The University of Manitoba

SPECIFICITY OF ANTIBODIES TO H-2 ANTIGENS CAPABLE OF AUGMENTING THE GRAFT-VERSUS-HOST REACTION

BY

ANGELA KEMP

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Master of Science

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SUMMARY

The aim of this work was to produce an alloantiserum which would modify the graft-versus-host (GVH) reaction, as has been done previously by several authors (33,34,35,36,37), and then to investigate the role of anti-Ia, anti-D and anti-K antibodies in producing these effects.

Of the eight alloantisera produced, only one was effective in altering the GVH reaction as measured by a spleen weight assay. A B10.BR anti-B10.D2 (anti-H-2^d) serum was capable of augmenting the spleen index produced by B10.BR spleen cells in adult (B10.D2 x B10.BR)F_1 hosts, over a wide range of antiserum doses. This effect could not be reproduced in newborn mice. The only known antigens controlled by the H-2 complex against which alloantibodies may be produced are H-2D, H-2K and Ia antigens. The activity of this antiserum could be absorbed out by lymphocytes, thymocytes and red blood cells. All these cell types express D and K antigens. Ia antigens are expressed on B lymphocytes in high concentration (25,42,53), thymocytes probably carry some Ia antigens in low concentrations (28,42,53), while red blood cells are believed to lack Ia antigens (25,28,42,64). Lymphocyte and thymocyte absorbtions completely removed all *in vitro* cytotoxicity for lymph node cells. However, the presence of some remaining cytotoxicity after absorbtion with red blood cells, supported the belief that anti-Ia activity remained in this sample (31,58).

From these results it was concluded that the antiserum component responsible for the spleen index augmenting activity was probably not anti-Ia antibody. Excluding the possibility of the presence of

antibody against an unknown antigen linked to H-2 and expressed on all the absorbing cells, it was further concluded that the effect on the spleen index was probably due to anti-D and/or anti-K antibodies. Of the non-antibody molecules considered as possible mediators of this activity, and which could possible have been present in an alloantiserum, the only one which is likely to be removed by all these absorbtions is a B10.BR helper factor (32) with specificity for B10.D2 (H-2^d) antigens, but there is no evidence either for or against this possibility. The mechanism of the augmentation of the spleen index is not known.

INTRODUCTION

The Graft Versus Host (GVH) Reaction

The graft versus host reaction results from the injection of allogeneic immunocompetent lymphoid cells into a host which is unable to reject the donor cells. The injected cells migrate to the lymphoid tissue of the host (1) where they are stimulated by the foreign host antigens. Spleen cells from a neonatally thymectomized animal (40), or anti-theta treated spleen cells (41), are unable to produce a GVH reaction, therefore the reaction is dependent on thymus derived lymphocytes (T cells). A GVH reaction can be produced in several ways, which include:

(a) A GVH reaction can be induced by injecting adult lymphoid cells into an animal in which the immune system is not yet mature and, therefore is unable to respond to the donor antigens. Newborn mice can be used because they do not become immunocompetent until several days after birth (2).

(b) F_1 hybrid animals express the histocompatibility antigens of both parental strains, therefore they do not recognize the antigens of either parent as foreign. When lymphocytes of either parental strain are injected into the F_1 animal, they are able to respond to the antigens of the other parental strain (1,3). The host cannot react against the histocompatibility antigens of the donor; however, it can make antibodies against the cell surface receptors which each parental strain carries for the recognition of the antigens of the other, and which are not expressed in the F_1 animal (44). There is some evidence that such an antibody response by the F_1 host may contribute to the eventual recovery of animal with GVH disease (45).

(c) Lethal irradiation of the host eliminates immunocompetent cells so

that the host cannot react against injected allogeneic cells (4).

The pathological characteristics of the GVHR are as follows. In the early stages the spleen, liver and lymph nodes enlarge, reaching a maximum size at eight to ten days (5,6). Meanwhile the thymus atrophies (7). Later all the lymphoid organs atrophy (2). The animals become emaciated or fail to grow and may die if the reaction is strong enough (2). The strength of the reaction depends on the strain combination (2) and on the dose of cells injected (19).

The donor lymphocytes begin to proliferate in response to host antigens within the first few days (8,9), and there is evidence that the host stimulating cell is lymphoid or hemopoietic tissue (10,11). By the time the lymphoid organs reach their maximum enlargement, most of the dividing cells are of host origin (8,9), therefore, the spleen enlargement is due mainly to host cells. The reason for the host cell proliferation is not known. If a host anti-donor reaction is ruled out, stimulation by contact with, or humoral factors from the activated donor cells, could be considered. A mitogenic factor released from activated lymphocytes in the mixed leukocyte reaction is able to cause transformation and thymidine incorporation (indicative of cell proliferation) of normal lymphocytes (51). The allogeneic effect factor (16), described later, may account for a proliferation of B cells. Activated lymphocytes also produce a chemotactic factor which attracts neutrophils and macrophages, and a macrophage inhibitory factor which inhibits the migration of macrophages out of the spleen and lymph nodes (51); either of these might contribute to an increased number of host cells in the spleen.

The response of donor lymphocytes against the host produces cytotoxic donor lymphocytes with specificity for host histocompatibility antigens. The host cells become nonspecifically cytotoxic, with maximum cytotoxicity

corresponding to the time of maximum spleen enlargement (11,12). The reason for the activity of host cells is unknown. It is unlikely that this is due to a reaction of host cells against donor antigens because the cytotoxicity is nonspecific, and GVH reactions only occur in a host which cannot reject the donor cells. This nonspecific cytotoxicity of host cells may contribute to the pathological symptoms of the GVH reaction.

It is not only T cells which are involved in the GVH reaction. A reduction of the spleen index has been achieved by removing macrophages from the donor cell preparation (14). It has also been found that peritoneal macrophages from F_1 mice injected with parental lymphoid cells show increased nonspecific cytotoxicity which reaches a maximum about 14 days after the injection (15). B cell activity may also be stimulated by a GVH reaction. An allogeneic stimulus, such as that presented to donor T cells in a GVH reaction, causes the T cells to produce a nonspecific factor which will enhance the antibody responses by host B cells to many antigens. This effect is known as the allogeneic effect, and the factor, as the allogeneic effect factor. There seems to be an optimum allogeneic cell dose above which the antibody production may be suppressed rather than enhanced (16). Plasma cells of host origin have been found in the lymph nodes of animals undergoing a GVH reaction, and host antibody production, especially of 7S antibody, is greatly augmented. Some of these host antibodies are autoantibodies (17). Autoantibodies have also been observed in hamsters during a GVH reaction (18). It may be that the allogeneic effect plays some part of this host B cell triggering. However, B cells do not appear to contribute to the spleen enlargement, since treatment of donor spleen cells with anti-β serum did not reduce the spleen index (14).

Measurement of the graft versus host reaction

Commonly used measurements of the GVH reaction are the mortality rate, phagocytic index, and spleen or lymph node enlargement. Mortality is not a very reliable method because times of death vary greatly in the same strain combination and using the same cell dose. Secondary bacterial and viral infections may be the cause of death, therefore environmantal factors affect mortality quite significantly. Mortality rate also has the disadvantage of taking a long time before the results are known. The phagocytic index is a measure of the rate of elimination of carbon particles from the circulation by activated macrophages. This is probably related to proliferation and/or nonspecific activation of reticuloendothelial cells (56). The increase in the weight of the spleen or a lymph node is due to the proliferating cells, mostly those of the reticuloendothelial system (56), and is measured at the time of maximum spleen enlargement, which occurs at about 7 to 10 days after injection of the allogeneic cells. The size of the appropriate lymph node is measured when the cell injection is made locally, such as into a footpad. Spleen size is measured when the cells are injected intraperitoneally or intravenously. Spleen enlargement is usually expressed as the spleen index, which is calculated as follows:

$$\text{Spleen Index} = \frac{\text{spleen weight} \div \text{body weight of test animal}}{\text{spleen weight} \div \text{body weight of control animal}}$$

The control animal is either uninjected or is injected with syngeneic cells. The spleen index is directly proportional to the logarithm of the number of cells injected (19).

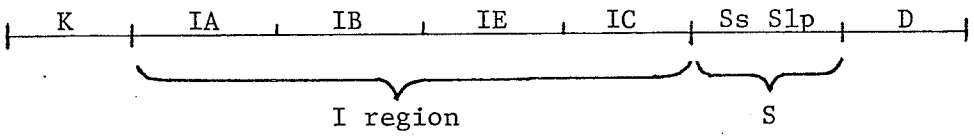
Genetics of the graft versus host reaction

The early studies of the GVH reaction showed only that the donor and recipient had to be of different inbred strains (20), or the donor had to

be from a parental strain and the recipient an F_1 animal (21). Later studies were done using congenic strains differing only at the mouse major histocompatibility locus (H-2). Incompatibility at H-2, which leads to the most rapid graft rejection, was found to be necessary for a GVH reaction. Differences at other histocompatibility loci usually only produce a measurable GVH reaction if the donors are immunized (22), although a one way GVHR and MLR occurs between Balb/c and DBA/2 which are apparently identical at H-2 (43).

The H-2 complex is situated in the ninth linkage group. A number of specificities controlled by loci at either end of the H-complex were discovered serologically, and these end regions were named D and K. An area next to D determines two specificities, known as Ss and Slp, which are present on a serum protein that is probably related to the complement system. The ability to respond to certain antigens is determined by immune response (Ir) genes situated between K and Ss-Slp. The relative positions of these loci were shown by recombination between strains which are congenic for H-2.

The H-2 Complex



Lymphocytes from mice differing only in the Ir region are able to stimulate each other's lymphocytes in a mixed lymphocyte culture (MLR) in spite of the fact that their D and K specificities are the same (24). Some pairs of strains, which differ at very small parts of this region, only stimulate one way in the MLR (42).

Klein and Park (13) used recombinant congenic mice to show that the strongest GVH reaction, as measured by spleen enlargement, occured when

the cell donor and host differed in the Ir region. Little or no spleen enlargement resulted when there were differences only in the D or K, or D plus Ss Slp regions. Because D and K region differences had been defined serologically, they became known as SD (serologically defined) differences. Ir region differences had been defined only by their GVH and MLR reactivity, therefore, they were named LD (lymphocyte defined) differences (24). Soon afterwards cytotoxic antibodies were produced against antigenic determinants controlled by genes in the Ir region, and these were named lymph node antigens (Lna) because they could be absorbed out most effectively by lymph node cells (25). When it became known that the Ir region carried genes controlling immune responses, GVH and MLR reactions, and lymphocyte antigens, it was renamed the I region (26). The Lna antigens became known as Ia (I region associated) antigens (27).

The tissue distribution of Ia antigens is not completely known. Conflicting reports may be due to different techniques and reagents used in different laboratories. They have been found consistently in high density on most B cells (25,28,29,39,42,49). They have also been found on macrophages, sperm cells and epidermal cells (28,53). There are a number of reports of Ia antigens on T cells and/or thymocytes, but in lower concentration than on B cells (28,30,42,49,65), while other investigators have not been able to show their presence on T cells or thymocytes (28,39,42). It is possible that at least some specificities are confined to certain subpopulations of lymphocytes (42). For instance, there is an Ia specificity which has only been found on T cells (30), also one anti-Ia serum could not be made to react with thymocytes or peripheral T cells (39). Some anti-Ia sera gave a biphasic curve when their cytotoxicity was titrated out against spleen or lymph node cells. One such antiserum reacted

with both T and B cells at high concentration, but only with T cells at low concentration. Absorbtion with B cells left activity against T cells. This suggests the presence of more than one population of anti-Ia antibodies (65).

Since Ir genes, MLR and GVH reaction genes, and Ia antigen genes are all present in the I region, it is of interest to know whether the Ia antigens have any functional reationship to MLR, GVH or Ir genes. Anti-Ia sera have been used to study this problem. Anti-Ia sera directed against the stimulating cells in the MLC reaction can block the reaction, but anti-Ia sera against the responding cells has no effect (28). Antibody against only one or two Ia specificities on the stimulating cell has a smaller effect. Elimination of Ia antigen bearing stimulator cells by anti-Ia serum and complement prevents the MLR, but similar treatment of responder cells has little of no effect. These results suggest that the Ia antigens may be the stimulus in the MLR, and that the responding cells in the MLR do not carry Ia antigens (28). Anti-Ia serum has also been used to block the <u>in vitro</u> formation of plaque forming cells against sheep red blood cells (28). It is also of interest that identity at I-A and I-B or some part of this region is necessary for successful T - B cell co-operation (28).

Ia antigens have been found on three factors presumably produced by T cells, and a possible function for Ia specificities on B cells has been suggested. The antibody response to the synthetic polypeptide (TG)-A-L, which consists of an alanine and lysine backbone with sidechains of tyrosine and glutamic acid, is controlled by genes in the I region. All high responder and most low responder mice produce an antigen specific T cell factor which can replace T cells in this thymus dependent antibody response, by stimulating antibody production by B cells in the presence of antigen. It has a molecular weight of about 50,000 and carries an antigen binding

site, but it does not react with anti-immunoglobulin sera, therefore, it does not carry immunoglobulin constant region determinants although the presence of idiotypic determinants is not ruled out. It can be absorbed by an anti-Ia immunoadsorbant column, therefore, it carries Ia antigens. It is possible that this factor is all or part of the T cell receptor controlled by an Ir gene in the I region. Only two known H-2 genotypes do not produce this helper factor (32). Most low responders do produce this factor, but have B cells which cannot respond to it. High responder strain B cells can absorb and respond to helper factor from low responder T cells. This implies that a second Ir gene in the I region controls a B cell receptor site for the T cell factor. Anti-H-2 serum directed against bone marrow cells can block their response to helper factor. Such a B cell receptor site could account for some Ia specificities on B cells (32).

A suppressor factor of molecular weight 35,000 to 55,000 was obtained from sonicated spleen cells or thymocytes taken from Balb/c mice primed with the carrier molecule KLH (keyhole limpet haemocyanin). This molecule was able to suppress the anti-DNP (dinitrophenol) IgG response to DNP-KLH, both in vitro and in vivo. It was shown to be carrier specific, and only acts in mice of the same H-2 allotype. Since suppression is only achieved in the presence of carrier primed T cells, it is assumed to act on these rather than on B cells directly. This molecule, like the helper factor, is not absorbed out by an anti-Ig immunoadsorbant, but is taken up by an anti-Ia immunoadsorbant, therefore, it does not carry immunoglobulin constant region determinants, but does carry Ia specificity (61).

The third factor is the allogeneic effect factor previously mentioned. It is not antigen specific, and it can replace T helper cell activity for B cells exposed to any antigen. Its activity can by absorbed out by an

anti-Ia immunoadsorbant, therefore, it carries Ia specificity (62).

The effect of alloantisera on the graft versus host reaction.

There have been several reports of either an increase or a decrease of the severity of the GVH reaction, produced by injections of donor anti-host sera into the host. These experiments have been done in adult and newborn mice, in F_1 and allogeneic hosts. The antisera have been injected before, simultaneously with, and after injection of the donor cells. Mortality rate, survival time and spleen index have been used as measurements of the severity of the GVH reaction. In most cases only a small amount of antiserum (0.1 ml or less) was required to produce a significant effect.

