma cells. Since L1210 had been maintained *in vitro* as a stable cell line for many years, the L1210 tumor may be a more easily labelled and perhaps also more easily lyseable target than normal spleen cells in the ADCC assay. However, three basic assumptions were made whose validity was unknown: 1) that L1210 bore all histocompatibility antigens present on P815, 2) that antibodies able to bind to P815 and mediate its lysis were also able to bind to L1210 and mediate its lysis, and 3) that L1210 bore no differentiation or tumor-associated antigens in common with P815.

In the first experiment (Tables XIV, XV), rabbit #11 serum, adsorbed once at 4^oC with pooled DBA/2 cells, was tested. The L1210 target was lysed by both the ADCC and complement-mediated cytolytic mechanisms ($p < .0005$), suggesting that the L1210 target was suitable for antiserum

Table XII: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against normal spleen cell targets

	Complement	PBL 115:1, E:T	No serum		Serum dilution		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	51.7	2914	--	--	--
saponin (SAP)	--	--	100	5636	--	--	--
complement (C')	+	--	57.5	3240	--	--	--
peripheral blood lymphocytes (PBL)	--	+	40.3	2270	--	--	--
normal rabbit serum alone	--	--	--	--	47.4	--	--
normal rabbit serum + PBL	--	+	--	--	43.6	--	--
unadsorbed #11 serum alone	--	--	--	--	43.3	34.4	52.2
unadsorbed #11 serum + C'	+	--	--	--	91.6*	96.4*	59.4
unadsorbed #11 serum + PBL	--	+	--	--	46.0	50.6	54.5
adsorbed #11 serum alone	--	--	--	--	45.5	46.1	51.5
adsorbed #11 serum + C'	+	--	--	--	43.2	51.0	52.7
adsorbed #11 serum + PBL	--	+	--	--	46.6	49.9	47.8

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells at 4°C, 18 hours.

* Significantly greater lysis than in the corresponding antiserum alone controls ($p < .001$).

Table XIII: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against normal spleen cell targets

	Complement	PBL 115:1, E:T	No serum		Serum dilution		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	5.17	2914	--	--	--
saponin (SAP)	--	--	100	5636	--	--	--
complement (C')	+	--	57.5	3240	--	--	--
peripheral blood lymphocytes (PBL)	--	+	40.3	2270	--	--	--
unadsorbed #11 serum + C'	+	--	--	--	91.6	96.4	59.4
adsorbed #11 serum + C'	+	--	--	--	43.2	51.0	52.7
unadsorbed #11 serum + PBL	--	+	--	--	46.0	50.6	54.5
adsorbed #11 serum + PBL	--	+	--	--	46.6	49.9	47.8

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver and bone marrow cells at 4°C, 18 hours.

Table XIV: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against L1210 targets

	comple-ment	PBL 115:1,E:T	no serum		serum dilution		
			%	counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	53.7	10,177	--	--	--
saponin (SAP)	--	--	100	18,948	--	--	--
peripheral blood lymphocytes (PBL)	--	+	40.2	7609	--	--	--
normal rabbit serum alone	--	--	--	--	29.2	34.2	36.2
normal rabbit serum + PBL	--	+	--	--	44.9*	34.9	29.0
unadsorbed #11 serum alone	--	--	--	--	24.3	31.3	34.3
unadsorbed #11 serum + C'	+	--	--	--	86.2*	79.2*	51.9*
unadsorbed #11 serum + PBL	--	+	--	--	71.8*	71.8*	71.0*
adsorbed #11 serum alone	--	--	--	--	28.8	25.3	26.3
adsorbed #11 serum + C'	+	--	--	--	37.4*	36.1*	36.6*
adsorbed #11 serum + PBL	--	+	--	--	57.6*	64.0*	50.1*

* significantly greater lysis than in the corresponding antiserum alone controls ($p < .0005$)

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells at 4°C for 18 hours.

Table XV: Specificity testing of once adsorbed rabbit #11 anti-P815 serum[1] against L1210 targets

	Complement	PBL 115:1, E:T	No serum		Serum dilution		
			%	Counts	1:6	1:60	1:600
spontaneous release (SR)	--	--	53.7	10,177	--	--	--
saponin (SAP)	--	--	100	18,948	--	--	--
peripheral blood lymphocytes (PBL)	--	+	40.2	7609	--	--	--
unadsorbed #11 serum + C'	+	--	--	--	86.2	79.2	51.9
adsorbed #11 serum + C'	+	--	--	--	37.4	36.1	36.6
unadsorbed #11 serum + PBL	--	+	--	--	71.8	71.8	71.0
adsorbed #11 serum + PBL	--	+	--	--	57.7	64.0	50.1

[1] Serum was adsorbed with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells at 4°C for 18 hours.

specificity testing experiments. Testing of once adsorbed serum showed markedly decreased ($p < .0005$) complement-mediated lytic activity against L1210, but only slightly but significantly ($p < .0005$) diminished ADCC activity against L1210. This indicated that one adsorption did not adequately deplete antibodies capable of mediating ADCC against the nonspecific L1210 target. Further serum adsorptions were made.

Twice adsorbed rabbit #11 anti-P815 antiserum, adsorbed at 4°C and 20°C, was compared with unadsorbed serum in ADCC tests against L1210 and P815. Both unadsorbed and adsorbed sera mediated ADCC against both L1210 and P815 targets (Tables XVI, XVII, XVIII, $p < .01$). Adsorption significantly ($p < .0005$) reduced cytolytic activity of rabbit #11 serum against L1210 targets at all dilutions tested. Against P815 targets, adsorption resulted in a significant reduction ($p < .0005$) in cytolytic activity detectable at the 1:600 and 1:6000 antiserum dilutions. In separate adsorption experiments in which xenogeneic sera were adsorbed with either L1210 or P815 tumor cells, adsorption resulted in the nonselective depletion of cytolytic activity directed against both L1210 and P815. Taken together, the data indicate that a large proportion of antibody activity in rabbit #11 anti-P815 antiserum appeared to be directed against membrane antigens (H-2?) shared by L1210, P815, and normal DBA/2 cells. Two adsorptions did not completely remove the nonspecific antibody activity from the anti-P815 antiserum.

Since two adsorptions (4°C, 20°C) failed to completely remove nonspecific ADCC activity directed against L1210, a third *in vitro* adsorption (37°C) was performed. Results were not substantially different from those of the twice adsorbed serum. However, the specificity criterion was fulfilled at a 1:6000 serum dilution, where ADCC of P815 but not L1210

Table XVI: Specificity testing of twice adsorbed rabbit #11 anti-P815 serum[1] against L1210 targets

	Complement	PBL 200:1, E:T	No serum		Serum dilution			
			%	Counts	1:6	1:60	1:600	1:6000
spontaneous release (SR)	--	--	37.1	5956	--	--	--	--
saponin (SAP)	--	--	100	16,041	--	--	--	--
unadsorbed rabbit #11 serum alone	--	--	--	--	23.7	29.2	44.3	29.9
unadsorbed rabbit #11 serum + C'	+	--	--	--	99.5*	76.2*	54.0*	52.2*
unadsorbed rabbit #11 serum + PBL	--	+	--	--	82.3*	81.0*	81.9*	47.6*
adsorbed rabbit #11 serum alone	--	--	--	--	19.2	20.3	19.9	17.2
adsorbed rabbit #11 serum + C'	+	--	--	--	35.1*	37.7*	27.8*	30.6*
adsorbed rabbit #11 serum + PBL	--	+	--	--	43.1*	52.8*	30.6*	18.1

* Significantly greater lysis than in the corresponding antiserum alone controls ($p < .025$).

[1] Serum was adsorbed at $4^{o}C$ and room temperature with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells.

Table XVII: Specificity testing of twice adsorbed rabbit #11 anti-P815 serum[1] against L1210 targets

	Complement	PBL 200:1, E:T	No serum		Serum dilution			
			%	Counts	1:6	1:60	1:600	1:6000
spontaneous release (SR)	--	--	37.1	5956	--	--	--	--
saponin (SAP)	--	--	100	26,041	--	--	--	--
unadsorbed rabbit #11 serum + C'	+	--	--	--	99.5	76.2	54.0	52.2
adsorbed rabbit #11 serum + C'	+	--	--	--	35.1	37.7	27.8	30.6
unadsorbed rabbit #11 serum + PBL	--	+	--	--	82.3	81.0	81.9	47.6
adsorbed rabbit #11 serum + PBL	--	+	--	--	43.1	52.8	30.6	18.1

[1] Serum was adsorbed at 4°C and room temperature with DBA/2 spleen, lymph node, thymus, blood, liver, and bone marrow cells.