Voisin and Kinsky, in 1962 (33), increased the survival time of newborn A strain mice suffering from runt disease induced by adult CBA spleen cells, by mixing 0.2 to 0.4 µl of CBA anti-A serum with the donor cells. In 1965 Batchelor and Howard (34) injected two 0.1 ml volumes of donor anti-host serum intraperitoneally into adult F_1 hosts, the first injection being given two hours before, and the second, one to four days after an injection of adult parental spleen cells. Three different strain combinations were used, and produced three different results. In the C57BL ⟶ (C57BL x CBA) F_1 combination, survival rate was decreased, but the phagocytic index, measured on day 11 was not altered. In the A ⟶ (A x C57BL)F_1 combination survival time and survival rate were both slightly decreased, but this was only evident if low numbers of donor spleen cells were used. In the C57BL ⟶ (A x C57BL)F_1 combination survival rate and survival time were both increased. In 1968 Voisin, Kinsky and Maillard (35) increased the survival rate of adult (B10.D2 x CBA)F_1 hosts by injecting 0.01 ml to 0.1 ml of B10.D2 anti-CBA serum intravenously, 24 hours before the injection of

B10.D2 spleen cells. Humphrey, Fitch and Coppleson, in 1972 (36) used A/He or CBA spleen cells injected into 3 - 9 day old (A/He x CBA)F_1 hosts. A 0.1 ml volume of A/He anti-CBA serum was also injected i.p., either one day before and three or four days after the cells, or mixed with the cells. The antiserum acted synergistically with cells of either parental strain to increase the spleen size, measured 10 to 14 days after the cell injection. In irradiated adults (360R), 0.5 ml of antiserum given i.p. one day before and three or four days after A/He spleen cells, also acted synergistically to decrease survival time. Jose, Stutman and Good (37) injected 5×10^7 CBA spleen cells into adult (CBA x DBA/2)F_1 mice, followed immediately by 0.1 ml i.p. of various dilutions of CBA anti-DBA/2 serum. The spleen index was measured on the eighth day. The antiserum produced a reduced spleen index over a limited range of dilutions, but at higher and lower dilutions it had no effect. IgG_2 and IgG_3 fractions of this antiserum were also able to mediate this effect, while the IgG_1 fraction was inactive and the IgM fraction produced a slight increase in the spleen index. As discussed later, the source of cells used as antigen in the production of the antisera may be important. Batchelor and Howard (34) state only that they used "normal and neoplastic" tissues as antigen in the production of their antisera, but in all the other studies spleen cells were used. Safford and Tokuda (38) were able to reduce mortality in A/J mice undergoing a GVH reaction by injecting the C57BL/K spleen cell donors with 0.1 ml of C57BL/K anti-A/J sarcoma (SaI), four to eight days before the spleen cells were used.

Many investigators measured mortality, of which the direct cause or causes are not fully understood. Others used a spleen weight assay, which is a measure of the proliferation of lymphocytes in the spleen.

In only one case (36) was the same antiserum used in both assays and in this case it had the same effect on the severity of the GVH reaction measured with either assay.

It is not known what determines whether an antiserum will increase or decrease the severity of the GVH reaction. It has been suggested that the immunization procedures, the class of antibody produced (36), the dose of antiserum (33,34), the strain in which the alloantibody is produced or the strain of the host (36), or a combination of these may be determining factors. There are no consistent trends to indicate what importance each of these factors may have. Only Jose, Stutman and Good (37) isolated the immunoglobulin classes responsible for the activity of their antiserum. They also noted that the protective effects of their sera were not related to their hemagglutination titers. Voisin, Kinsky and Maillard found that the hemagglutinating ability of their sera could be absorbed out without completely removing its protective effect (35).

The mechanism of the action of these antisera remains unknown, but it has been compared to the enhancement or facilitation by antisera of tumors and allografts. Covering of antigenic sites on host alloantigens is the simplest explanation for protection against runt disease, but where very small amounts of antiserum have been used, or a critical dose is required, this is unlikely (37,38). The critical dose observed by Jose et al. (37) suggests that the formation of antigen-antibody complexes at a critical ratio of antigen to antibody may be required in order to suppress the donor cells. Safford and Tokuda provided good evidence in favour of a direct effect on donor cells of antibody, which might perhaps turn off specific responding cells or stimulate the formation of suppressor cells (38). When Jose et al. preincubated donor cells with their

antiserum the GVH reaction was not altered (37), but the incubation may not have been long enough to affect the cells irreversibly. It has not been ruled out that some component of antiserum other than antibody might be involved in increasing or decreasing the severity of the GVH reaction.

Mechanisms of enhancement

It has been suggested that alloantisera might protect against runt disease by the same unknown mechanism as that by which alloantisera enhance the survival of allogeneic tumours (47,48) or normal tissue allografts (50) *in vivo*, or protect tumour cells from cell mediated lympholysis *in vitro* (47,48). There are several hypotheses to explain the action of enhancing antisera. The antibody may cover antigenic sites on the graft or suppress the expression of cell surface antigens, so that either the normal lymphocyte cannot become sensitized or the sensitized lymphocyte cannot kill the target cell. Antibody or immune complexes may act directly on the immunocompetent cells to either prevent their sensitization or inhibit their cytotoxic activity.

There is really no conclusive evidence for the prevention of sensitization or killing by covering antigenic sites, although grafted tissues do take up the relevant antibody (46). It is doubtful whether the amount of antibody necessary for enhancement would be sufficient to cover enough antigenic sites to prevent sensitization or killing. French and Batchelor (50) found that in rats with long surviving kidney allografts established 5 months earlier by using enhancing alloantiserum, there are no circulating antibodies, and the kidneys from these rats can take up as much radiolabelled alloantibody as kidneys which had been newly transplanted without enhancing alloantiserum; therefore, the antigenic sites are not all covered. However, during the first few weeks after transplantation, during which there is circulating

antibody, masking of antigenic sites cannot be ruled out. In contrast, Thoenes et al. (57) found circulating antibody in rats with long established kidney allografts.

Takasugi and Klein (47) and Takasugi and Hildeman (48) were able to extend the survival of an allogeneic tumor in vivo and protect tumor cells from destruction by sensitized lymphocytes in vitro, using anti-H-2 serum. They observed that lymphocytes taken from mice injected with antibody as well as tumor cells were less cytotoxic to tumor cells in vitro than cells taken from mice injected only with the tumor. When the antibody was injected six days after the tumor, by which time the animals were already sensitized, there was a reduction in lymphocyte cytotoxicity which was not a result of the removal of the antigenic stimulus, because the removal of the tumor did not have the same effect on the sensitized lymphocytes. Thus, there appears to be a central inhibitory effect on the sensitized lymphocytes, although it has not been ruled out that antibody may also act at other levels.

French and Batchelor (50) also produced evidence for a central inhibitory effect of alloantisera. They made a rat kidney, F_1 to parental strain, graft accompanied by enhancing antigraft alloantibody. Later, without the use of any additional antiserum, a second F_1 kidney graft was accepted by the first host, and the first kidney graft was transferred to a second host where it was rejected. The enhancement was associated with the host which was given antiserum, and not transferred with the transplanted kidney. Rats bearing enhanced F_1 kidneys are capable of producing antibody against donor strain and their lymphoid cells can produce a GVHR in the F_1 strain. However, they make only a weak cell mediated immunity against a later skin graft from the same F_1 donor

strain as the kidney. Even following two injections of fifty million spleen cells to induce a response, neither the skin grafts nor the kidney are completely rejected.

Duc et al. (59) were able to produce tumour blocking activity by mixing alloantiserum with tumour antigen in the right ratio. This suggests that antigen antibody complexes may be important for enhancement.

Several mouse tumour enhancing alloantisera have been analysed to find the Ig class responsible for their activity. Takasugi and Hildemann (52) found that IgG_2 enhanced, IgG_1 had no effect, and IgM was cytotoxic. Takasugi and Klein (47) found most activity in the IgG_2 fraction, some in the IgG_1 fraction but none in the IgM fraction. Rubinstein et al. (63) were able to enhance with all three fractions, if used in the correct doses, but high concentrations of IgM or IgG_2 inhibited tumour growth.

Gillespie (46) used Fab, $F(ab)_2$ and Fc fragments of IgG_2 alloantibodies to protect tumour target cells from lysis in an in vitro cell mediated cytotoxicity test. He showed that Fab fragments protected the tumour target cells when preincubated with the tumour cells, but not when preincubated with the normal thymocytes which were used as the source of T cells. Conversely, the Fc fragment protected when incubated with the lymphocytes, but not with the tumour cells. It was suggested that the Fab portion of the molecule may mediate enhancement at the target cell level, while the Fc end mediates a central inhibition of the lymphocyte. This hypothesis is difficult to reconcile with the immune complex theory unless enhancing antisera have several modes of action. It is also possible that the action of Ig fragments in vitro may not accurately reflect the action of whole molecules in vivo.

Two authors have produced evidence that it is the anti-Ia component of an enhancing alloantiserum which may be responsible for its activity. D and K specificities are present on red blood cells and platelets, but Ia specificities are believed to be absent (25,60,23) therefore, anti-D and anti-K antibodies can be removed from an antiserum by red blood cells or platelets, leaving anti-Ia antibodies. Jansen et al. (58) found that the IgG_2 fragment of a $B6AF_1$ anti-B10.D2 serum would enhance B10.D2 skin grafts on $B6AF_1$ mice. After absorbing with B10.D2 red blood cells he found that all the cytotoxicity had been removed but the enhancing activity remained. A similar experiment by Davies (60) involved the use of rat anti-Ag-B serum, (Ag-B is the major histocompatibility complex of rats), to enhance (Wag x Agus)F_1 to Agus heart transplants. When the antiserum was absorbed with Wag red cells or platelets, the enhancing activity was not diminished. In both these cases the anti-Ia component of the antiserum was able to produce the same enhancing effect as the whole antiserum.

The mechanism of enhancement is not completely clear. There is a lot of evidence in favour of a central inhibitory effect, but peripheral effects cannot be ruled out. It is likely that antigen-antibody complexes are required. Most authors have found activity in the IgG fractions of enhancing antisera, and some have implicated the anti-Ia component.

OBJECTIVE OF THE PRESENT STUDIES

The studies discussed in the previous section demonstrated that donor anti-host sera may in some cases suppress or augment GVH reactions. The mechanism of these effects is not clear, but may be tentatively attributed to antibodies directed against some antigens of the major histocompatibility complex , as suggested by the fact that all the antisera used by the authors quoted above contained anti-H-2 antibodies and that H-2 incompatibility between donor and host is necessary for the development of GVH reactions. Since different antisera, all containing anti-H-2 antibodies, have been shown to produce opposite effects and frequently may have no effect at all on the intensity of a GVH reaction, this raises the problem as to what characteristics of the antisera are responsible for these different effects. Some of the previous authors have investigated the possibility that the antibody class or subclass may be critical for determining the effects of an antiserum on GVH. While this possibility deserves further investigations, another possibility is suggested by the recent progress in our knowledge of the H-2 antigens. It has been shown that GVH reactions are at least in part due to differences of LD antigens determined by the I region. Thus, antibodies directed against the same antigens would be expected to interfere with the reaction and induce either its suppression or its augmentation. As mentioned previously, Ia antigens may be related to LD antigens which are determined by the same H-2 region. The possibility that anti-Ia antibodies contained in the anti-H-2 sera were the factors which affected GVH reactions in previous experiments should be taken into serious consideration. This possibility was not considered at the

time when most of the experiments quoted above were done, because the existance of Ia antigens was not known. Moreover several authors have omitted in their papers, details about the methods of preparation of their antisera which would retrospectively supply some information about the role of Ia antigens. The possible role of anti-Ia antibodies is further supported by the similarities of the suppression of GVH reactions with antisera and immunological enhancement of allografts, which has been recently suggested to be due to anti-Ia antibodies (58,60).

On the basis of these considerations, it was decided to investigate the role of anti-Ia antibodies in the modification of GVH reactions with antisera, in the hope that this might shed some light on the role of Ia antigens in GVH reactions. Anti-Ia antibodies may be obtained in two ways, i.e. (i) by the use of congenic recombinant strains of mice different only for part of the I region of H-2 and identical for the K and D regions, and (ii) by absorbing the anti-K and anti-D antibodies from an antiserum produced against the whole spectrum of H-2 antigens with tissues which contain these antigens but do not contain Ia antigens. The first approach presents the advantage that anti-K and anti-D antibodies would not contaminate the antiserum, but has the disadvantage that some anti-Ia antibodies critical for the elicitation of the desired biological effect may not be represented in the antiserum depending on the point of the H-2 chromasome where the recombination which distinguishes the two strains has occured. The second approach does not present the latter problem but presents several technical problems related to the choice of tissue for absorbtion and to the difficulty of determining when the absorbtion is complete. Since the appropriate congenic recomb-

inant strains were not available in this laboratory, only the second approach was possible for us.

The first step toward the objective stated above was to reproduce the results of previous studies, i.e. the augmentation and/or suppression of GVH reactions with antisera to H-2 antigens. As shown in the Results section, the different antisera tested either produced no effect or induced an augmentation of the GVH reaction. Thus, it was not possible to study, as originally planned, the effect of anti-Ia antibodies in the suppression of GVH reactions.

MATERIALS AND METHODS

Chemical and biological materials.

1. Agarose was obtained from Marine Colloids, Inc., Rockland, Maine.
2. Alsevers solution was prepared as follows: dextrose 20.5 g/1, sodium citrate (dihydrate) 8.0 g/1, citric acid (monohydrate) 0.55 g/1, sodium chloride 4.2 g/1. This solution was autoclaved at 15 p.s.i. for 15 minutes.
3. Chromium 51 was obtained from Atomic Energy of Canada Ltd. or from the Radiochemical Centre, Amersham, England, in the form of a sodium chromate solution. The pH was corrected to approximately 7.4 by adding 0.2N HCl dropwise, using phenol red as in indicator. Then the concentration was adjusted to 1000 µCi/ml with Dulbecco's buffer.
4. (Ethylenedinitrilo) tetraacetic acid, tetrasodium salt (EDTA), was obtained from Matheson, Coleman and Bell, Cincinnati, Ohio.
5. Foetal calf serum (FCS) was obtained from Microbiological Associates, Bethesda, Maryland. Complement was heat inactivated at 56°C for 30 minutes.
6. Hanks balanced salt solution (HBSS) and RPMI were both obtained in powder form from Grand Island Biological Co., Grand Island, New York. They were made up according to the accompanying instructions, except that in both cases sodium bicarbonate was replaced by Hepes buffer at a final concentration of 0.01M. 0.025M Hepes was sometimes used in RPMI for tumor cell cultures.
7. N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) buffer was obtained from Calbiochem, San Diego, California. A 1.0 M stock solution was prepared and corrected to pH 7.3 with 2N NaOH.

8. Madin and Darby's solution was prepared as follows: NaCl 8.0 g/l, KCl 0.4 g/l, $NaHCO_3$ 0.58 g/l, dextrose 1.0 g/l, EDTA 0.2 g/l.
9. Normal mouse serum (NMS) was obtained from pooled blood collected from various strains of mice. B10.BR NMS for experimental control purposes was collected from mice being killed as cell donors. These sera were heat inactivated at 56°C for 30 minutes and stored at -28°C in 1 ml aliquots.
10. Pertussis vaccine containing approximately 15×10^9 killed organisms per ml was obtained from Connaught Laboratories Ltd., Toronto, Canada.
11. Dessicated penicillin-streptomycin was obtained from Difco Laboratories, Detroit, Michigan. It was dissolved in sterile water and added to culture media at a concentration of 100 units of penicillin and 100 µg of streptomycin per ml.
12. Phosphate buffered saline was prepared as follows: NaCl 7.659 g/l, Na_2HPO_4 0.725 g/l, KH_2PO_4 0.212 g/l.
13. Trypan blue was used at a concentration of 0.25% in normal saline.
14. Dessicated bacto-trypsin was obtained from Difco Laboratories, Detroit, Michigan. It was dissolved in distilled water at a concentration of 5% and stored at -20°C. For use it was diluted to 0.5% in Madin and Darby's solution.
15. Turk's solution was 0.01% methylene blue in 1% acetic acid.

Sterilization.

The solutions and sera were sterilized when necessary by passing them through 0.45 µ Millipore filters which had been sterilized by autoclaving.

Animals.

B10.D2 new, B10.BR, C57BL/6 and $B6AF_1$ mice, and New Zealand White

rabbits were obtained from Jackson Laboratories, Bar Harbour, Maine. (B10.D2 x B10.BR)F_1 mice were bred in the Animal Care Centre, Basic Medical Sciences Building, University of Manitoba.

Tumors.

The B10.D2 sarcoma cells were obtained from a frozen sample of a tumor previously produced in this laboratory by one subcutaneous injection of 1 mg of methylcholanthrene in oil.

The P815 tumor was originally obtained from Dr. K. T. Brunner. At the time these experiments were performed, the tumor had been carried by serial intraperitoneal transplantation in DBA/2 mice for four years in this laboratory.

Preparation of cell suspensions.

(a) For production of antisera in B10.D2 and B10.BR mice. Spleens, lymph nodes and thymuses were taken from the donor mice and collected in HBSS over ice. These organs were broken up in a loose fitting homogenizer, the debris was allowed to settle for 5 minutes, and the supernatant containing cells was pipetted off. This was repeated using more HBSS and the second supernatant was added to the first. The cell suspension was adjusted to the required volume with HBSS. The cells were not washed.

(b) For production of antisera in A/J and C57BL/6 mice, and for graft versus host reactions. Mice were killed by cervical dislocation, their spleens were removed and put in a petri dish containing HBSS. The spleens were teased apart with two needles and the remaining lumps were broken up in a loose fitting homogenizer. The cells were separated from the debris by passing them through a 200 gauge stainless steel mesh, then washed twice and resuspended in more HBSS. A 50 µl sample was taken, using a 50 µl capillary pipette, and diluted 20 times in Turk's solution

for a total cell count, or in trypan blue for a viable cell count. At least 100 cells were counted each time and two separate counts were made for each cell preparation. The cells were counted in a Neubauer hemacytometer. The required number of cells was centrifuged once more and resuspended in the appropriate volume of HBSS.

Production of antisera.

(a) B10.BR andi-B10.D2 (1) and B10.D2 anti-B10.BR (1). 0.5 ml of a suspension of spleen, lymph node and thymus cells in HBSS was injected intraperitoneally (i.p.) into each mouse. Ten donor mice were used for fifty recipients. A total of seven such injections were given at ten day intervals. Fifteen to twenty drops of blood were taken from the tail vein of each mouse immediately before the last three injections and the animals were bled out ten days after the last injection. The sera from all the bleedings were heat inactivated, pooled and stored at -20°C in 2 ml aliquots.

(b) B10.BR anti-B10.D2 (2) and B10.D2 anti-B10.BR (2). 0.5 ml of a suspension of spleen, lymph node and thymus cells in HBSS was injected intraperitoneally into each mouse. Ten donor mice were used for fifty recipients. A total of eight injections were given. The second injection was given fourteen days after the first, and the rest at seven day intervals. The animals were tail bled immediately before the last two injections and bled out seven days after the last injection. The serum from each bleeding was heat inactivated and stored separately at -80°C.

(c) C57BL/6 anti-A/J; C57BL/6 anti-$B6AF_1$; A/J anti-C57BL/6; A/J anti-$B6AF_1$. The immunization procedure used was that described by Jose, Stutman and Good (37). On day 0, 2×10^7 viable spleen cells mixed with 10^9 killed

pertussis organisms in 0.5 ml HBSS, were injected i.p. into each mouse. On day 14, 2×10^7 viable spleen cells only were injected i.p. in 0.5 ml HBSS. The animals were tail bled on day 18 and bled out of day 19. The sera from both bleedings were pooled, heat inactivated and stored at -80°C in 2 ml aliquots.

(d) B10.BR anti-P815. P815 cells which had been cultured for at least 16 days were used to immunize 10 B10.BR mice. After washing 5 times, the following numbers of viable cells were injected i.p. into 0.5 ml of HBSS: Day 0 - 1×10^6; Day 10 - 3.8×10^6; Day 20 - 3.6×10^6; Day 30 - 13×10^6. The animals were tail bled immediately before the last injection and bled out 10 days after the last injection. The serum was pooled, heat inactivated and stored at -80°C.

Tumor cell cultures.

All culture procedures were carried out in a laminar flow cabinet using sterile equipment. The cells were cultured in 75 cm^2 sterile plastic disposable flasks with 15 to 20 ml of RPMI + 5% FCS in each.

P815.

One P815 bearing DBA/2 mouse was killed, the fur was shaved off and the skin was sterilized with 70% alcohol. The tumor cells were removed using a syringe with an 18 gauge needle, washed twice in HBSS, resuspended in RPMI and counted. Approximately 10^6 cells were put into each of ten culture flasks. The cells were removed from the flasks for transfer or immunization by removing the RPMI and adding 10 ml of 0.5% trypsin in Madin and Darby's solution. As soon as the cells were free they were removed from the flask, washed once in HBSS + 5% FCS to separate clumped cells and then 5x more in HBSS only.

B10.D2 sarcoma.

A vial of frozen cells was thawed and the cells were counted. Into each of three culture flasks were placed, 2.8×10^5 viable cells, and 2.8×10^5 viable cells were injected subcutaneously into each of two adult B10.D2 male mice. The cells in the flasks did not grow, therefore, as soon as small tumours appeared in the mice, one was excised under sterile conditions, cut into small pieces, mixed with 10 ml of 0.5% trypsin in Madin and Darby's solution in a trypsinizing flask, and stirred with a magnetic stirrer. After about 30 minutes, the supernatant was taken off and the cells contained in it were washed 5 times. Although there appeared to be few viable cells, several culture flasks were set up and eventually the cells grew well. When it became necessary to transfer the cells, 0.5% trypsin in Madin and Darby's solution was used to free the cells, which were then washed well in HBSS. It was later found that Madin and Darby's solution alone would free the cells, therefore, this method was used on one occasion.

The cytotoxicity test.

Preparation of rabbit complement. The rabbit was anaesthetized with nembutal and bled out by heart puncture. The whole absorbtion process was carried out at 4°C in order to avoid activating the complement sequence. The rabbit serum was diluted 1:2.5 in HBSS. Calcium and Magnesium ions are necessary for the activation of complement, therefore, these were removed by adding 2 ml of 0.1M EDTA per 100 ml of diluted serum. Then 80 mg of agarose per ml of the undiluted serum was added, and the mixture was stirred gently at 4°C for 30 minutes. The agarose was removed by centrifuging at 4340 g for 10 minutes at 4°C, and the

supernatant was centrifuged again at 12000 g for 10 minutes at 4°C. The Ca^{++} content was restored by adding 2 ml of 0.1M $CaCl_2$ per 100 ml of diluted absorbed serum. A sample was titrated in the two step cytotoxicity test and the rest was stored at -80°C in 1 ml aliquots.

Chromium 51 labelling of lymph node cells.

A mouse of the appropriate strain was killed and the lymph nodes were removed. A cell suspension in HBSS was prepared by pressing the lymph nodes through a 200 gauge stainless steel mesh using a rubber tipped syringe plunger. The cells were washed once, resuspended in a small volume of RPMI + 5% FCS and a sample was counted in trypan blue. The required number of cells were taken and diluted to a concentration of 10^7 viable cells per ml. ^{51}Cr was added to give a concentration of 100 μCi/ml. The cells were incubated with the ^{51}Cr at 37°C for one hour, mixing every 10 minutes. The labelled cells were washed 6 times in HBSS, resuspended in HBSS + 2% NMS, counted in trypan blue, and diluted to a concentration of 10^6 cells per ml in HBSS + 2% NMS.

Procedure.

The one step test was used for the preliminary testing of the rabbit complement. The two step test was used to test the final absorbed complement preparation, all the mouse antisera and some of the B10.BR anti-B10.D2 (2) absorbed samples. The three step test was used for the final testing of the absorbed antisera. In the early tests, U bottomed plates were used, but it was later found that V bottomed plates gave more accurate results and were used in all tests on absorbed mouse sera. Plastic adhesive sheets were used as covers. Serial dilution of 0.1 ml of antiserum

in HBSS were made in the plates, and 0.1 ml HBSS containing 10^5 ^{51}Cr labelled lymph node cells was added to each well. The plates were covered and left at room temperature for 15 minutes. At this stage the procedure varied:

(a) In the one step test 0.05 ml of 1/5 complement was added and the plate was incubated at 37°C for 40 minutes, centrifuged and 0.1 ml of supernatant was removed for counting.

(b) In the two step test the plate was centrifuged, 0.16 ml of the supernatant was removed and discarded and 0.2 ml 1/20 complement was added. After covering the plate and resuspending the cells, the plate was incubated at 37°C for 40 minutes, centrifuged and 0.12 ml supernatant was removed for counting.

(c) The three step test was the same as the two step test, but the cells were washed in 0.2 ml HBSS before adding complement.

All centrifuging was done at 216 g for 8 minutes. Counting was done in a Nuclear Chicago gamma counter for 2 minutes. Samples were tested in triplicate if possible, or in duplicate if only small volumes were available, as was the case with some absorbed sera.

Since the complement had some cytotoxicity remaining even after agarose absorption, wells containing cells plus complement were used as the 0% control. Previously tested sheep anti-mouse lymphocyte serum (ALS) was used at a dilution of 1/40 for the 100% control. Two concentrations of the test serum without complement were included, but these readings were always below the 0% readings.

Graft versus host reactions.

Injections. Newborn mice were injected intraperitoneally, using a 30 gauge

needle inserted through the hind leg muscles. Adults were injected intravenously via a tail vein. Antiserum dilutions were made in phosphate buffered saline and the volumes used were 0.05 ml for the newborn mice and 0.1 ml for the adults. Cells were injected into the newborn mice in 0.1 ml of HBSS, and into adults in 0.3 ml of HBSS. Cells were always injected 30 minutes to 4 hours after the antisera. The control mice were not injected.

Measurement. Ten days after the cells were injected, the mice were killed and weighed. The spleens were then removed, rinsed in saline, blotted with gauze to remove excess moisture, and weighed. Weights were measured correct to the nearest tenth of a milligram.

Absorbtion of B10.BR anti-B10.D2 serum.

Absorbtion with lymphocytes. The spleen and lymph nodes were removed from B10.D2 mice and a cell suspension was prepared as described on page 23. The cells were washed 5 times in HBSS, centrifuged at 375 g for 5 minutes in a graduated tube, and the volume of packed cells was noted. The cells were resuspended in a sample of antiserum diluted with HBSS and rotated gently at 4°C for 1 hour. The suspension was then centrifuged at 375 g for 5 minutes, then the supernatant was removed and centrifuged again at 1000 g for 5 minutes to make sure all the cells were removed. Two samples were absorbed as follows:

	Serum Vol.: Packed Cell Vol.	Dilution after absorbtion
Preparation I	1 : 1.75	1/10
Preparation II	1 : 4	1/2.5

Absorbtion with thymocytes. B10.D2 thymuses were broken up by teasing

with needles in HBSS and then pressing them through a 200 gauge stainless steel mesh, using a rubber tipped syringe plunger. The cells were washed 5 times, centrifuged at 375 g for 5 minutes in a graduated tube, and the packed cell volume was noted. The cells were resuspended in the diluted antiserum sample and rotated at 4°C for 1 hour. The absorbed antiserum was separated by centrifuging at 375 g for 5 minutes, followed by 5 minutes at 1000 g. The following three absorbtions were made.

	Serum Vol.: Packed Cell Vol.	Dilution after absorbtion
Preparation I	1 : 3.5	1/10
Preparation II	1 : 3	1/10
Preparation III	1 : 3	1/10

Absorbtion with red blood cells (RBC's). The method described by Hauptfeld, Hauptfeld and Klein (31) was used. B10.D2 mice were bled into Alsever's solution, using approximately two volumes of Alsever's solution to one volume of blood. The red blood cells were then washed 6 times in HBSS, removing the buffy coat from the surface of the red cells each time. The cells were then divided into several equal aliquots and centrifuged at 1000 g for 5 minutes. The antiserum sample was diluted 10x in HBSS and absorbed with each aliquot in turn, by rotating gently at 4°C for 15 minutes. The cells and serum were separated be centrifuging at 1000 g for 5 minutes. This procedure was carried out on the same antiserum sample on three separate occasions. Between absorbtion the antiserum was frozen while a small sample was tested in a cytotoxicity test, the results of which are shown in figure 10. The details of the absorbtions are as follows:

1st absorbtion:- 5 aliquots of cells, each with an antiserum to to packed cell volume ratio of 1:1

Total of this absorbtion = 1:5

2nd absorbtion:- 3 aliquots of cells, each with an antiserum to packed cell volume ratio of 1:1

Total of this absorbtion = 1:3

Total of all absorbtions = 1:8

3rd absorbtion:- 3 aliquots of cells, each with an antiserum to packed cell volume ratio of 1:1.33

Total of this absorbtion = 1:4

Total of all absorbtions = 1:12

Absorbtion with B10.D2 sarcoma cells. The tumour cells were allowed to grow until they covered the bottom of the flasks. The same sample of antiserum was absorbed on three separate occasions with tumour cells and its cytotoxicity was measured after each absorbtion. The results of these cytotoxicity tests are shown in figure 9.

1st absorbtion:- The medium was removed from 5 flasks and the adhering cells were washed 6 times with HBSS. One ml of antiserum was diluted 10x with HBSS and put into each of the 5 flasks in turn for 10 minutes with rocking. After removing the antiserum from the fifth flask it was centrifuged at 1000 g for 5 minutes to remove any tumour cells and stored frozen.

2nd absorbtion:- Two 4.5 ml volumes of the previously absorbed diluted antiserum were each passed through twelve flasks of cells as described for the first absorbtion, centrifuged at 1000 g for 5 minutes and stored frozen.

3rd absorbtion:- For this absorbtion the cells were removed from 29 flasks by replacing the culture medium in each flask with Madin and Darby's solution and rocking. The cells were washed 5 times in HBSS and centrifuged at 375 g for 5 minutes in a graduated tube. The 1.45 ml of packed cells were resuspended in 9 ml of the previously absorbed diluted antiserum and rotated at 4°C for 30 minutes. The cells were removed by centrifuging at 375 g for 5 minutes and then at 1000 g for 5 minutes.

RESULTS

Preparation of rabbit complement

In order to measure the cytotoxicity of the mouse alloantisera, it was necessary to obtain a preparation of complement effective in lysing antibody treated murine lymph node cells, but not cytotoxic for the target cells if used alone. Rabbit serum can be a good source of complement for use with mouse alloantisera (55), but the sera from individual rabbits may vary both in their complementary activity and in their content of naturally ocurring antibody cytotoxic to mouse cells. To select a proper donor of complement, serum samples were taken from the ears of four New Zealand White rabbits and their cytotoxicities against ^{51}Cr labelled B10.A lymph node cells were measured, using the rabbit serum as the source of both antiserum and complement. It has been shown that agarose absorbtion can remove much of the cytotoxic antibody against mouse cells from both guinea pig (54) and rabbit (55) sera. Therefore, a serum sample from each rabbit was absorbed with agarose, following the method of Cohen and Schlesinger (54), and the cyototoxicities of the absorbed sera were measured together with those of the unabsorbed sera. The absorbtions were carried out at 4°C in the presence of EDTA, which chelates the divalent cations, Ca^{++} and Mg^{++}, and thus prevents the activation of the complement system by the antigen-antibody complexes formed during absorbtion. After the agarose had been centrifuged out, the cation content was restored by adding $CaCl_2$, so that the complement system could be activated when the serum was used in the cytotoxicity test.