Table XVIII: Specificity testing of twice adsorbed rabbit #11 serum tested in ADCC against L1210 targets

	PBL	No serum		Serum dilution		
		%	Counts	1:60	1:600	1:6000
spontaneous release (SR)	--	12.7	3536	--	--	--
saponin (SAP)	--	100	27,788	--	--	--
peripheral blood lymphocytes (PBL)	+	14.2	3944	--	--	--
unadsorbed rabbit #11 serum	+	--	--	95.3*	89.4*	81.0*
twice adsorbed rabbit #11 serum	+	--	--	50.4*	36.6*	2.60*

* Significantly greater lysis than in the corresponding NRS-PBL controls ($p < .01$).

[1] SR, SAP, and PBL control data are expressed in terms of percent lysis ($\frac{E}{SAP} \times 100\%$) and counts; ADCC experimental data are expressed in terms of percent specific lysis $\left\{\frac{E - (NRS - PBL)}{SAP - (NRS - PBL)} \times 100\%\right\}$

was observed. This low serum dilution was not considered useful for the in vivo experiments which follow since insufficient lysis (13.7%) was observed at this dilution.

Since in vitro adsorptions of rabbit anti-P815 serum were incomplete, in vivo adsorptions were undertaken. In this procedure, DBA/2 mice were injected with rabbit anti-P815 antiserum, then bled hours later. In theory, serum antibodies would freely circulate. Those antibodies specific for normal mouse tissue antigens would bind to cells of the mouse. Over a period of time, anti-normal tissue antibodies would be depleted from mouse serum, leaving specific anti-P815 antibodies behind. In fact, in vivo adsorption resulted in a nonselective depletion of cytolytic activity against both P815 and L1210 targets ($p < .0005$). At no serum dilution was activity against L1210 completely removed.

In conclusion, a specific anti-P815 antiserum, defined operationally by the ability of the serum to mediate ADCC against P815 and not L1210 targets, was not obtained. Results of the in vitro and in vivo adsorption studies using xenogeneic rabbit anti-P815 antisera showed that the adsorption process efficiently removed antibodies directed against normal cell antigens and mediating ADCC. However, the adsorption process did not remove completely the non-specific cytolytic activity of the antisera against the L1210 tumor target.

E. Titration of Twice Adsorbed Rabbit #11 Anti-P815 Antiserum Against P815 Targets

Rabbit #11 anti-P815 antiserum, adsorbed at 4^oC and room temperature, was titrated against P815 targets in the presence of excess human PBLs (230:1 effector to target cell ratio). Maximum lysis plateaued at 1:6,

1:12, and 1:24 serum dilutions (Figure 8). This experiment suggested that maximum protection in the *in vivo* experimentation phase which follows might be achieved using antiserum dilutions less than 1:24.

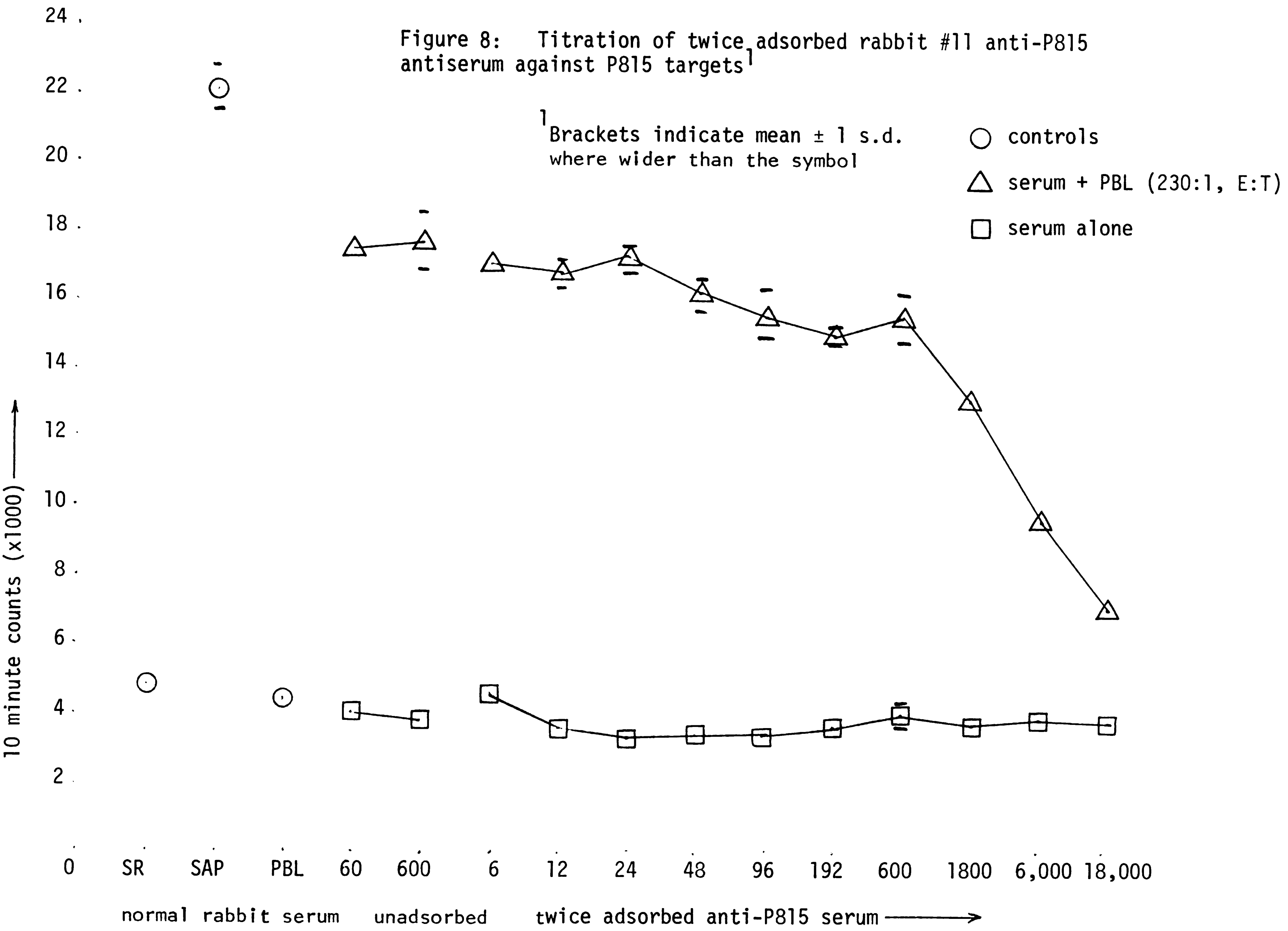
F. In Vivo Experiments

The purpose of the *in vivo* experiments was twofold: 1) to characterize the growth of the P815 tumor in DBA/2 mice as a function of tumor cell dose, and 2) to assess whether the growth of P815 tumor *in vivo* could be deterred by treatment with anti-P815 antiserum and PBL effector cells.

1. P815 dose response determination

These experiments were designed to 1) characterize tumor growth in these animals, and 2) to determine the number of injected tumor cells (intradermal route) which resulted in either tumor takes in 50% of mice (TD_{50}) or death in 50% of mice (LD_{50}). The first P815 dose response experiment measured tumor growth parameters (rapidity of tumor appearance, percent tumor takes, percent regression, day of mouse death) and correlated them with tumor cell number injected. Groups of DBA/2 mice (10 mice per group) were injected for each of the following tumor cell doses: 10^6, 10^5, 10^4, 10^3, and 10^2 cells per mouse. As the tumor cell dose increased from 10^2 to 10^6 cells, there was corresponding step-wise decrease in the mean time of tumor appearance from 12.1 to 4.0 days (Table XIX). That is, the higher the tumor cell number injected, the sooner palpable tumor appeared. Other parameters of tumor growth (number of tumor takes or regressions, mean day of mouse death, and number of mouse deaths), did not differ among groups.

Figure 8: Titration of twice adsorbed rabbit #11 anti-P815 antiserum against P815 targets[1]



[1] Brackets indicate mean ± 1 s.d. where wider than the symbol

Table XIX: Measure of tumor parameters as a function of injected P815 cell number

cell number injected	tumor takes	mean day of tumor appearance	number of mouse deaths	mean day of mouse death	regressors
10^6	10/10	4.00	10/10	31.8	0
10^5	10/10	5.80	9/10	29.3	1/10
10^4	10/10	8.20	9/10	28.4	1/10
10^3	9/10	9.89	9/10	32.5	0
10^2	9/10	12.2	9/10	32.0	0

In a dose response experiment designed to determine the TD_{50} and LD_{50} dose, groups of mice were injected with 10, 30, 40, 50, 60, 70, 90 and 110 P815 cells/mouse. The percentage of tumor takes decreased with decreasing cell numbers injected (Table XX). By extrapolation, the TD_{50} was calculated to be 80 cells per mouse and the LD_{50} to be 83 cells per mouse. There was no difference in mean day of tumor appearance between groups. Note also that regression of tumor growth was rare; out of 126 mice injected, only two grew tumors which later regressed.

2. In vivo ADCC protection against P815 tumor growth

a. Syngeneic DBA/2 spleen cell effectors

In the first pilot ADCC protection experiment using DBA/2 spleen cell effectors, protection appeared to occur. P815 tumor cells were mixed with unadsorbed rabbit anti-P815 antiserum (1:3 dilution) plus spleen cells (610:1, E:T ratio), then injected into mice at a dose of 10^4 cells per mouse. Out of 10 mice injected with this mixture, only one mouse developed tumor and this mouse subsequently died. The 10% take rate in this experimental group is significantly lower than the 90% take rate observed in the control group receiving 100 cells/mouse (see Table XIX).

In the second experiment, the following mouse groups were injected:

1. P815 alone (83 cells/mouse)
2. P815 + DBA/2 anti-P815 serum (1:2 dilution) + spleen cells (1000:1, E:T)
3. P815 + unadsorbed rabbit #11 serum (1:2 dilution + spleen cells
4. P815 + NRS (1:2 dilution) + spleen cells

Table XX Measure of tumor parameters as a function of injected P815 cell number

P815 cell number injected per mouse	tumor takes		mean day of tumor appearance (± s.d.)	mouse deaths		regressors
	number	%		number	%	
110	12/15	80	11.6 ± 1.73	11	73	1
90	9/15	60	12.2 ± 0.67	9	60	0
70	6/15	40	12.0 ± 0	5	33	1
60	4/15	27	16.5 ± 6.4	4	27	0
50	0/15	0	--	0	0	0
40	1/15	6.67	12.0 ± 0	1	6.67	0
30	1/21	4.76	12.0 ± 0	1	4.76	0
10	0/15	0		0	0	0

5. P815 + NMS (1:3 dilution) + spleen cells

Results (Table XXI) show that neither syngeneic nor xenogeneic anti-P815 sera were capable of mediating in vivo ADCC against P815 when compared with normal mouse serum and normal rabbit serum controls. Therefore, confirmation of the initial protection observed in the first experiment was not achieved.

b. Human PBL effector cells

Since human PBLs were highly active in in vitro ADCC assay against the P815 tumor target, these effector cells were examined for their possible ability to prevent tumor growth in vivo.

In the first experiment using human PBL effector cells, groups of mice (20 per group) were injected as described in Section II.G.2. The tumor cell dose was 83 cells per mouse (LD_{50} dose); the effector to target cell ratio was 300:1; and the sera used were NRS and twice adsorbed (4°, 20°) rabbit #11 anti-P815 antiserum at a 1:2 dilution (1:8 final dilution). The order of mouse group injection was as follows:

1. P815 alone
2. P815 + rabbit #11 serum (1:2 dilution)
3. P815 + NRS (1:2 dilution)
4. P815 + PBL
5. P815 + NRS + PBL
6. P815 + rabbit #11 serum + PBL

Results, (Table XXII) show that the percent tumor takes dropped steadily with time as mouse groups were injected. The latter groups injected developed fewer tumors than the earlier groups.

Since the time of group injection was long (3 hours), it was

Table XXI: *In vivo* ADCC protection experiment against P815 in the presence of mouse splenic effector cells

Group	tumor takes		mouse deaths		regressors
	number	%	number	%	
P815 alone	7/10	70	7/10	70	0
P815 + DBA/2 -P815 + S.C. *	9/10	90	9/10	90	0
P815 + unads. rabbit anti-P815 + S.C.	9/10	90	9/10	90	0
P815 + normal rabbit serum (NRS)+S.C.	8/9	88.9	7/9	77.8	1
P815 + normal mouse serum (NMS) + S.C.	8/11	72.7	8/11	72.7	0

* spleen cells were added at a 1000:1 effector to target cell ratio.

Table XXII: First *in vivo* ADCC protection experiment against P815 in the presence of human PBL effector cells

Group	tumor takes		regressors
	number	%	
P815 alone	16/19	84	0
P815 + R#11	13/20	65	0
P815 + NRS	6/20	30	0
P815 + PBL	4/20	20	0
P815 + NRS + PBL	2/20	10	1
P815 + R#11 + PBL	0/20	0	0

hypothesized that adherence of cells to the tube walls may have contributed to the decrease in tumor takes among groups correlating with time of injection. Although preliminary experiments failed to demonstrate P815 cell adherence to tubes with time, it was decided to minimize the adherence factor in the next experiment by 1) using siliconized tubes to decrease cell attachment, 2) decreasing the time of injection to 1 hour, 20 minutes, and 3) injecting rabbit #11 anti-P815 serum groups prior to the NRS groups.

Results of the second experiment (Table XXIII)(300:1, E:T; 1:2 serum dilution) showed that the percent tumor takes among the groups did not differ significantly and ranged from 75-90% takes. Therefore, it was concluded that no protection occurred.

In order to maximize the probability of obtaining an in vivo ADCC effect in the third experiment, three mouse groups were injected:

1. P815 alone (83 cells/mouse = LD_{50})
2. P815 + unadsorbed rabbit #11 anti-P815 serum (1:2 dilution) + PBL (1000:1, E:T)
3. P815 + NRS (1:2 dilution) + PBL (1000:1, E:T)

Results (Table XXIV) show a pronounced decrease in tumor takes in the group receiving unadsorbed anti-P815 antibody plus human peripheral blood lymphocytes. This group also demonstrated a marked decrease in the percent of mouse deaths (44%) compared to the control groups.

However, when the above experiment was repeated using identical conditions (Table XXV), no difference in tumor takes, mouse deaths, or tumor regressions was observed between groups.

In summary, the TD_{50} for P815 was calculated to be 80 cells per mouse; the LD_{50} was 83 cells per mouse. As the tumor cell dose was

Table XXIII: Second *in vivo* ADCC protection experiment against P815 in the presence of human PBL effector cells

group	tumor takes	
	number	%
P815 alone (83 cells/mouse)	15/20	75
P815 + R#11	16/20	80
P815 + NRS	18/20	90
P815 + PBL	17/20	85
P815 + NRS + PBL	15/20	75
P815 + R#11 + PBL	16/20	80

Table XXIV: Third *in vivo* ADCC protection experiment against P815 in the presence of human PBL effector cells

group	tumor takes		mouse deaths		regressions
	number	%	number	%	
P815 alone (83 cells/mouse)	9/9	100	8/9	89	1
P815 + unads R#11+PBL (1000:1, E:T)	5/9	56	4/9	44	1
P815 + NRS + PBL (1000:1, E:T)	8/8	100	8/8	100	0

Table XXV: Fourth <u>in vivo</u> ADCC protection experiment against P815 in the presence of human PBL effector cells

group	tumor takes		mouse deaths		regressors
	number	%	number	percent	
P815 alone(75 cells/mouse)	19/20	95	19/20	95	0
P815 + PBL (1000:1, E:T)	19/19	100	--	--	0
P815 + R#11 unadsorbed	20/20	100	18/20	90	2
P815 + NRS	18/20	90	--	--	1
P815 + unads R#11+PBL	20/20	100	19/20	95	1
P815 + NRS + PBL	20/20	100	19/20	95	1

increased from 10^2 to 10^6 cells, the mean time of tumor appearance correspondingly decreased from 12.2 to 4.0 days. No change in number of tumor takes or regressions, number of mouse deaths, or mean day of death was observed as the dose increased. Neither syngeneic DBA/2 spleen cells nor xenogeneic human PBLs reproducibly protected mice from the growth and progression of antibody-sensitized P815 tumor cells.