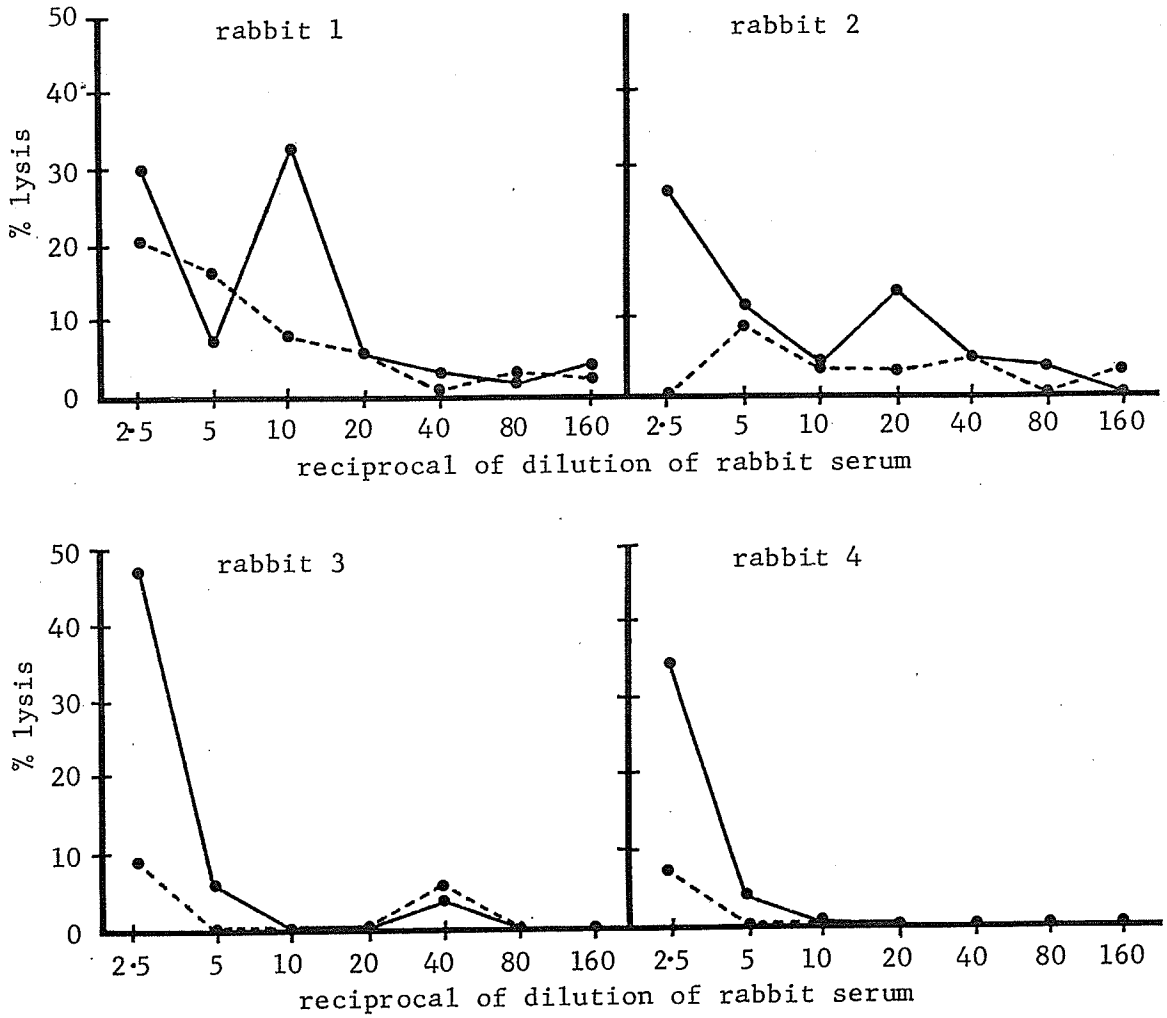
The results of the cytotoxicity tests with the absorbed and unabsorbed samples of rabbit serum are shown in Fig.1. The sera of rabbits 3 and 4 had practically no cytotoxicity when diluted to 1/5 and their

cytotoxic activity was easily absorbed out with agarose. Having shown that it was possible to obtain from rabbits 3 and 4 sera without significant cytotoxic activity, it was necessary to examine these sera for their complement activity. This was done by additing serial dilutions of the rabbit sera to ^{51}Cr labelled B10.A cells treated with C57-anti-(C57 x A)F_1 serum in a concentration of 1/20 which had previously been shown to induce 100% lysis. The results are shown in Fig. 2. Maximal cytotoxicity was obtained, although the values did not reach 100% lysis. While the absorbed samples did not show any cytotoxicity at a dilution of 1:5, their complement activities at this concentration were good.

Rabbits 3 and 4 were bled out by heart puncture under nembutal anaesthesia, and all the serum from rabbit 3 and a sample of serum from rabbit 4 were absorbed. Two step cytotoxicity tests were carried out with the use of these three sera as the source of complement. 10^5 ^{51}Cr labelled B10.A lymph node cells in 0.1 ml of HBSS were added to 0.1 ml of a 1/20 dilution of C57 anti-B6AF_1 serum. The plates were left at room temperature for 15 minutes. To eliminate any nonspecific anti-complementary activity of the mouse alloantiserum, most of the alloantiserum was removed by taking 0.16 ml from each well after centrifuging. 0.2 ml of each of a series of rabbit serum dilutions in HBSS were added. The plates were incubated at 37°C for 40 minutes, centrifuged, and 0.1 ml was removed for counting. The results shown in Fig. 3 indicate that absorbed serum from rabbit 3 produces maximal lysis down to a dilution of 1/40, with a small amount of cytotoxicity due to the rabbit serum itself, whereas the complement activity of even unabsorbed serum from rabbit 4 was lower.

Figure 1

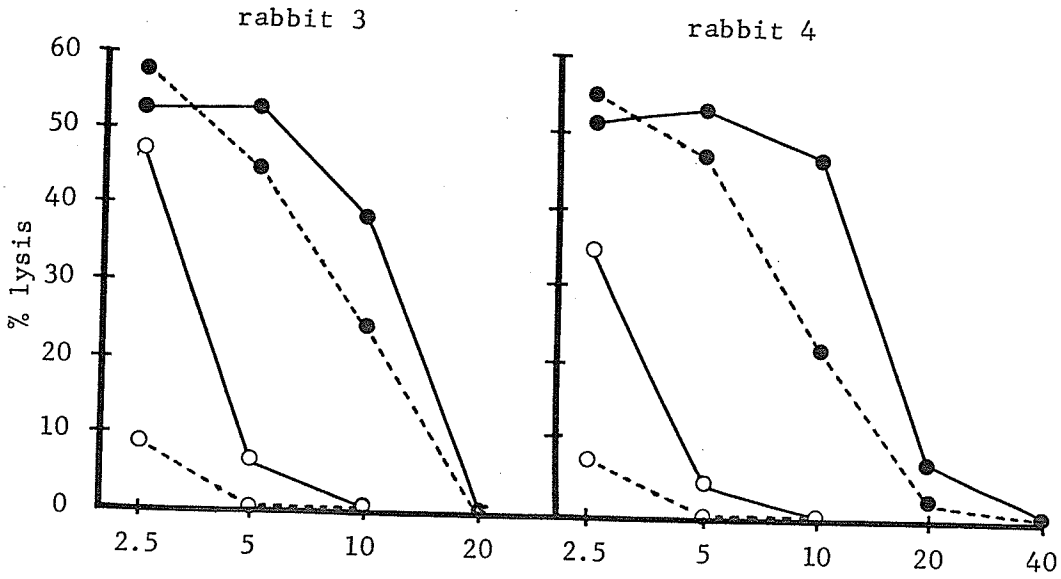
Cytotoxicity of rabbit serum for ^{51}Cr labelled B10.A lymphocytes



Each well contained 0.1 ml of HBSS containing 2% NMS, plus 10^5 ^{51}Cr labelled B10.A lymphocytes in 0.1 ml of HBSS containing 2% NMS, plus 0.05 ml of each dilution of rabbit serum.

Figure 2

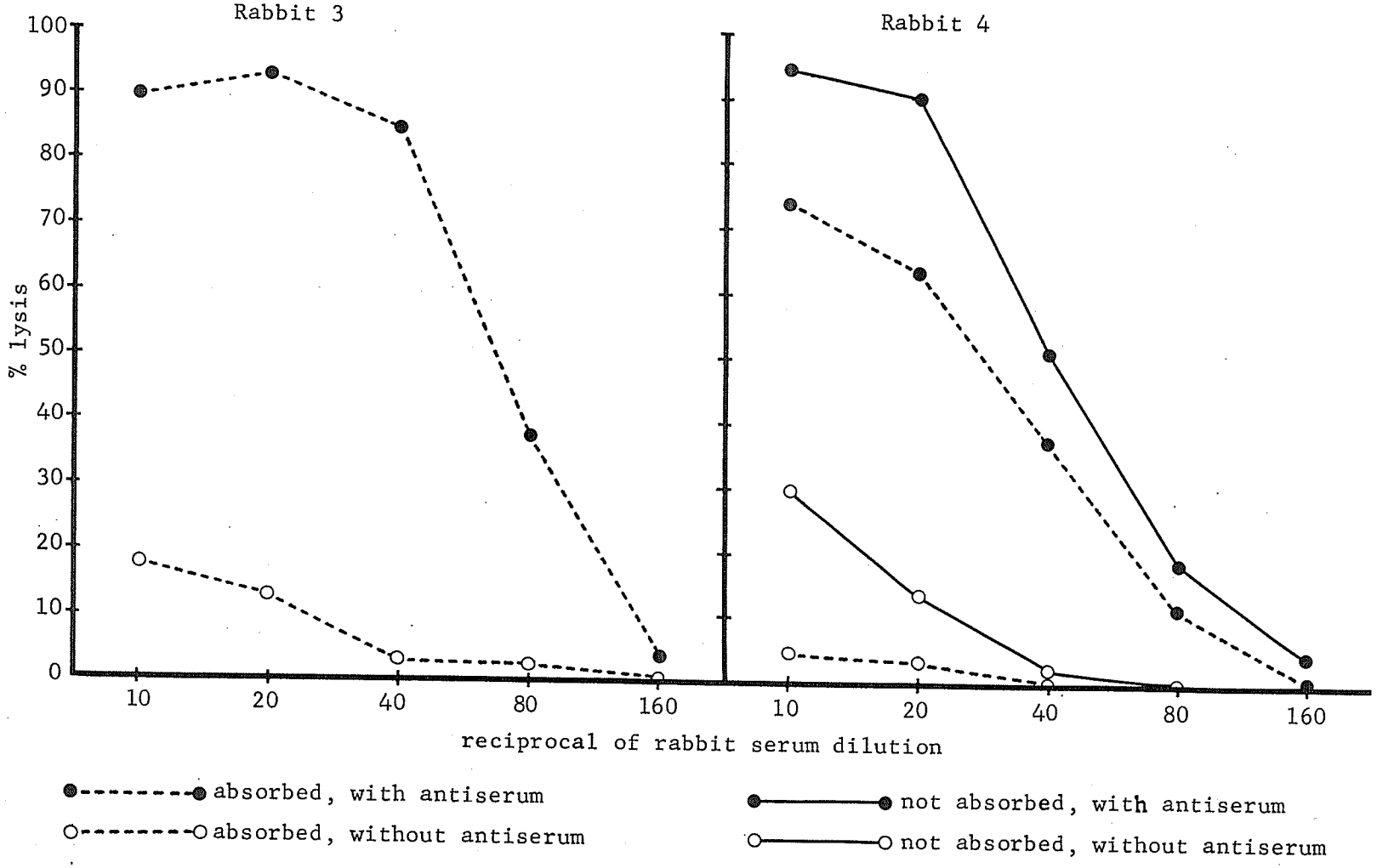
Titration of complement activity in rabbit serum samples



Each well contained 0.1 ml of a 1/20 dilution of C57 anti-(C57 x A/J)F_1 in HBSS or 0.1 ml of HBSS containing 2% NMS, plus 10^5 ^{51}Cr labelled B10.A lymphocytes in 0.1 ml of HBSS containing 2% NMS, plus 0.05 ml of each rabbit serum dilution in HBSS. A one step cytotoxicity test was used.

Figure 3

Titration of complement activity and cytotoxicity of rabbit sera, using a two step cytotoxicity test



Since to obtain reproducible cytotoxicity it was considered advisable to provide complement in excess of that required for 100% cytotoxicity, 0.2 ml of a 1/20 dilution of absorbed serum from rabbit 3 was used as a source of complement in all subsequent cytotoxicity tests. The absorbed serum still retained some cytotoxic activity, therefore, a control with rabbit complement only was performed in all assays. The number of counts produced by this control was subtracted from all values to calculate the percentage of lysis of the target cells.

Titration of the alloantisera.

The different alloantisera to be used in the experiments on GVH reactions were titrated in the two step cytotoxicity test, using ^{51}Cr labelled, donor strain lymph node cells as target cells. The cytotoxic activity of the antisera was considered to be an adequate measure of the cytotoxic anti-H-2 antibodies, since H-2 antigens are well expressed on lymph node cells. The results of these cytotoxic tests are reported in Figs. 4-8. As an index of antibody concentration, the antiserum dilution capable of inducing 50% lysis of the target cells was determined graphically. These indices are reported in Table 1:

TABLE I

Antiserum	Reciprocal of highest antiserum dilution which gives at least 50% lysis
B10.D2 anti-B10.BR (1)	160
B10.D2 anti-B10.BR (2)	640
B10.BR anti-B10.D2 (1)	80
B10.BR anti-B10.D2 (2)	320 – 640
A/J anti-C57BL/6	160
A/J anti-$B6AF_1$	80
C57BL/6 anti-A/J	320
C57BL/6 anti-$B6AF_1$	160
B10.BR anti-P815	80

Figure 4

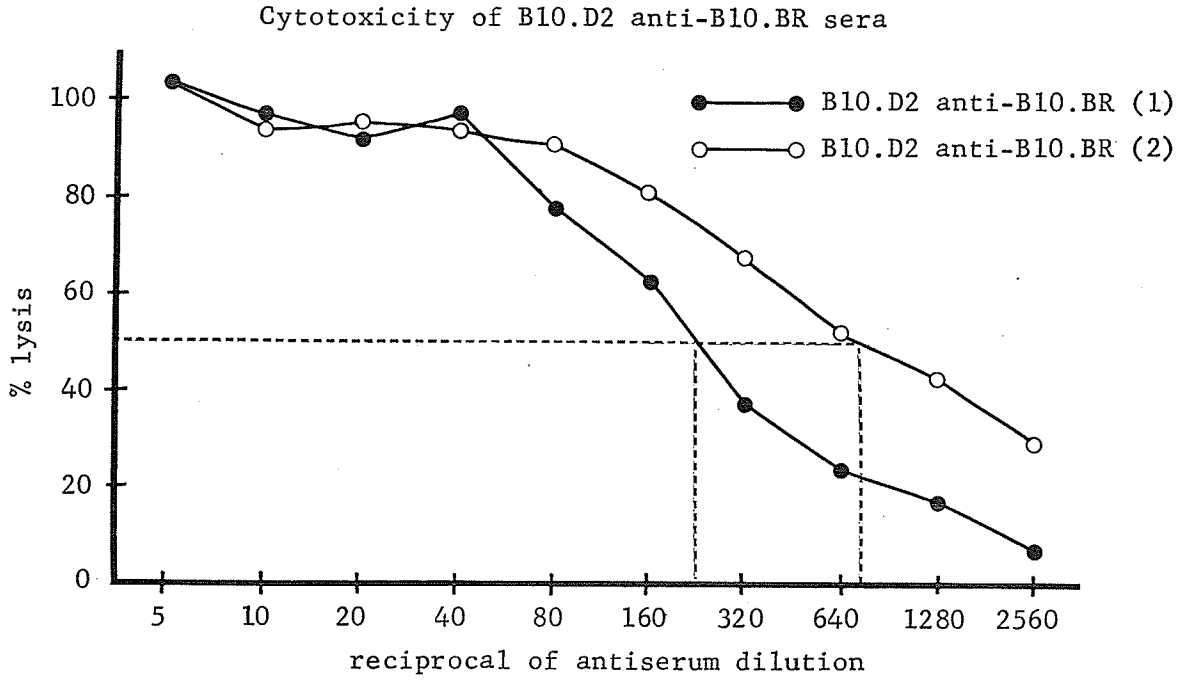


Figure 5

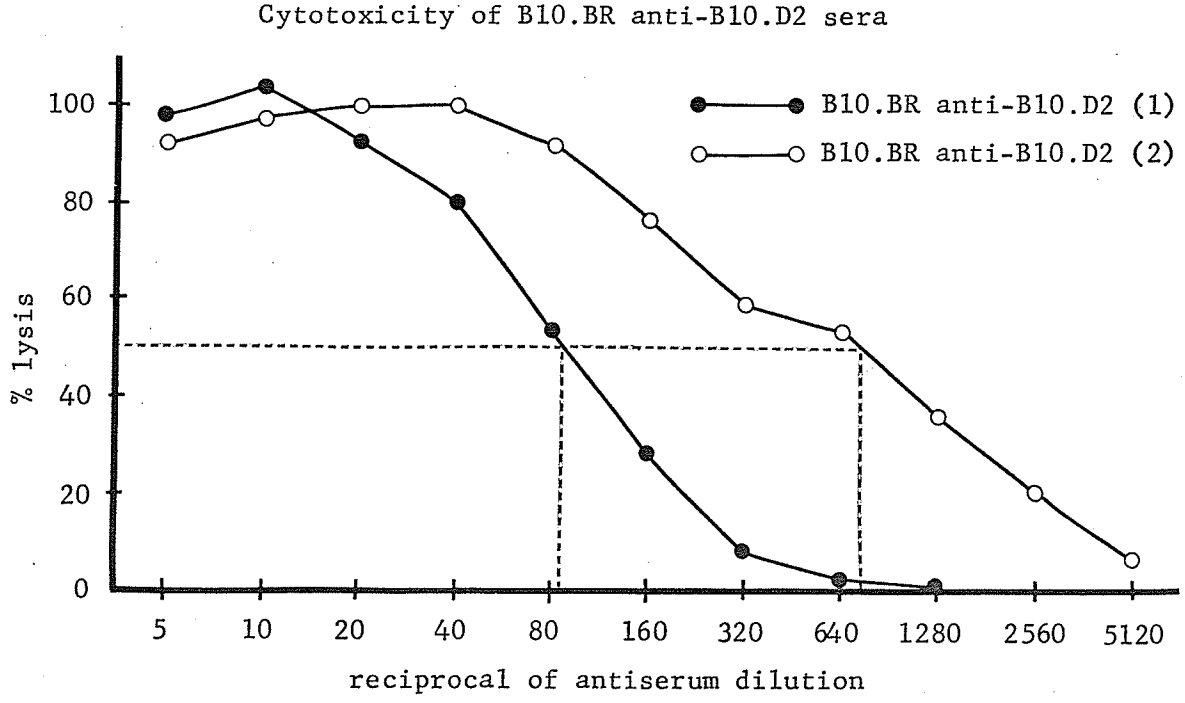


Figure 6

Cytotoxicity of A/J anti-C57BL/6 and A/J anti-$B6AF_1$ sera.