IV. DISCUSSION

A. Raising of Anti-P815 Antisera

To obtain anti-P815 antisera, the following hosts were immunized with P815 cells: xenogeneic rabbits, allogeneic mice, and syngeneic mice. Sera from immunized animals were tested for their ability to support complement-mediated lysis and ADCC of P815 targets. Xenogeneic and allogeneic sera but not syngeneic sera mediated complement lysis of P815. Xenosera but neither allosera nor syngeneic sera mediated ADCC of P815 targets.

1. Xenogeneic New Zealand rabbit anti-P815 antisera

Prior to immunization, rabbit sera contained no detectable antibody lytic activity against P815 targets. After immunization, rabbit sera contained antibodies capable of mediating complement-mediated cytolysis and ADCC of P815 targets. Anti-P815 antibody activity was detectable as early as 5 days post-boost. Serum antibody activity peaked between 10 and 13 days post-boost, then fell to low levels by day 16 or 18.

The ADCC pathway was distinguishable from the complement-mediated cytolysis pathway in its antibody requirements. ADCC required significantly less antibody to effect target cell lysis compared to the complement-mediated cytolytic pathway. A 1:3072 dilution of anti-P815 antiserum supported maximal plateau levels of ADCC, whereas complement lysis levels at this serum dilution were minimal. The relatively low antibody requirements of ADCC is consistent with data of Perlmann and Holm (140) who found that antiserum concentrations too low to support complement-mediated lysis were even more effective than higher concentrations in

mediating ADCC. In addition, Hersey et al. (73) discovered, in testing anti-HL-A antisera that not only was ADCC more sensitive than the complement assay method (as measured by lytic titers), but also that a greater number of antigeneic specificities were detected utilizing the ADCC method.

2. Allogeneic C57B1/6 anti-P815 antisera

C57B1/6 anti-P815 antisera were found to contain antibodies capable of mediating complement lysis of P815 targets. Further testing showed these sera were unable to mediate ADCC of P815, although they supported both PBL-mediated and complement-mediated lysis of L1210 tumor targets. These results generally concur with those of Greenberg, Shen, and Medley (52) who reported that C3H anti-P815 alloserum and normal DBA/2 splenic effector cells did not support lysis of P815, although they did support lysis of SL-2 lymphoma targets. Our results differ from those of Greenberg, et al. in the complement-mediated lysis experiments. Our C57B1/6 antiserum does mediate lysis of P815, whereas the Greenberg et al. C3H antiserum does not.

Our data and those of Greenberg et al. indicate that the anti-P815 antibodies present in the allosera bind to P815 target cells but fail to recruit effector cells able to lyse the antibody-sensitized targets. While the anti-P815 antibodies in C57B1/6 serum bind to P815 cells in amounts adequate to activate the complement-mediated lytic pathway, the antibodies do not trigger ADCC. This and further evidence to be discussed in Section IV, D. indicate that the anti-P815 alloantibodies may be of a class or subclass which possess Fc regions unable to activate the ADCC mechanism.

3. Syngeneic DBA/2 anti-P815 sera

If syngeneic anti-P815 serum were available, it would offer several advantages: 1) serum would contain antibodies specific for tumor-associated antigens (and perhaps also antibodies to most cell specific antigens), 2) serum would lack antibodies directed against mouse histocompatibility antigens, and therefore would not need adsorption, and 3) syngeneic mouse antitumor antibody might possess Fc portions with higher affinities for mouse effector cell Fc receptors than xenogeneic antibodies. However, neither syngeneic tumor bearer sera nor immune sera mediated complement-mediated lytic or ADCC activity against P815 targets *in vitro*.

Our ability to raise an antibody response to P815 in syngeneic mice may be attributed to either a lack of immunogenicity or weak immunogenicity of tumor-associated antigens on P815; the DBA/2 mouse may be a genetic nonresponder to P815 neoantigens. Bertschmann and co-workers (12) reported difficulty in obtaining an immune response to P815 in the syngeneic host: a detectable immune response was only observed when tumor cells were injected intradermally; other routes of immunization (subcutaneous or intraperitoneal) with treated or untreated tumor cells, tumor cell homogenates, or tumor cell membranes were ineffective in raising an immune response to P815. The immune response which was demonstrable was primarily cell mediated; examinations of humoral immune responsiveness by complement-mediated cytotoxicity and immunofluorescence were negative (11). The weak immunogenicity of P815 was confirmed by Hawrylko *et al*. (58) and led Nordin, Cerrotini and Brunner (122) to conclude that P815 may lack tumor associated neoantigens.

The weak immunogenicity of P815 TAAs may be attributed to the

hypothesis, proposed by Schierman and McBride (156), that if the TAAs on P815 act as haptens, an antibody response could not be elicited in the syngeneic host (151). They argued that antibodies could be made to TAA haptens in xenogeneic or allogeneic hosts if the H-2 antigens on the tumor cell were recognized as carriers for the TAAs. Our xenogeneic and allogeneic serum data did not establish the presence of specific antibodies directed against P815 TAAs, therefore the Schierman and McBride hypothesis remains an unvalidated possible explanation.

B. Testing of Effector Cell Populations in the Lysis of Antibody-coated P815 Targets

1. Mouse effector cells

Syngeneic DBA/2 spleen cells at a 1000:1 effector to target cell ratio were unable to lyse xenogeneic antibody-coated P815 target cells although the antiserum concentration was sufficient to support target cell lysis by the complement-dependent pathway, and although media conditions were those which adequately supported human PBL-induced lysis of antibody-sensitized P915 targets and allosensitized C57B1/6 spleen cell-mediated cytolysis of P815. The same source of DBA/2 spleen cells were able to lyse antibody-sensitized CRBC targets.

The resistance of the sensitized P815 tumor to lysis by murine effector cells has been substantiated by other investigators. As noted previously, Greenberg *et al*. (52) reported the resistance of P815 to ADCC. Similar findings were reported by Greenberg and Wolosin (53) in the L5178Y lymphoma system of DBA/2 mice: L5178Y was resistant to cytolysis mediated by tumor specific serum and unsensitized spleen cells. Independently, Lovchik and Hong (102) reported an inability to lyse

rabbit anti-P815 sensitized cells by normal mouse spleen, lymph node, peritoneal or peripheral blood leukocytes. The mechanism of resistance to lysis is unknown and will be discussed in greater detail in Section IV. D.

In contrast to the inability of DBA/2 spleen cells to lyse P815 targets through an antibody-dependent pathway, this same source of effector cells was able to lyse sensitized CRBC targets. This apparent paradoxical finding may be explained by data from several laboratories which have clearly established that different target cells (CRBC or non-erythrocyte) are capable of being lysed by different effector cell populations. Antibody-sensitized CRBC targets are lysed by two populations of cells: one population is an adherent, phagocytic cell, macrophage in nature, while the other is a non-phagocytic and nonadherent cell, perhaps of the lymphocyte or monocyte lineage (26, 44, 52, 103, 105, 158). Non-RBC targets appear to be lysed exclusively by nonadherent effector cells (52). Removal of phagocytic or adherent macrophage-like cells resulted in unchanged or enhanced ADCC activity (41, 80, 108). Thus, an explanation for our ability to demonstrate intact murine spleen cell-mediated ADCC function against CRBC targets may be that anti-CRBC ADCC is mediated by adherent phagocyte effectors (see Section 111.B.1.), which are inactive against tumor targets such as P815.

Since normal spleen cells failed to lyse antibody-coated P815 targets, we examined the ability of alloactivated splenic T cells to lyse antibody-coated P815 targets; results of these experiments were also negative. The experimental conditions employed were those of Kimura, Rubin, and Andersson (84) who showed that <u>in vivo</u> alloactivated Fc-receptor bearing cytotoxic T cells were able to lyse antibody-sensitized

CRBC targets. Since we did not use CRBC targets in these experiments, it is possible that alloactivated T cells may be capable of killing sensitized CRBC but not P815 tumor targets.

While reports of the ability of alloactivated T cells to mediate ADCC are becoming more prevalent, it is unclear whether these activated T cells are capable of lysing tumor targets. Recently, Evans and coworkers (37) reported the ADCC activity of allosensitized human T cells against chromium-labelled heterologous CRBC or autologous PBL targets. The MLC-activated T cells expressed both E-rosetting ability and the T_{H_2} differentiation marker. IgM-induced human lymphocyte-mediated cytotoxicity reported by Wahlin _et al_. (174) and by Fuson and Lamon (42) may also be indicators of T cell-mediated ADCC. However, since none of these studies utilized tumor targets, it has been established that activated T cells kill sensitized tumors.

Reports by Lamon _et al_. (91-96) in the murine Moloney sarcoma virus (MSV) system suggested that unsensitized thymus-derived lymphocytes (thymocytes and splenic T cells) were cytotoxic for IgM and IgG sensitized MSV tumor cells _in vitro_ in a microcytotoxicity assay. These results must be interpreted with caution, however, since microcytotoxicity assays measure other parameters besides cell death, such as cytostasis and detachment of tumor cells from microtiter plate wells. In view of the complexity of the micro-cytotoxicity assay and its corresponding difficulties in interpretation, Lamon _et al_. (96) re-examined the ADCC potential of murine thymus cells in a chromium-51 release assay using SRBC targets; thymocytes, particularly cortisone-resistant thymocytes, were found to be cytolytic. Since effector cells for erythrocyte targets may be incapable of lysing tumor targets, it would be of interest to

determine whether murine thymus-derived cells are also capable of killing tumor cells in a short-term radioisotope release assay.

Since murine spleen cells did not lyse sensitized P815 targets, DBA/2 peritoneal exudate cells were examined: Thioglycollate-induced activated peritoneal exudate cells at a 250:1 effector to target ratio lacked cytolytic activity against antibody-coated P815 targets. This result contrasts with that of Ojo and Wigzell (125) who recently reported that Corynebacterium parvum-induced peritoneal exudate cells were able to lyse alloantibody-coated P815 and CRBC targets. The mode of induction of the peritoneal exudate may possibly explain the apparent discrepancy in results.

Another murine effector cell population, peripheral blood lymphocytes (PBLs), has also been shown to mediate ADCC by other investigators (9). The finding that murine PBLs, but not murine spleen cells, lyse antibody-sensitized tumor targets may indicate that PBLs 1) contain an ADCC effector cell not present in spleen, 2) contain greater numbers of ADCC effector cells than spleen, or 3) lack a suppressor cell for ADCC found in spleen. Murine PBLs were not tested in our study because the low quantities of lymphoid cells isolatable from mouse blood were inadequate for the *in vitro* and *in vivo* studies.

2. Rat spleen cell effectors

In the absence of demonstrable murine cell-mediated lysis of antibody-sensitized targets, rat spleen cell effectors were tested. Spleen cells from both Fisher 344 and Lewis strains were able to mediate significant lysis of sensitized P815 targets (Tables V and VI). This finding has been confirmed by Lovchik and Hong (102) who found rat spleen

cells capable of lysing antibody-coated P815 and erythrocyte targets. Also consistent with our data is the report by Kedar, de Landazuri, and Fahey (80) that rat spleen cells were more effective than mouse cells in lysing EL-4 tumor targets.

3. Human PBL effectors

Human peripheral blood mononuclear cells isolated by Ficoll-Hypaque density centrifugation and depleted of phagocytic cells by iron were highly cytotoxic to antibody-sensitized P815 cells. Specific lysis occurred at an effector to target cell ratio as low as 8:1. Maximal PBL-induced lysis occurred at a 71.5:1 to 132:1 effector to target ratio. A comparison between human PBL and rat spleen cell effectors (Figure 5) revealed human PBLs to possess greater cytolytic activity at the 1090:1 effector to target cell ratio tested. Primarily for this reason, human PBLs were chosen as the effector cells for the in vitro and in vivo ADCC experiments which follow.

The ability of human PBLs to lyse both sensitized RBC and tumor targets has been extremely well documented by a number of investigators whose work has been reviewed (22, 140, 145). The cytolytic cell present in the peripheral blood lymphocyte population has been termed "K" cell, for its killer activity. The K cell is nonadherent, nonphagocytic, and is neither classical T nor B cell. Human K cells bear C3 receptors in addition to their Fc receptors (146). At present, the nature of the K cell lineage is controversial. That the K cell may be a member of the T cell lineage is suggested by evidence from Schlossman's laboratory which indicates that K cells possess Ia antigens in common with activated T cells (24, 78). That the K cell may be a nonphagocytic, nonadherent

monocyte is suggested by work by Greenberg in the mouse (49) and by Papamichail and Temple in man (133). Further marker studies may resolve this point.

C. Adsorption and Testing of Rabbit Anti-P815 Antisera

Xenogeneic rabbit anti-P815 antisera was expected to contain antibodies specific for normal mouse tissue antigens (e.g., anti-H-2 antibodies) in addition to antibodies specific for P815 tumor associated antigens. The anti-P815 sera were adsorbed *in vitro* with DBA/2 mouse bone marrow, thymus, blood, liver spleen, and lymph node cells in order to remove antibodies specific for normal cell antigens, particularly H-2 antigens (33). The adsorbed sera were then tested for their ability to mediate lysis (complement-mediated cytolysis or ADCC) of nonspecific targets, either normal spleen cell targets or syngeneic L1210 tumor targets. If the adsorption process removed antibodies binding normal cell membrane antigens alone, the adsorbed sera should lose the ability to lyse the nonspecific target while retaining the ability to lyse the specific target, P815.

When adsorbed rabbit #11 anti-P815 antiserum was tested on normal spleen cell and P815 targets, it was found that adsorption resulted in the removal of complement-mediating lytic activity against both normal spleen and P815 targets. A probable explanation for these results is that the complement fixing antibodies contained in the xenogeneic antiserum consisted primarily of antibodies specific for mouse tissue histocompatibility antigens.

Since ADCC was not observed utilizing normal spleen cell targets, another target was selected for further serum specificity tests, L1210,

a B-lymphocytic leukemia syngeneic to DBA/2. The rationale for the choice of this target was the following: 1) L1210 was syngeneic to DBA/2 and should therefore have the identical H-2 and non-H-2 antigens as P815; 2) L1210 was a tumor which had been used widely and had been chosen as the standard for testing of new chemotherapeutic and immunotherapeutic regimens (114); 3) L1210 was available as a stable _in vitro_ line in the laboratory of Dr. David Martin and might be a more easily labelled and lysed target than normal spleen cells; and 4) the possibility that L1210 shared tumor-associated antigens with P815 appeared unlikely since both tumors were chemically induced and of different histiological types.

As predicted, L1210 was easily labelled with chromium-51 and was lyseable by the ADCC mechanism using human PBL effectors. Evaluation of rabbit anti-P815 antisera adsorbed with DBA/2 tissue cells at $4^{o}C$, $20^{o}C$, and $37^{o}C$ showed that the adsorption process non-selectively removed antibodies able to mediate ADCC and complement-mediated lysis against both L1210 and P815 targets. Since adsorption of xenogeneic sera with normal mouse tissue greatly reduced the anti-P815 and anti-L1210 ADCC titers (19,000 to 1500 and 9,500 to 190 respectively), it was concluded that most of the cytolytic antibody activity present in the xenosera was directed against normal mouse tissue antigens, probably H-2 antigens.

Consistent with this hypothesis was the fact that xenogeneic anti-P815 antiserum was found capable of blocking lysis of P815 by allosensitized C57Bl/6 anti-P815 spleen cells (see Section III.B.1). It has been clearly demonstrated using allogeneic (22, 120, 100) and trinitrophenyl-modified syngeneic (19, 157) targets that inhibition occurs only in the presence of antisera directed against products of the H-2 region or more specifically against products of the H-2K and H-2D loci. Therefore, the

conclusion that xenogeneic anti-P815 sera contain anti-H-2 activity appears valid.

While most of the anti-P815 activity in xenosera was directed against histocompatibility antigens, it is possible that a small fraction of anti-P815 antibodies were directed against tumor-associated antigens. This possibility is supported by our evidence which shows that after adsorption, xenoserum at a 1:6000 dilution lysed only P815 and not L1210 targets; however, at this dilution only 14% lysis of P815 was observed. Therefore, it was suggested that the *in vitro* adsorbed sera might be partially but not entirely specific for P815, still containing a large amount of residual anti-H-2 antibody in addition to a small amount of anti-P815 TAA antibody. An alternative explanation which cannot be excluded is that P815 and L1210 share tumor-associated antigens (TAA) and that the residual antibody activity present in adsorbed sera is mainly directed against these TAAs. In support of this explanation is evidence that mammary tumors and many chemically-induced leukemias of DBA/2 mice bear a common tumor-associated antigen called the ML antigen (168). If the ML antigen is shared by the P815 mastocytoma and the L1210 leukemia, then anti-ML antibody raised against P815 would also react with L1210. This hypothesis remains to be substantiated.