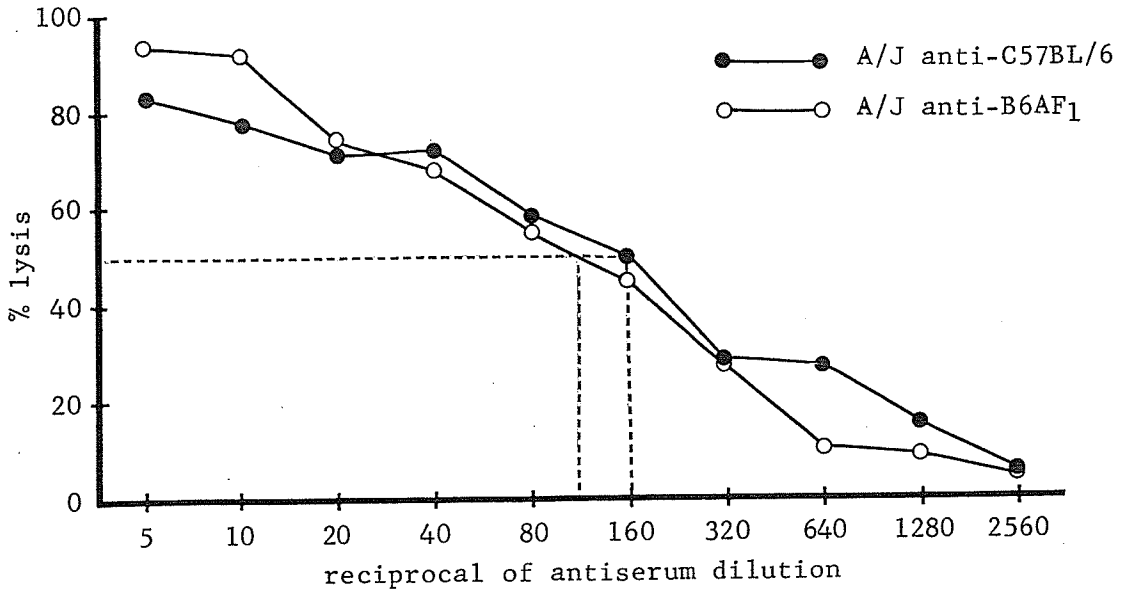


Figure 7

Cytotoxicity of C57BL/6 anti-A/J and C57BL/6 anti-$B6AF_1$ sera.

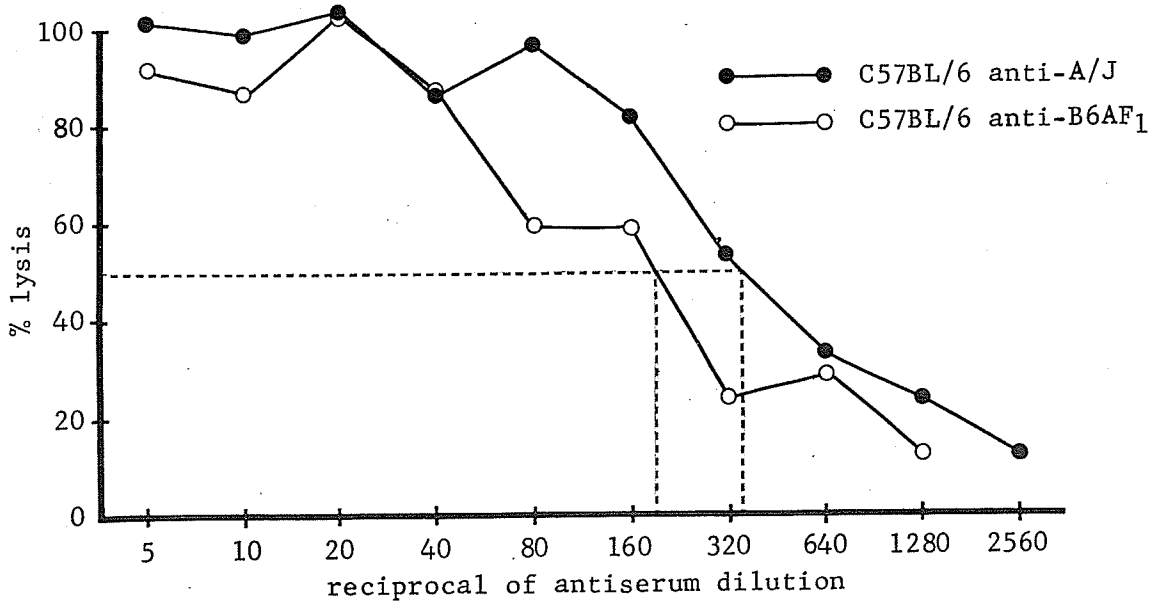
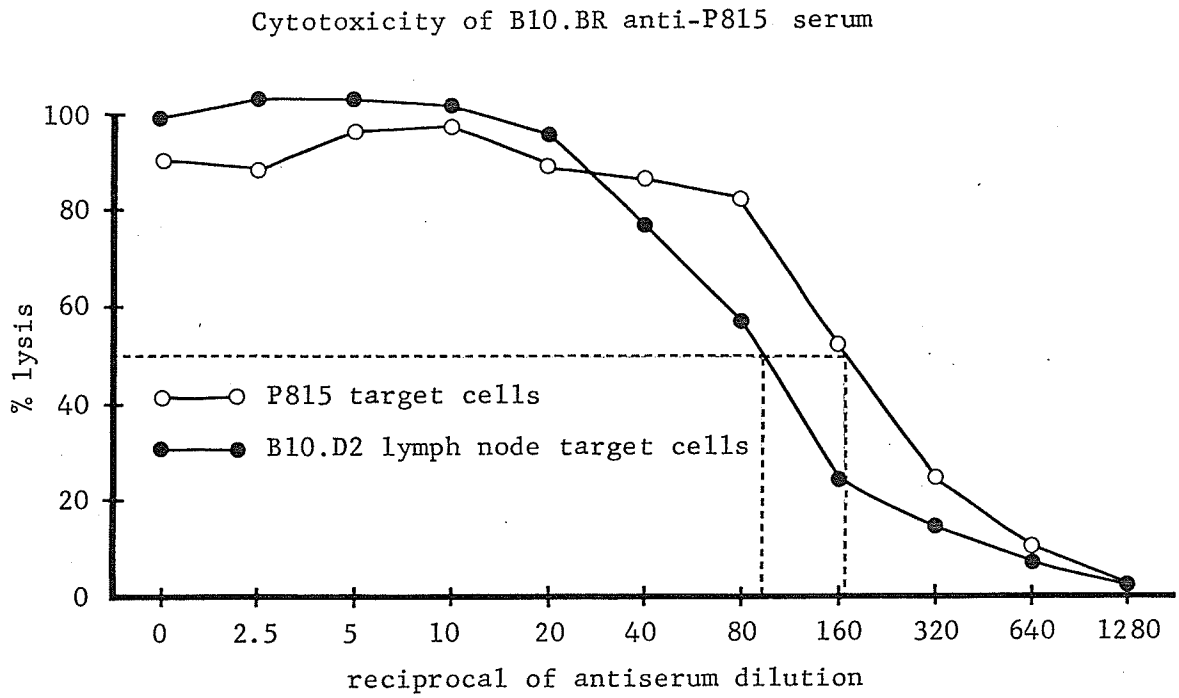


Figure 8


Cytotoxicity of B10.BR anti-P815 serum
100
80
60
40
20
0
% lysis
P815 target cells
B10.D2 lymph node target cells
0
2.5
5
10
20
40
80
160
320
640
1280
reciprocal of antiserum dilution

Absorbtion of the B10.BR anti-B10.D2 (2) serum.

Antisera produced against lymphocytes are known to contain antibodies to both Ia antigens and antigens determined by the D and K loci. To study the effect on the GVH reaction of anti-Ia antibodies, it was necessary to remove the latter type of antibodies, using for absorbtion, cells which carry H-2^d antigens other than Ia antigens. For this purpose a B10.D2 methylcholanthrene induced sarcoma was chosen, because it arose from connective tissue cells from which Ia antigens are believed to be absent, (personal communication by D.C. Shreffler to Dr E. Sabbadini). The tumour cells were cultured for several weeks in order to obtain a sufficiently large supply of tumour cells not contaminated by lymphocytes or any other type of cell which might carry Ia antigens. These tumour cells adhere to the culture flask as a monolayer, therefore their numbers are limited by the area of the flask. The first two absorbtions were carried out by rocking the antiserum over the monolayer of cells in the flask, in order to avoid the use of trypsin which may remove surface antigens or damage the cells. However, this method became impractical when it became evident that many flasks of cells were required. It was found that the cells could be removed from the flasks using Madin and Darby's solution alone. Therefore, the final absorbtion was carried out in a test tube with a suspension of tumour cells.

The results of the absorbtions with tumour cells are shown in figure 9. After using 58 flasks of tumour cells, the titre was 1/40 and a 1/10 dilution of the antiserum was able to kill 72% of the target cells. The end point had not been reached, therefore, tumour cell absorbtion was abandoned in favour of red blood cell absorbtion (31) because of the time taken to grow the many flasks of cells required.

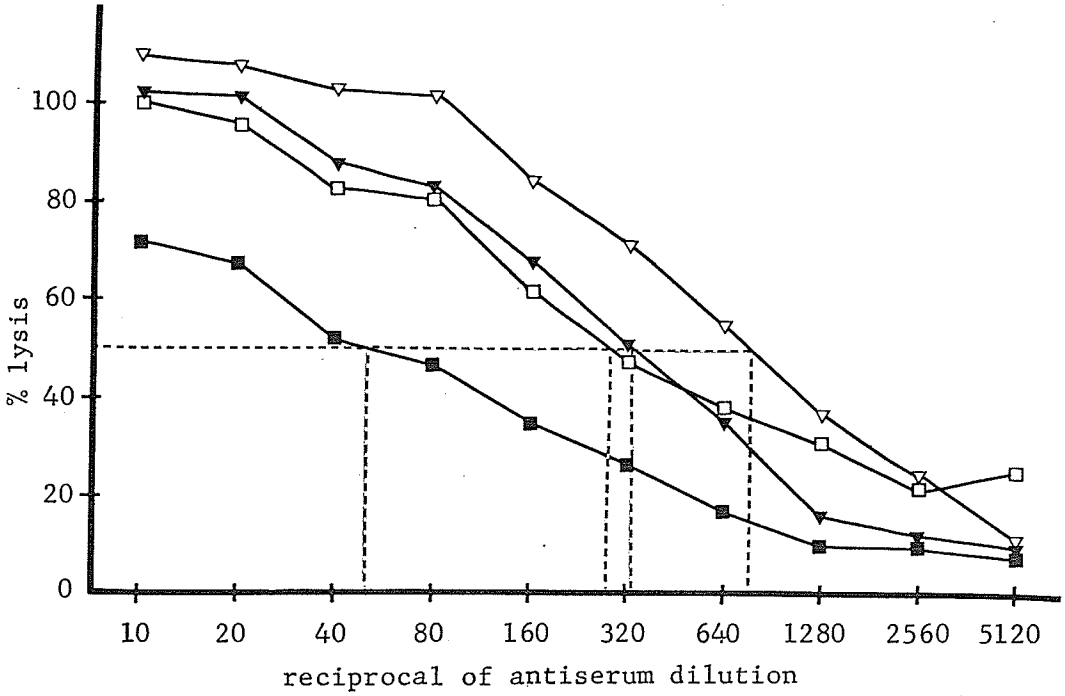
B10.D2 red blood cells were used to absorb the antiserum because Ia antigens are believed to be absent from them (25), and anti-Ia sera have previously been prepared by this method (31). RBC's are also easily available in large numbers. The results are shown in Fig. 10. The observation of two consecutive absorbtions with no decrease in the titre was taken as evidence that the absorption was complete. The titer of the antiserum absorbed with RBC's was 1/40 and a 1/10 dilution was able to kill about 80% of the lymph node target cells.

Thymocytes were also used to absorb the antiserum. Early evidence indicated that Ia antigens are either absent from (29,39) or present in very low concentration (53,64) on thymocytes. There is now more evidence that at least a subpopulation of thymocytes do carry Ia antigens, many of which are probably common to both B and T cells (65,49,53), while other may be specific for T cells (29,65,39). Because of the early failures to detect Ia antigens on thymocytes, Ia antigens on T cells are considered to be in such low concentrations compared to D or K antigens, that absorption with thymocytes has previously been used to produce an anti-Ia serum from an anti-H-2 serum (31). When absorbed with thymocytes, using an antiserum to packed cell volume ratio of 1:3, the antiserum had no cytotoxic activity at a dilution of 1/10 in a cytotoxic test with lymph node target cells (Fig. 11). This observation indicates that probably anti-Ia antibodies had been removed during absorption and is in agreement with the presence of Ia antigens on thymocytes.