Since *in vitro* adsorbed sera, adsorbed with normal murine cells and tumors (P815, L1210) failed to show specificity, *in vivo* adsorption of xenogeneic sera was performed. DBA/2 mice were injected with antiserum i.p., then bled several hours later. *In vivo* adsorbed sera lacked specific anti-P815 activity. These results contrast with those of Bertschmann *et al*. (12) in which *in vivo* adsorption of xenogeneic sheep and allogeneic C3H anti-P815 sera removed complement-dependent anti-H-2

activity from the sera.

The difference in results might be attributed to differences in the compositions of rabbit compared to sheep and mouse sera. For example, rabbit sera may possess greater amounts of antibodies with specificities for H-2 antigens, whereas sheep and mouse sera may contain greater quantities of anti-P815 TAA antibodies. These differences in serum composition might be explained by differences in recognition of P815 TAAs in the immunized host species. Alternatively, variation in the expression of TAAs on P815 sublines which exist in different laboratories may account for the discrepancies in serum composition observed. A possible source of subline variation might be differences in extent of tumor antigenic modulation. The modulation of loss of TAAs has been documented using several tumors (2, 128). For example, the 141 lymphoma of New Zealand mice loses TAAs upon _in vitro_ cultivation (Kondo, Sawada, Roubinian, and Talal, unpublished observations). Evidence that P815 tumor cells also lose expression of TAAs when grown _in vivo_ (Biddison and Palmer, 13), and _in vitro_ (Faanes and Choi, 38) has been reported. If our particular P815 subline modulates TAAs more rapidly or to a greater extent than other sublines prior to rabbit immunization, the antibodies elicited would be expected to be primarily directed against the normal histocompatibility antigens remaining on the P815 cells.

In summary, xenogeneic rabbit anti-P815 antisera were adsorbed _in vitro_ with normal DBA/2 tissue cells or tumor cells (L1210 or P815) or _in vivo_ in DBA/2 mice. The adsorption process removed antibodies specific for normal tissue antigens from the sera; however, the remaining antibodies appeared to lack specificity for P815 TAAs. Possible explanations for this observation were provided.

D. Mechanisms of Resistance of P815 Targets to Lysis

1. Documentation of the resistance to lysis phenomenon

It appears paradoxical that the P815 tumor, one which has been widely employed as the classic neoplasm for the study of T cell-mediated cytotoxicity *in vitro*, should be resistant to ADCC. Yet our data clearly indicate that P815 is a difficult target cell to lyse by means of the ADCC mechanism although it is easily lysed by the complement-mediated and T cell-mediated cytolytic pathways. Normal DBA/2 spleen cells which lyse antibody-sensitized CRBCs were found incapable of killing antibody-coated P815 cells. Alloactivated C57Bl/6 spleen cells which kill P815 directly were inhibited by the addition of xenogeneic rabbit anti-P825 antiserum. While syngeneic murine effector cells (spleen and peritoneal exudate) were incapable of lysing xenoantibody-sensitized P815 targets, xenogeneic rat spleen cell and human PBL effectors were able to lyse the targets. However, human PBL effectors were unable to lyse alloantibody-coated P815 targets, although the same alloantibody permitted complement-mediated lysis of the P815 tumor and PBL-induced ADCC of the L1210 tumor.

The resistance of P815 tumor to murine effector cell-mediated ADCC has been confirmed by Greenberg *et al*. (52) and by Lovchik and Hong (102). The resistance of other nonerythrocyte targets to antibody-dependent murine effector cell lysis has been reported by Halloran and Festenstein (54) and more recently by Berger and Amos (9). Neither murine bone marrow, thymus, mesenteric lymph node, nor peritoneal exudate cells were sources of effector cell activity for antibody-sensitized EL-4 targets; however, DBA/2 mouse peripheral blood lymphocytes were cytolytic against the same targets. On the other hand, other investigators have reported low levels of ADCC against tumor targets utilizing mouse splenic effector

cells. Zighelboim et al. (186) found that normal C57Bl/6 or Balb/c splenic adherent and non-adherent cell populations were cytotoxic when tested against antibody-coated EL-4 targets. Forman and Moller (41) reported spleen cell-induced ADCC of isologous antibody sensitized YAC tumor cells. Several laboratories have confirmed mouse effector cell-induced lysis of Moloney sarcoma targets via ADCC. Finally, in addition to the Berger and Amos report described above, others have shown murine ADCC against the P815 target. Kiessling and Klein (81) demonstrated that spleen cells from nude mice could kill anti-YAC antibody-coated P815 targets. More recently, Ojo and Wigzell (125) reported that 1) spleen cells from young CBA/H mice, and 2) Corynebacterium parvum-induced peritoneal exudate cells were able to lyse alloantibody-coated P815 and CRBC targets. ADCC against sensitized P815 targets was associated with natural killer (NK) cell activity against YAC-1 targets: both were detectable in normal spleen of mice 2 months of age, but both were absent in 10 day old and 12 month old mouse spleens. These results may indicate that ADCC and NK effector cells and/or regulator cells arise transiently in the spleen, then disappear. Since the DBA/2 mice used in our experiments were adults, 3-6 months of age, splenic ADCC activity and NK activity may have vanished by this age. Whether spleens from 2 month old DBA/2 mice contain cells able to mediate ADCC against P815 is not known. The fact that DBA/2 mice possess NK active spleen cells (83) may suggest that associated ADCC effector cells also exist in the spleen.

Although the apparently conflicting data concerning the ability or inability of murine effector cells to lyse antibody-coated tumor targets might be explained by quantitative differences in ADCC effector cells present in lymphoic organs, the data might also indicate the presence of

ADCC regulator cells in these organs. The possibility that murine spleen cells contain a suppressor cell population which inhibits ADCC function was presented by Herrick and Pollack (39). Other studies in which C. parvum-induced suppressor cells prevented natural killer cell activity may indirectly support the suppressor cell theory since the cell population mediating NK activity has been indistinguishable from the population mediating ADCC thus far. Data negating the suppressor cell hypothesis was presented by Berger and Amos (unpublished data). When they mixed murine spleen cells and PBLs, they failed to observe either antagonism or synergism, suggesting the absence of ADCC regulator cells in spleen. They concluded that the inability of murine spleen cells to lyse sensitized tumor targets in their experiments was best attributed to a quantitative or qualitative defect in ADCC effector cells present in spleen rather than to the presence of suppressor cells. Thus, evidence in support of the suppressor cell hypothesis remains inconclusive.

2. Possible mechanisms of resistance to lysis

The mechanisms which determine the resistance of tumor cell targets such as P815 to ADCC are unknown. Resistance to lysis may be expressed at the level of the target cell, the antibody, or the effector cell. At the target cell level, three hypotheses explaining the resistance of P815 to lyse are tenable: 1) the P815 membrane antigen density may be important in determining the targets' susceptibility to ADCC as it is in determining susceptibility to complement-mediated lysis, 2) P815 may possess a high membrane turnover rate, shedding antigen and antibody-antigen complex, and 3) P815 may be capable of repairing damage induced by ADCC, preventing lysis.

No evidence exists which allows evaluation of the first hypothesis. Existing evidence supports the second hypothesis. Shedding of tumor antigens by P815 in vitro was reported by Faanes and Choi (38). Antigen shedding may explain the finding by Biddison and Palmer (13) that tumor cells from P815 ascites tumor bearing mice become resistant to cytolysis by syngeneic effector cells. The authors concluded that tumor cells may escape immune attack by the loss of expression of cell surface tumor-associated antigens. As mentioned previously, this phenomenon of "antigenic modulation" has been well characterized, particularly in the thymus-leukemia tumor system (127, 128, 132) where antibody was shown to trigger the reversible loss of tumor antigenicity. Finally, the third hypothesis, that P815 resistance to lysis occurs through membrane repair mechanisms, is probably incorrect in view of our data which show that PBLs are capable of mediating lysis of P815 in the presence of xenoserum, although alloserum cannot mediate ADCC using the same PBL effectors. This data might indicate that the defect in ADCC lies at the antibody level rather than at the target cell level.

At the antibody level, ADCC of target cells requires fulfillment of two criteria: 1) the Fab portion of antibody must be able to bind to antigens on the target cell, and 2) once the antibody binds to a target cell, the Fc portion of antibody must recruit an effector cell capable of lysing the target. P815 resistance to lysis cannot be attributed to an inability of the antibodies in anti-P815 sera to bind to membrane antigens on P815 targets since we showed that both xenoserum (which fails to mediate murine spleen cell-mediated P815 lysis) and alloserum (which fails to mediate human PBL-mediated P815 lysis) are able to mediate antibody-dependent complement lysis of P815 targets as well as ADCC of L1210

targets. In addition, data from Greenberg et al. (52) demonstrated that while P815 and SL-2 tumors both bound the same amount of anti-P815 IgG antibody as measured by immunofluorescence, only P815 tumor was resistant to murine spleen cell-induced lysis. Therefore, it is unlikely that resistance of P815 to lysis is attributable to a failure of antibody to bind to P815.

However, the resistance of P815 to lysis may be due to the inability of antibodies to bind to membrane Fc receptors on the effector cells. Effector cell membrane Fc receptors differ in binding affinity for the Fc regions of various antibodies depending on 1) the species in which the antibody is raised, and 2) the class or subclass of antibody involved.

An explanation for our data that xenogeneic rabbit and allogeneic mouse anti-P815 antisera failed to mediate murine spleen cell and human PBL mediated lysis, respectively, of P815, might be that the species-specific amino acid residues of the anti-P815 antibodies present in the antisera interfered with the binding of the antibody to membrane Fc receptors on the cells. Evidence for the importance of the antibody source in the recruitment of ADCC effector cells was obtained by Imir, Saksela, and Makela (79, 154) who examined the species specificity of ADCC against 4-hydroxy-5-iodo-3-nitrophenyl acetyl (NIP) or (4-hydroxy-3, 5-dinitrophenyl) acetyl (NNP) hapten-conjugated CRBCs. Antisera directed against NIP and NNP were prepared in rabbits, rats, chickens and mice; then sera were standardized with respect to their hapten binding capacities and compared for their relative abilities to recruit various ADCC effector cells. Mouse antibody, raised in CBA x C57B1/6 F1 hybrids, was more efficient than fowl or rabbit antibodies with mouse effector cells.

On the other hand, rabbit antibody was at least ten times more effective than mouse, fowl, guinea pig, or rat antibody when human PBLs or spleen cells served as effector cells. These results are consistent with our data.

It is possible that if syngeneic DBA/2 anti-P815 antibodies were raised, these antibodies may have been able to recruit murine effector cells to lyse antibody-coated targets. Lovchik and Hong (102) postulated that perhaps recruitment of murine ADCC effector cells requires more stringent Fc recognition or Fc receptor-induced cell activation than that of human or other xenogeneic effector cells. This hypothesis evolved from the observation that the feature common to most systems in which mouse effector cells were shown to kill mouse tumor cell targets was that isologous sensitizing antisera were always employed. However, there are exceptions to the isologous serum rule: 1) the Zighelboim (185) findings that mouse spleen cells (both adherent and non-adherent) were cytotoxic to rabbit antibody-coated EL-4 targets, and 2) the report by Berger and Amos (9) that mouse PBLs lysed alloserum-coated targets. In addition, previously cited work by Greenberg and Wolosin (53) demonstrated that isologous antibody does not always insure recruitment of ADCC function: in their particular L-5178Y system, spleen cell-induced cytostasis rather than cytotoxicity was observed.

Alternatively, the inability of the anti-P815 sera to recruit murine or human effector cell populations may be explained by the restricted immunoglobulin class or subclass composition of the serum antibodies. These antibodies may differ in their relative abilities to bind to cellular Fc receptors and to recruit various effector cell populations. In support of this concept are inhibition studies in which ADCC of erythrocyte

and nonerythrocyte targets were blocked by known IgG subclasses. Chicken erythrocyte target lysis was inhibited to a greater extent by IgG_1 and IgG_3 than by IgG_2 and IgG_4 (Wisloff et al., 181; Larsson et al., 99). This data is compatible with evidence that monocytes, which bear Fc receptors that preferentially bind IgG_1 and IgG_3 subclasses (59, 77), lyse sensitized CRBC targets. On the other hand, Chang tumor target cell lysis was inhibited to a greater extent by IgG_2 and IgG_4 (MacLennan et al., 110), suggesting that the nonphagocytic, nonadherent K cell effectors which lyse these targets (52) bear membrane receptors for IgG_2 and IgG_4.

Finally, at the effector cell level, resistance of P815 to ADCC may be attributed to a defect of the antibody-recruited effector cell to lyse the P815 target. Supporting this hypothesis is evidence that ADCC effector cells possess restricted ranges of lysis, depending on the targets examined. For example, phagocytic effector cells lyse sensitized erythrocyte targets but lack the ability to lyse tumor targets. Similarly, phagocytic effector cells may lyse one class of tumor target but not another, depending on the nature of the cytotoxic machinery the effectors possessed. This may explain the selective ability of murine spleen cells to lyse anti-P815 antibody-coated SL-2 targets but not P815 targets (52).

E. In vivo ADCC Protection Experiments

1. P815 dose response experiments

The reported lethality (12) of the P815 tumor was confirmed in the first experiment. When mice were injected with P815 cell doses ranging from 10^2 to 10^6 cells per mouse, all mice died within approximately one month. As the dose increased from 10^2 to 10^6 cells, the time of tumor

appearance decreased from 12.2 to 4 days after injection.

In the next dose response experiment, the tumor dose range was reduced to one from 10 to 100 cells per mouse. As the number of injected cells per mouse increased, the percent tumor takes and mouse deaths correspondingly increased. The TD_{50} and LD_{50} was calculated at 80 and 83 cells per mouse respectively. Once tumor growth had become sufficiently large to be detected, it almost inevitably progressed to kill the mouse; regression of P815 tumor growth was rare (2%).

Our P815 tumor line appeared to be more lethal and less prone to regression than that of Bertschmann _et al._ (10, 11). The three identifiable lines of P815 which exist are the Berne, Lausanne, and Sloane Kettering lines. These various lines may vary in the expression of a number of biological parameters such as lethality, metastasizing potential, doubling time, or expression of tumor associated antigens. This may explain the differences observed between our Lausanne line and the Berne line of Bertschmann.

2. _ADCC protection experiments_

In the ADCC protection experiments, antibody-coated P815 cells were mixed with effector cells (human PBLs or mouse spleen cells), then the mixture was injected intradermally into syngeneic mice. While preliminary experiments indicated that protection possibly occurred, subsequent verification in controlled experiments was not achieved.

The first experiment using DBA/2 mouse spleen cell effectors suggested their ability to prevent growth of rabbit anti-P815 antibody-coated tumor cells. However, when the experiment was repeated with controls, no ADCC protection was observed.

Similar results were obtained with human PBL effectors. Initially it appeared that the observed decrease in tumor takes may have been attributable to ADCC. However, when the order of injection was changed and the time decreased, no protection was observed. When the effector to target cell ratio was increased to 1000:1 to maximize any possible ADCC effect, no reproducible protection was observed.

Therefore, it was concluded that no reproducible cell-dependent inhibition of rabbit anti-P815 antibody-sensitized P815 tumor cell growth occurred. Our inability to consistently demonstrate cell-mediated suppression of antibody-coated tumor growth may be explained by difficulties encountered at one or more of three levels: 1) the P815 tumor cell level, 2) the xenogeneic anti-P815 tumor serum level, or 3) the effector cell level.

First, the P815 tumor may simply be resistant to cytolysis by means of the ADCC mechanism. Our <u>in vitro</u> results indicated that the tumor is not lysed by murine spleen cells. This may explain the lack of <u>in vivo</u> protection observed with murine effectors. It is possible that another murine tumor (e.g., L1210 or SL-2) may be more easily killed by the ADCC mechanism <u>in vitro</u> and <u>in vivo</u>. However, this does not explain the inability of human PBL effectors, which lyse P815 <u>in vitro</u>, to prevent tumor growth <u>in vivo</u>. The observed <u>in vivo</u> resistance of P815 to ADCC mediated by both human PBLs and murine spleen cells may be explained by the phenomenon of antigenic modulation. Biddison and Palmer (13) have shown that the P815 tumor loses TAAs upon <u>in vivo</u> injection, and thus becomes resistant to lysis by cytolytic T effector cells. Since antibody triggers the modulation process (127, 128, 132), our anti-P815 antibodies may have accelerated the loss of histocompatibility antigens and TAAs

from the tumor cells, causing them to be resistant to lysis by antibody-dependent effector cells.