As a control, the antiserum was absorbed with B10.D2 lymph node and spleen cells, against which the antiserum was produced. The first

Figure 9

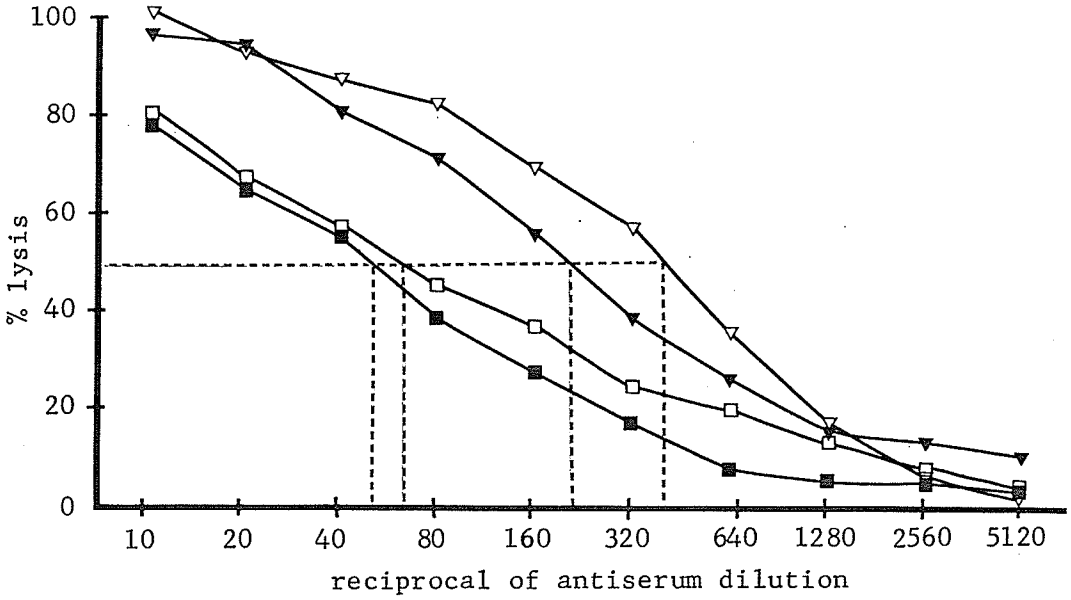
Absorbtion of 1 ml of B10.BR anti-B10.D2 (2) serum with B10.D2 tumour cells



	Total number of flasks	Estimated packed cell volume	Titre
▽——▽	Not absorbed		1/320 to 1/640
▼——▼	5	0.25 ml	1/320
□——□	29	1.45 ml	1/160 to 1/320
■——■	58	2.90 ml	1/40

Figure 10

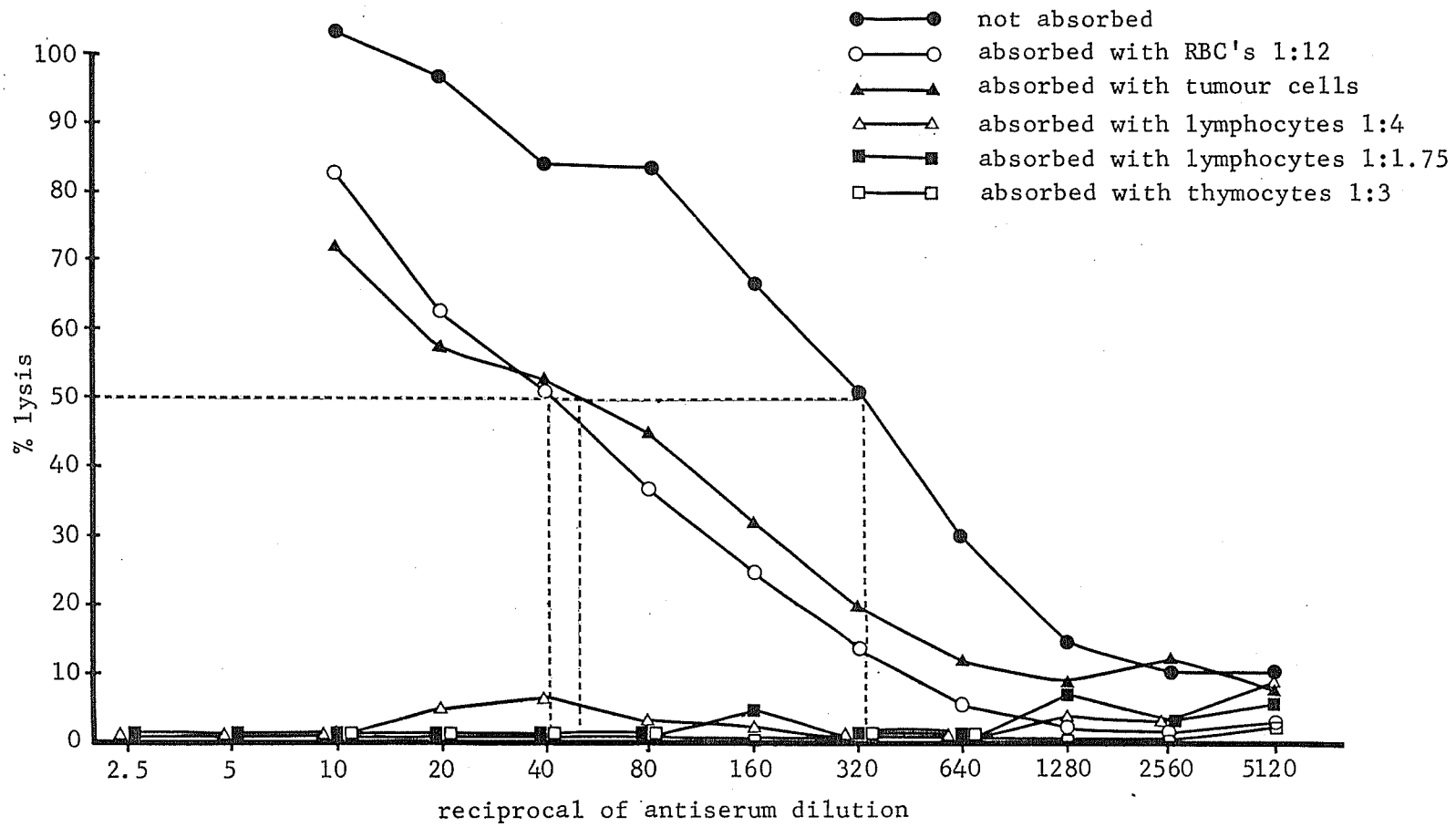
Absorbtion of B10.BR anti-B10.D2 (2) with B10.D2 red blood cells



	Antiserum vol : packed cell vol.	Titre
▽——▽	Not absorbed	1/320 to 1/640
▼——▼	1 : 5	1/160
□——□	1 : 8	1/40
■——■	1 : 12	1/40

Figure 11

Cytotoxicity of absorbed B10.BR anti-B10.D2 (2) samples



sample was absorbed at a dilution of 1/10, using an antiserum to packed cell volume of 1:1.75. No cytotoxicity was detectable after this absorption, however, some augment activity on the GVH reaction remained in the antiserum. It could not be known whether any cytotoxicity would have been detectable at a higher concentration of antiserum because it was diluted at the time of absorption. A second sample was absorbed at a dilution of 1/2.5, using an antiserum to packed cell volume ratio of 1/4. The second sample was only diluted to 1/2.5 so that any residual cytotoxicity would be more likely to be detected. The cytotoxicity tests on both these samples are shown in figure 11. Figure 11 shows the result of a cytotoxicity test carried out on the unabsorbed and all absorbed samples on the same occasion.

Experiments on the graft-versus-host reaction.

When the preparation of several antisera was completed, it became possible to study their effects on GVH reactions, For this purpose it was necessary to establish the conditions for a reproducible measurement of GVH reactions. It had been demonstrated by several authors that there is a linear relationship between the logarithm of the spleen cell dose and the spleen index, up to a maximum spleen index, after which, increasing the spleen cell dose does not increase the spleen index (17). Cell dose response curves were studied for all donor recipient combinations, so that a dose could be chosen which lay in the linear portion of the graph and gave a significant spleen enlargement. The spleen cell doses chosen for use in the experiments with antisera are marked on the cell dose response curves (figures 12 to 16). When (B10.D2 x B10.BR)F_1 mice were used as recipients, spleen cells from both parental strains

produced linear dose response curves over a wide range of cell doses. The activity of B10.BR cells appeared to be somewhat higher and produced a steeper dose response curve than B10.D2 cells. Moreover, when adult F_1 hybrids were used, higher cell doses were required than for newborn animals, as would be expected.

When injecting C57BL/6 cells into 10 week old $B6AF_1$ mice (Fig.15), the maximum spleen index was reached at a very low spleen cell dose, with only one point lying below the maximum spleen index. It was not possible to obtain a staight line for the ascending part of the curve, therefore, the experiment was repeated using lower numbers of spleen cells. Both the donors and recipients used in the second experiment were ten weeks older than those used in the first experiment, and the spleen indices were much higher than those produced by the same number of cells when using the younger mice. This indicated that the ages of the mice used may be an important factor in determining the degree of spleen enlargement in GVH reactions. Several other experiments with $B6AF_1$ recipients of different ages were performed. All the C57BL/6 donor mice were aged between 8.5 and 10 weeks. $B6AF_1$ mice aged 6 weeks or more were obtained from Jackson Laboratories and were all female. $B6AF_1$ mice from newborn to 4 weeks of age were bred in the Animal Care Centre of this University and were of both sexes. A cell dose response curve was produced for each recipient age group and the spleen indices were plotted against cell dose per gram of body weight at the time of injection, since mice of different ages had very different weights (fig,17). The spleen indices in newborn mice were larger than those of older mice when the cell dose was small, but newborn mice could not develop large spleen indices even when large parental spleen

Figure 12

Cell dose response curve - B10.BR adult spleen cells injected into newborn (B10.D2 x B10.BR)F_1 mice.

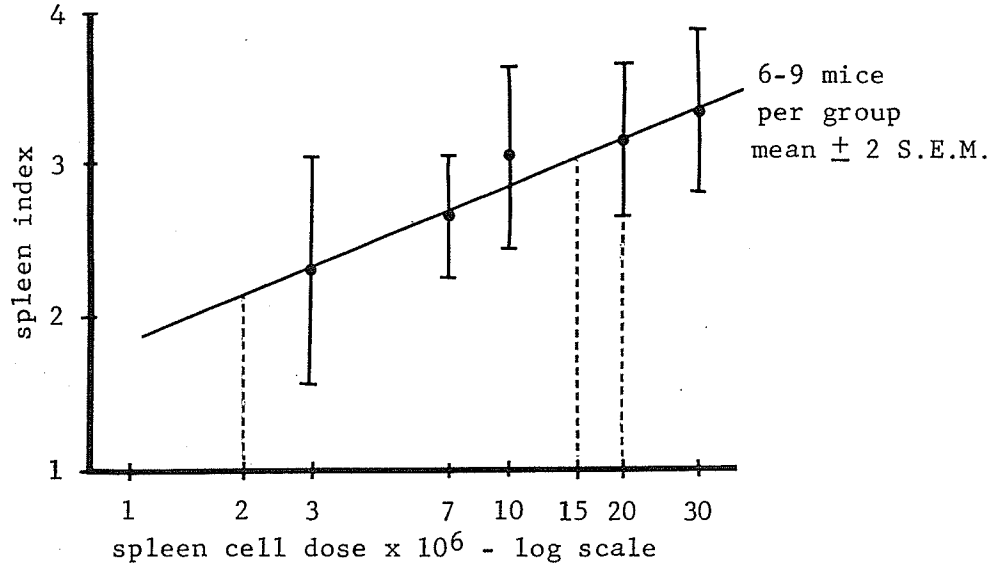


Figure 13

Cell dose response curve - B10.D2 adult spleen cells injected into newborn (B10.D2 x B10.BR)F_1 mice.

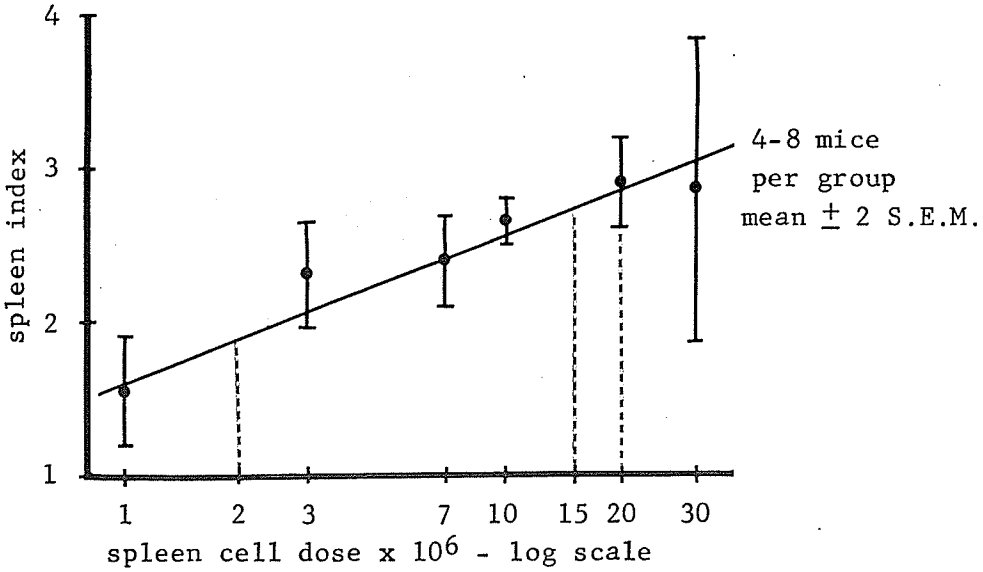
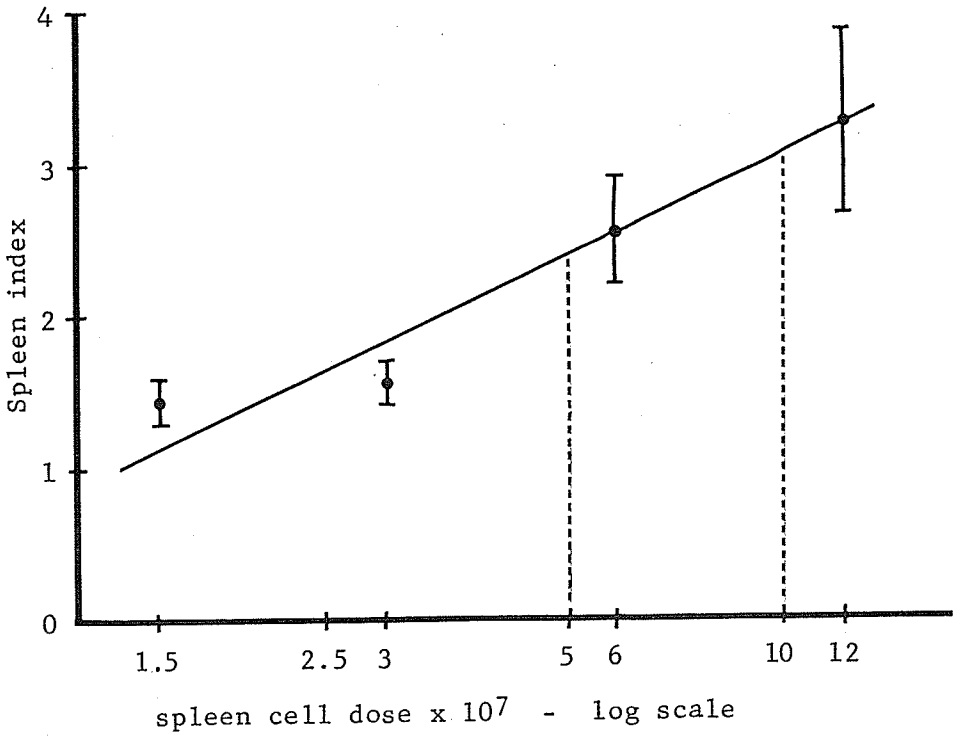


Figure 14

Cell dose response curve - B10.BR adult spleen cells injected into (B10.D2 x B10.BR)F_1 adult mice



Each group contains 5 mice.

Fig.15

Cell dose response curves - C57BL/6 spleen cells injected into $B6AF_1$ mice.

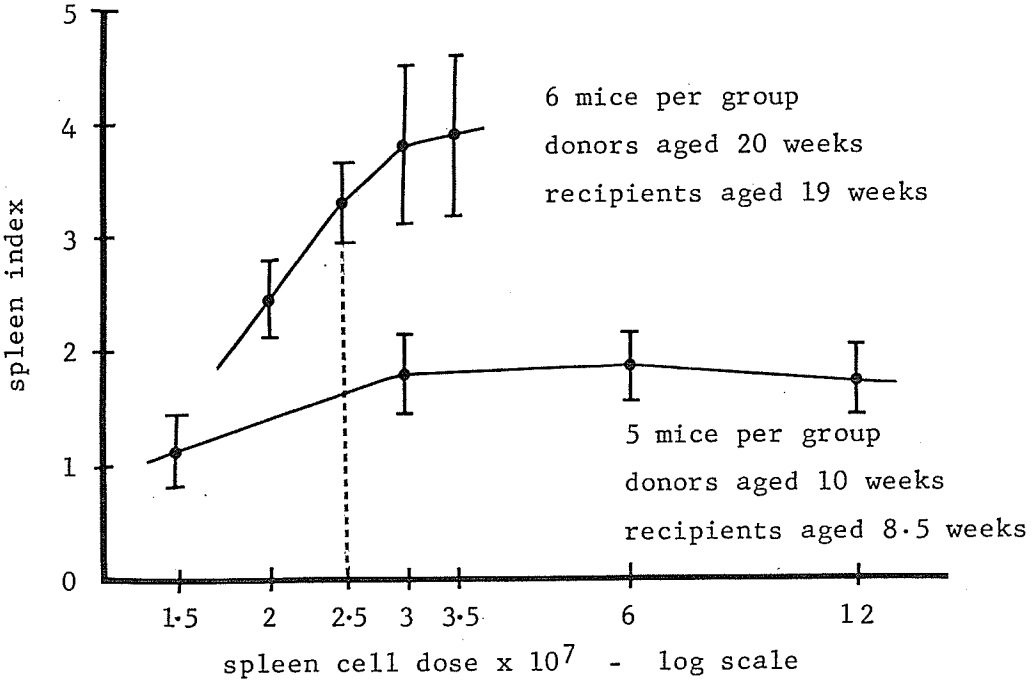


Fig.16

Cell dose response curve - A/J spleen cells injected into $B6AF_1$ adult mice.

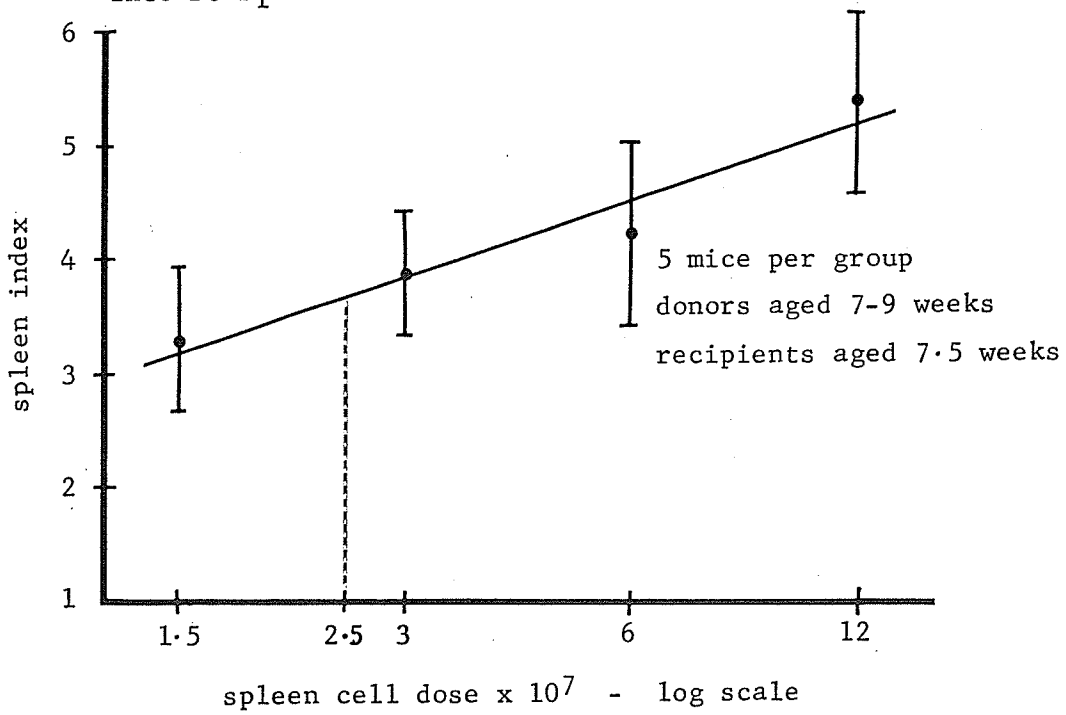
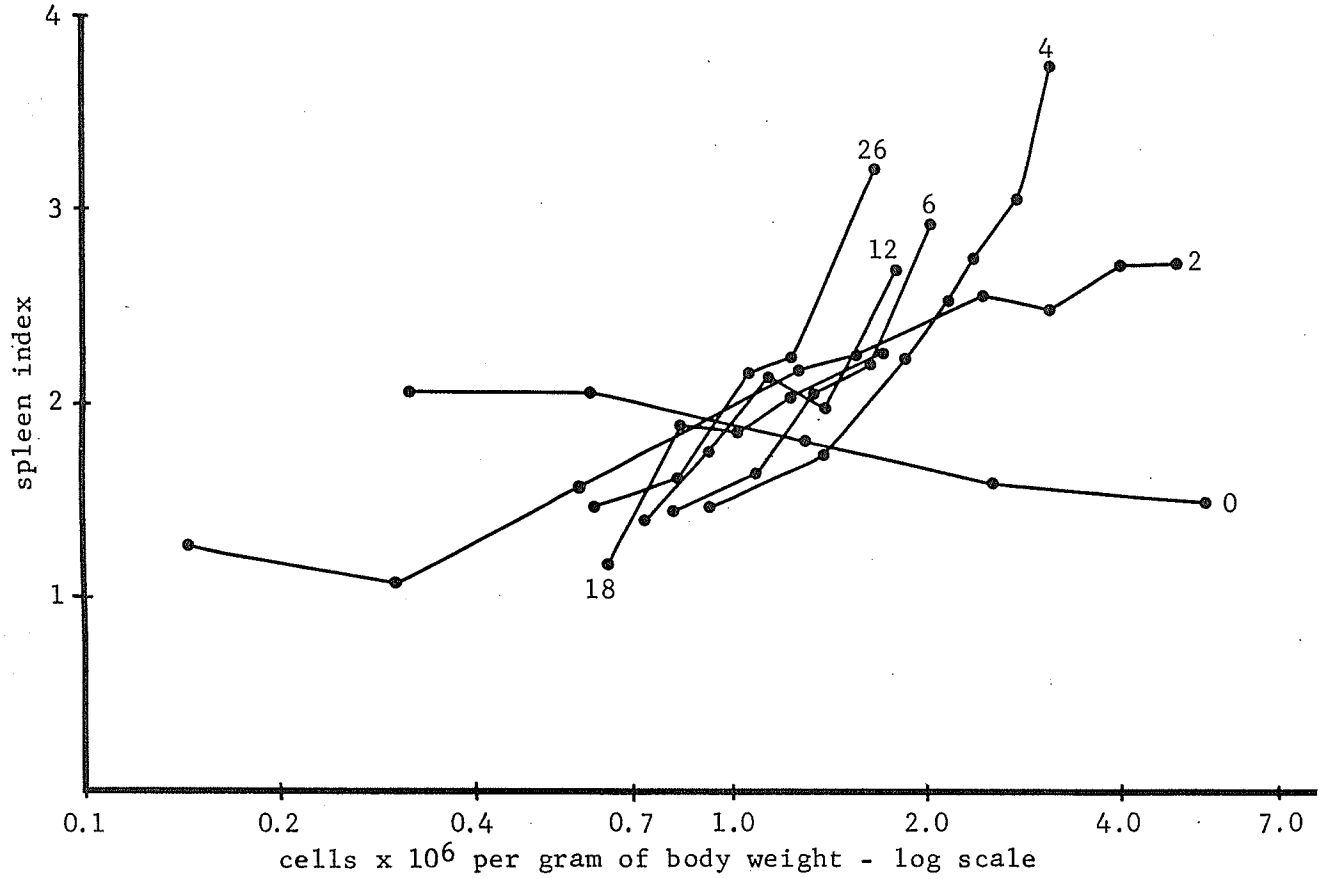


Figure 17

Influence of recipient age on the spleen index in the C57BL/6 donor to $B6AF_1$ recipient strain combination



cell doses were used. In fact the spleen indices in newborn mice were slightly reduced when the cell dose was increased. The reason for this might have been that the spleen cell doses in newborn mice were too high to show the ascending portion of the dose response curve, in which case all the doses used would have been in the plateau region. At the age of 2 weeks the recipients seemed to be able to develop larger spleen indices. From 4 weeks of age up to 26 weeks, there is only a slight increase in the spleen indices for the same cell dose. Therefore, the age of the recipients cannot account for the markedly different curves shown in figure 15 and the different spleen indices obtained in figure 20(c). The fact that the inclusion of 27 week old donor cells with some 12 week old donor cells produced a much higher spleen index than 13 week old donor cells alone in figure 20(c), suggests that the age of the donors may be important. Another possibility is that both old donor cells and old recipients may be required for the production of high spleen indices. This demonstrates that conditions for experiments with GVH reactions have to be carefully controlled.

There were smaller age differences between the different experiments using $B6AF_1$ recipients with A/J donors. The results were reproducible and higher spleen indices were produced (figure 16).

The effect of antisera on the spleen indices.

The effects of the antisera on the spleen indices are shown in figures 18 to 28. Adult mice received intravenous injections of 0.1 ml of the antisera in several dilutions, followed by 0.3 ml of HBSS containing parental spleen cells. Newborn mice received intraperitoneal injections of 0.05 ml of several dilutions of antiserum, followed by

Fig.18

The effect of B10.D2 anti-B10.BR (1) serum on the spleen index produced by 2 x 10^7 B10.D2 spleen cells in (B10.D2 x B10.BR)F_1 newborn mice.

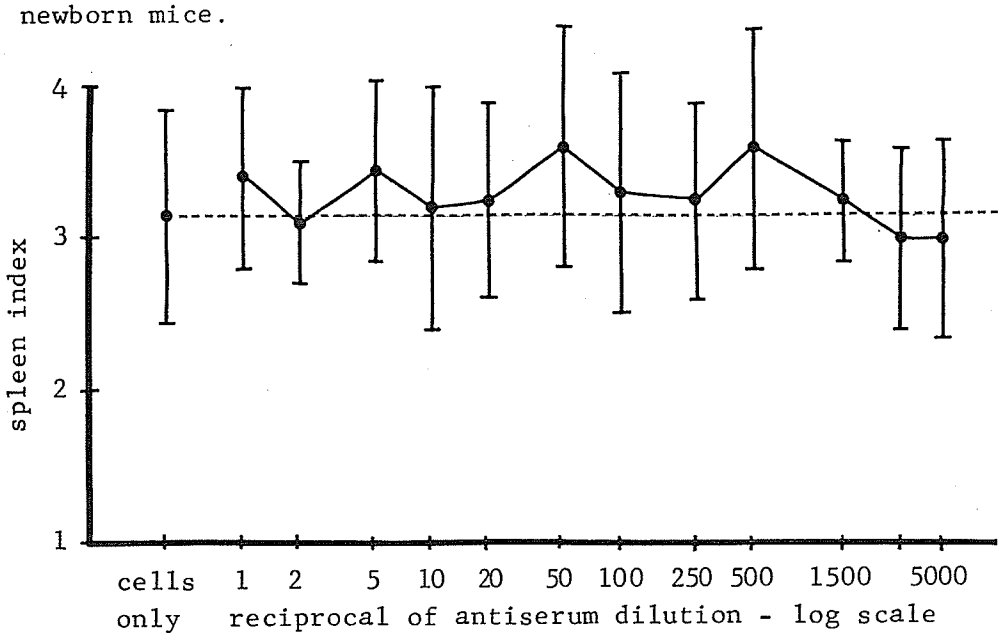


Fig.19

The effect of B10.BR anti-B10.D2 (1) serum on the spleen index produced by 2 x 10^7 B10.BR spleen cells in (B10.D2 x B10.BR)F_1 newborn mice.

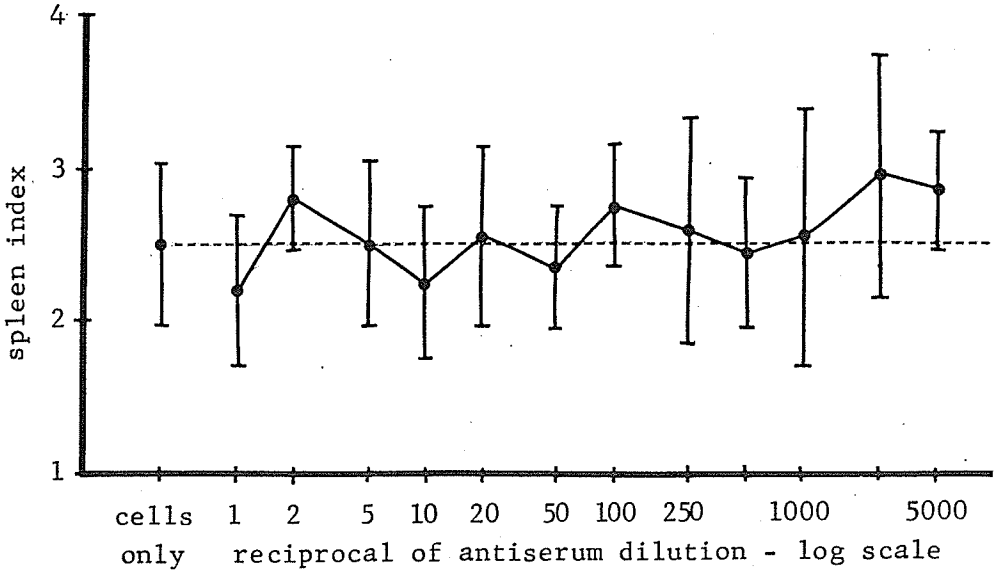


Fig.20 Effect of antisera on the spleen indices produced by C57BL/6 spleen cells in adult $B6AF_1$ mice

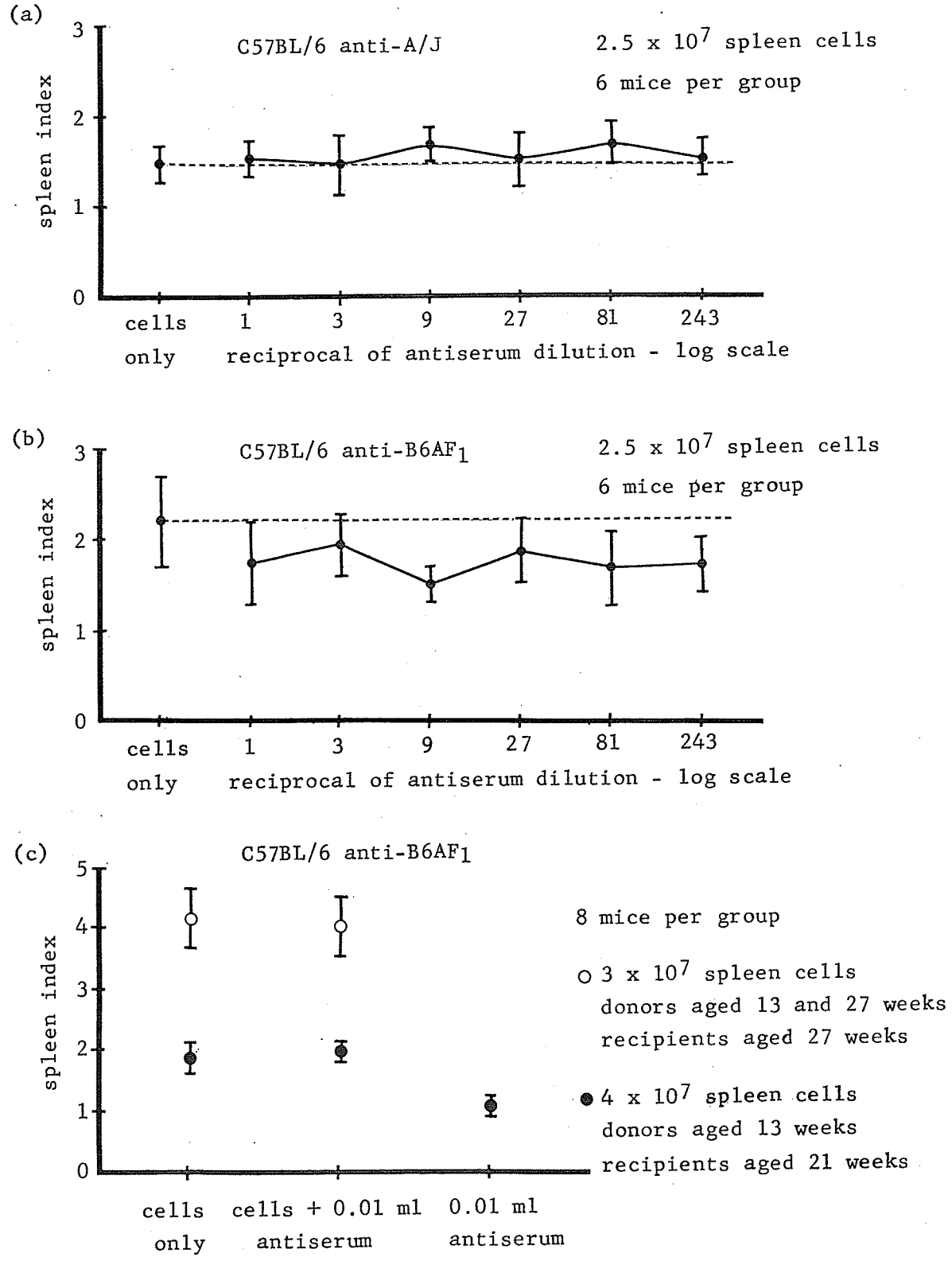


Figure 21

The effect of antisera on the spleen index produced by A/J spleen cells in adult $B6AF_1$ mice.