Second, lack of in vivo protection may be explained by the failure of the anti-P815 antibodies present in the xenosera to recruit cytolytic effector cells in vivo. If the xenogeneic serum contains a mixture of different classes or subclasses of antibodies with specificity for P815 antigens, then some of these antibodies might "arm" effector cells to lyse the tumor while other antibodies might "block" effector cell binding to tumor targets. The balance between arming and blocking antibodies in xenosera might be shifted towards blocking by the in vivo microenvironment. Alternatively, the xenosera may recruit murine host cells bearing Fc receptors which are incapable of lysing the tumor. These host cells would compete with injected PBL donor cells for Fc sites on tumor target cells, preventing the contact of PBLs with their targets and preventing subsequent target cell lysis.

Finally, difficulties at the effector cell level may explain the inability to demonstrate consistent ADCC protection in vivo. Although human PBLs were capable of lysing P815 in vitro, they may have been inactivated by the xenogeneic mouse host environment, or they may have been blocked from contact with P815 tumor cells by soluble or cellular factors in the foreign host environment. Syngeneic 3-6 month old mouse spleen cells were found to lack ADCC activity in vitro for P815. Therefore, their failure to consistently mediate ADCC in vivo is not surprising. If spleens from two month old mice were used as an effector cell source, perhaps in vitro and in vivo ADCC might have been observed.

F. Summary and Conclusions

The original goals of this project were to establish an *in vitro* ADCC tumor model system in the DBA/2 mouse, and to determine whether the ADCC mechanism was also effective in protecting DBA/2 mice from tumor growth *in vivo*. The first goal was partially fulfilled, but the second was inconclusive.

The following points were successfully established: 1) Xenogeneic rabbit anti-P815 antisera were raised and found to mediate ADCC and complement mediated lysis against chromium-51 labelled P815 and L1210 tumor targets. 2) Allogeneic C57B1/6 anti-P815 antisera were found to mediate complement-dependent lysis of both P815 and L1210; however, these sera only mediated ADCC of L1210, not P815. This observation suggested that P815 targets may be resistant to ADCC and that the resistance to lysis defect may be localized to the level of the alloantibody. 3) Human peripheral blood lymphocyte and rat spleen cell effectors were able to lyse xenogeneic rabbit anti-P815 antibody sensitized P815 targets in the chromium release assay for ADCC. 4) While syngeneic adult DBA/2 spleen cells were able to lyse antibody-coated chicken red blood cell targets, these same effectors were unable to lyse antibody-sensitized P815 targets. This indicated a defective ADCC mechanism, possibly at the effector cell level. 5) Alloactivated murine spleen cells lysed unsensitized P815 targets, and this lysis was blocked by the addition of xenogeneic rabbit anti-P815 antiserum. This suggested that a) our particular P815 subline was sensitive to direct cell-mediated cytolysis by allosensitized murine spleen cells in contrast to its resistance to indirect cell-mediated cytolysis by normal spleen cells and anti-P815 antibody, and b) alloactivated splenic T cells were unable to mediate ADCC

against P815. 6) In vivo P815 dose response experiments indicated the P815 mastocytoma to be a highly lethal tumor with a TD_{50} and an LD_{50} of 80 intradermally injected cells per mouse and 83 cells per mouse respectively.

The experimental results also raised questions, one of which is why is P815 resistant to lysis? It was shown that while xenoserum-sensitized P815 targets were lysed by human PBL and rat spleen cell effectors, they were resistant to lysis by murine spleen and peritoneal exudate cell effectors. Moreover, while allogeneic C57Bl/6 anti-P815 antisera were incapable of mediating ADCC of P815 targets, these same sera were able to mediate not only complement-dependent lysis of P815 targets but also both ADCC and complement-dependent lysis of L1210 tumor targets.

One might attempt to answer the question of the resistance of P815 to lysis by two approaches. In the first approach, the ability of C57Bl/6 anti-P815 serum to mediate ADCC against L1210 but not P815 tumor targets might be explored, allowing the evaluation of the hypothesis that resistance to lysis is due to a failure of P815 cells to bind cytotoxic antibodies. In these cold target cell inhibition experiments, optimum levels of alloserum and PBL-mediated lysis of radiolabelled L1210 targets would first be determined, then competing cold P815, L1210 or normal DBA/2 or C57Bl/6 cells would be added to the alloserum-PBL-radiolabelled L1210 target cell mixture. If cold P815 binds cytotoxic antibodies capable of mediating ADCC against the L1210 target, then one would expect the level of ADCC against labelled L1210 would be diminished.

In the second approach, the observation that xenogeneic but not allogeneic serum mediates ADCC against P815 might be explored. In these ex-

periments, limiting dilutions of xenoserum and alloserum would be mixed with radiolabelled P815 cells; and the ability of alloserum to block or inhibit xenoantibody-mediated ADCC would be assessed. If blocking occurred, then it might be concluded that 1) the antibody specificities present in the alloserum and xenoserum are similar, and 2) while alloantibodies bind to P815 targets, they lack the ability to recruit cytotoxic effector cells. If blocking did not occur, then it might be concluded that antibody specificities in the alloserum and xenoserum differ.

Since the _in vivo_ ADCC protection experiments were inconclusive, the question remains whether the ADCC mechanism is able to prevent tumor growth. Based on this experiment, a list of criteria might be presented for the optimization of ADCC experimental conditions: First, the experimental animal chosen for study must be inbred and possess nonlethal inherited or induced defects in the expression of anti-tumor mechanisms other than ADCC. For example, the C5-deficient DBA/2 mouse permits the study of ADCC apart from the complicating antibody-dependent complement-mediated lytic system. Alternatively, the nude mouse allows the study of ADCC isolated from the mature T cell-mediated cytolytic mechanism. Defects in immune mechanisms might also be induced by treatment; for example, natural killer cell activity in mice might be suppressed by strontium irradiation, and macrophage activity might be depressed by silica or caragheenan injection.

Second, the choice of tumor appears critical. It must be easily maintained _in vitro_ and exhibit a low spontaneous release rate upon labelling. The tumor-associated antigens must be immunogenic and elicit antibody production in the syngeneic host. The TAAs must remain

relatively stable on the tumor membrane and not modulate. Finally, the syngeneic anti-tumor antibody-coated tumor targets must be susceptible to lysis by syngeneic effector cells.

With the optimized tumor system described above, the hypothesis that ADCC effector cells are capable of preventing tumor growth could be evaluated in the effector cell antibody-sensitized tumor cell mixing experiments previously described. If the ADCC mechanism did prevent tumor growth in these artificial mixing experiments, then it is possible to investigate the more intriguing question: can antibody-armed effector cells be employed therapeutically to regress established neoplasms?

V. APPENDIX

A. Guide to Tables

All tables of complement-mediated cytotoxicity and ADCC data have been arranged according to a standard format to facilitate inspection and analysis. Row headings, located along the left-hand margin, indicate the control or experimental groups examined. Column headings at the top of each table describe the serum source, whether complement or effector cells (e.g., PBLs) were added, and the serum dilutions tested. For most control groups (such as the spontaneous release (SR), total counts (T), and saponin detergent lysis (SAP), complement alone (C'), and peripheral blood lymphocytes alone (PBL) controls) which are situated first in each table, results appear in the "no serum" column and are expressed in terms of percent lysis (percent lysis = $\frac{\text{experimental counts}}{\text{saponin lysis counts}} \times 100\%$) and counts, either 4 or 10 minute values. Subsequent control and experimental group data are generally expressed as percent lysis values. In a few tables (such as in Table), some data are expressed in terms of percent specific lysis, as defined in each of these tables.

Each datum in the tables and figures represents the mean of triplicate or quadruplicate counts. The statistical analyses employed in the tables were the parametric unpaired one-tailed t-test (Zar, J.H., Biostatistical Analysis, Prentice-Hall, Inc.: Englewood Cliffs, New Jersey, 1974) and the nonparametric Friedman two-way analysis of variance by ranks test (Siegel, S. Nonparametric Statistics: for the Behavioral Sciences, McGraw-Hill Book Co.: New York, 1956). Data in the experimental groups which were determined to be significantly different from data in the control groups are asterisked, and p values are displayed.

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