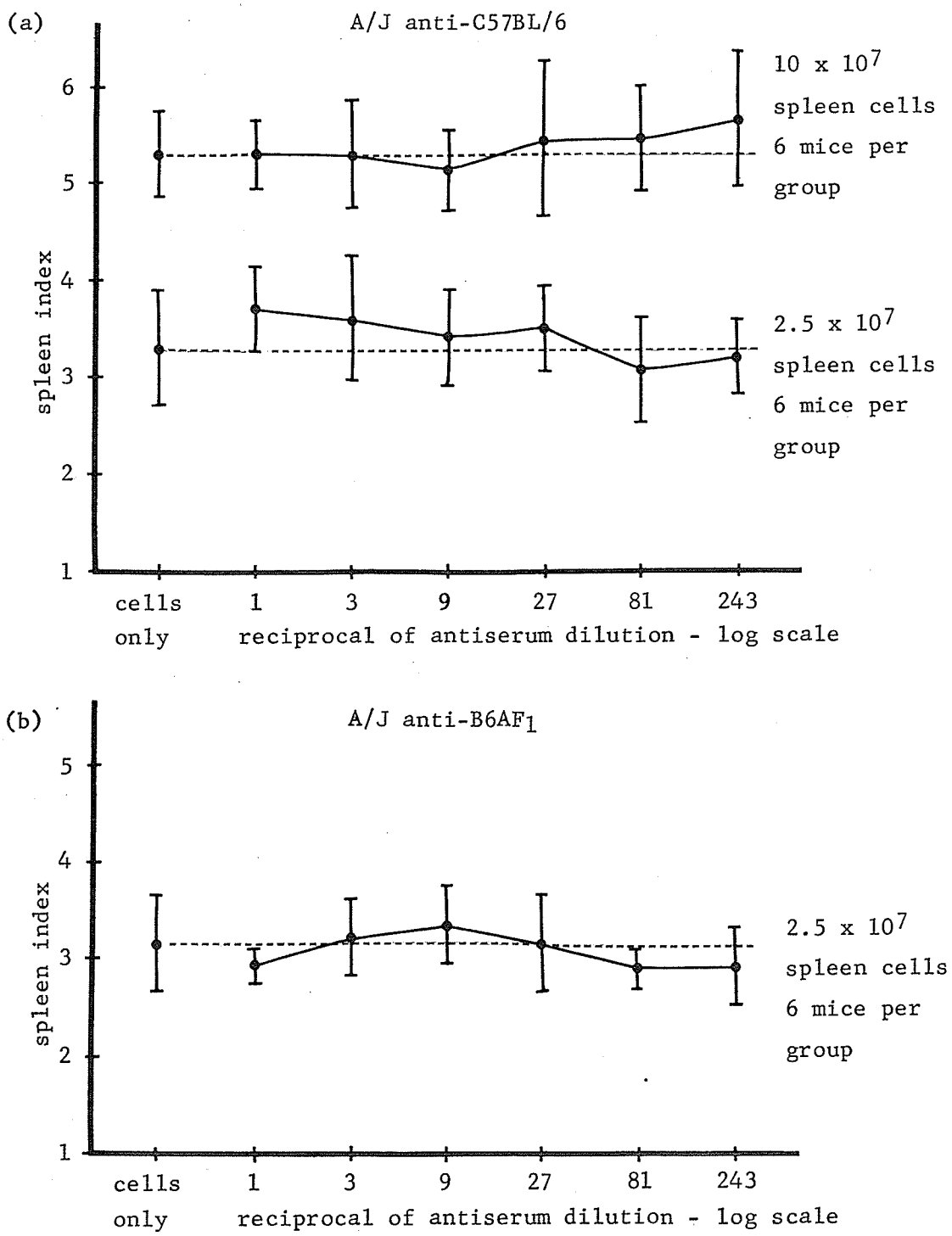


Fig.22

The effect of B10.D2 anti-B10.BR (1) serum on the spleen index produced by 10^8 viable B10.D2 spleen cells in adult (B10.D2 x B10.BR)F_1 mice.

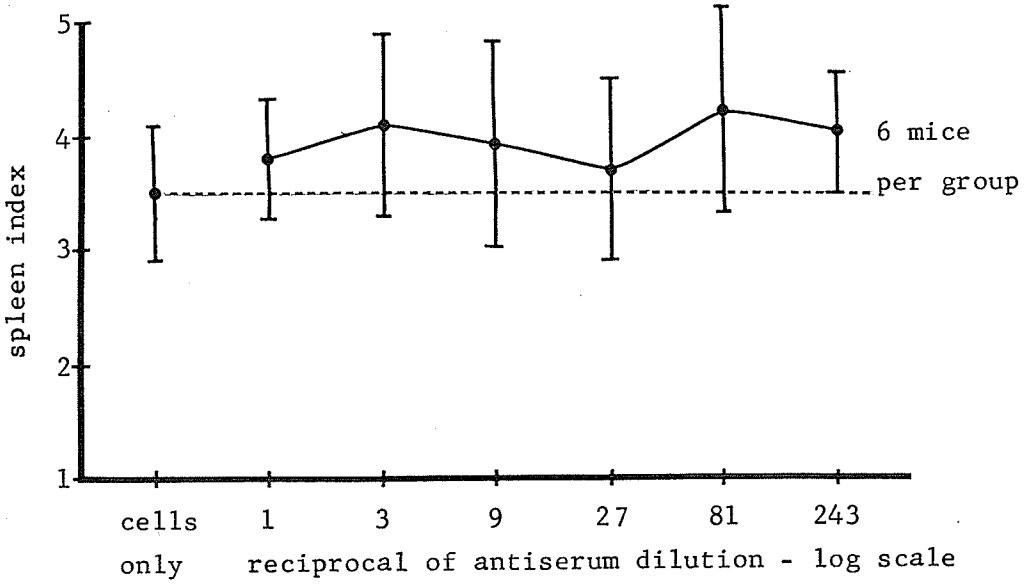


Fig.23

The effect of B10.BR anti-B10.D2 (1) serum on the spleen index produced by 10^8 viable B10.BR spleen cells in adult (B10.D2 x B10.BR)F_1 mice.

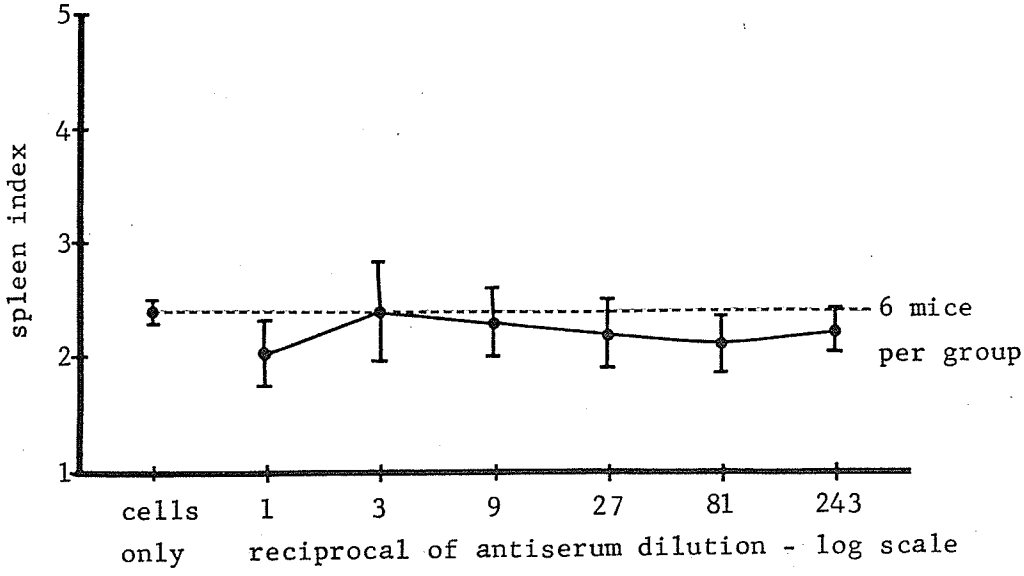


Figure 24

The effect of B10.D2 anti-B10.BR (2) serum on the spleen index produced by 5 x 10^7 viable B10.D2 spleen cells in adult (B10.D2 x B10.BR)F_1 mice.

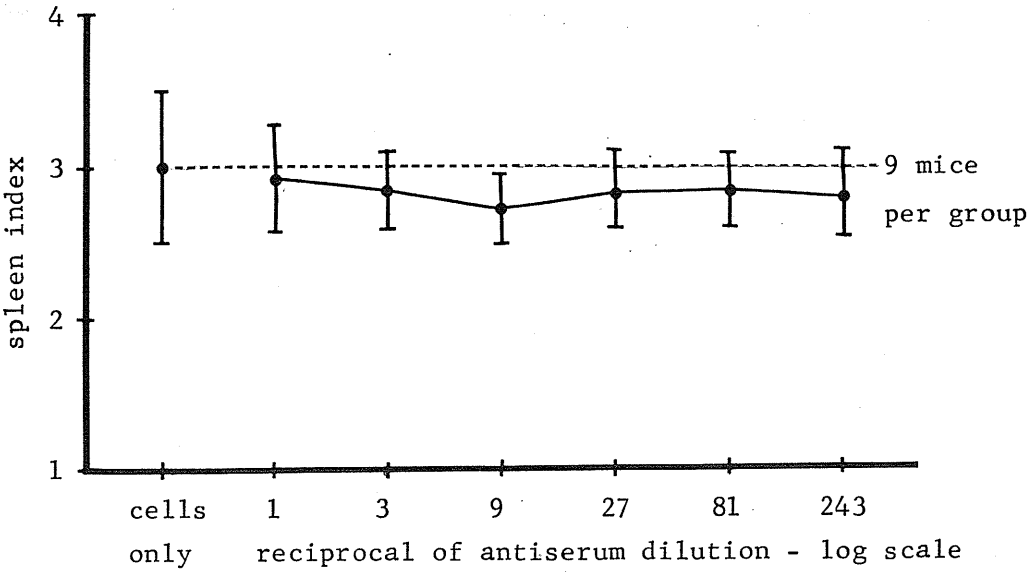


Figure 25

The effect of B10.BR anti-B10.D2 (2) serum on the spleen index produced by 5 x 10^7 viable B10.BR spleen cells in adult (B10.D2 x B10.BR)F_1 mice.

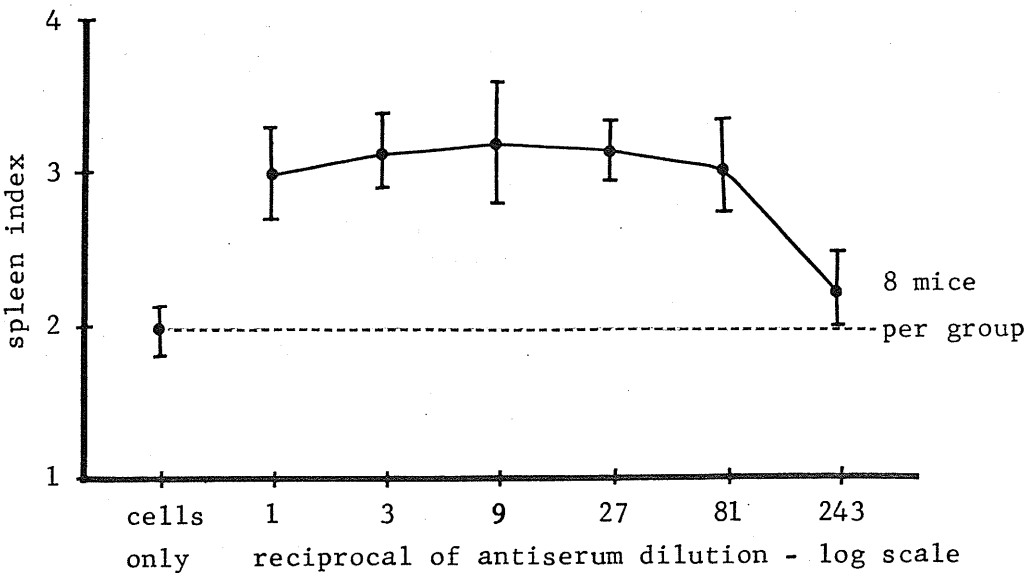


Fig.26

The effect of B10.BR anti-B10.D2 (1) serum on the spleen index produced by 5×10^7 viable B10.BR spleen cells in adult (B10.D2 x B10.BR)F_1 mice.

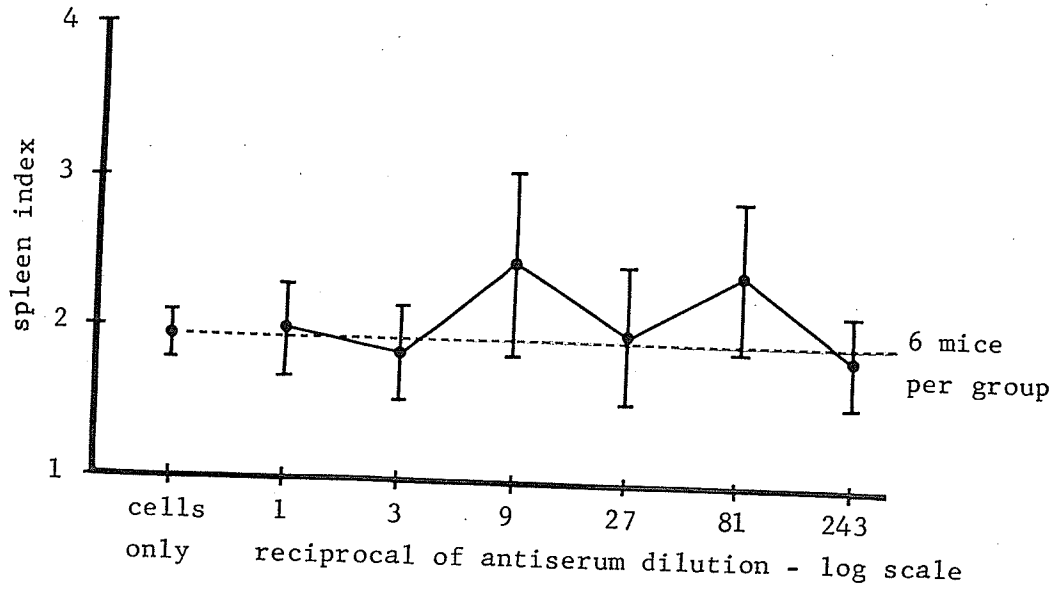


Fig.27

The effect of B10.BR anti-B10.D2 (2) serum on the spleen index produced by B10.BR spleen cells in newborn (B10.B2 x B10.BR)F_1 mice.

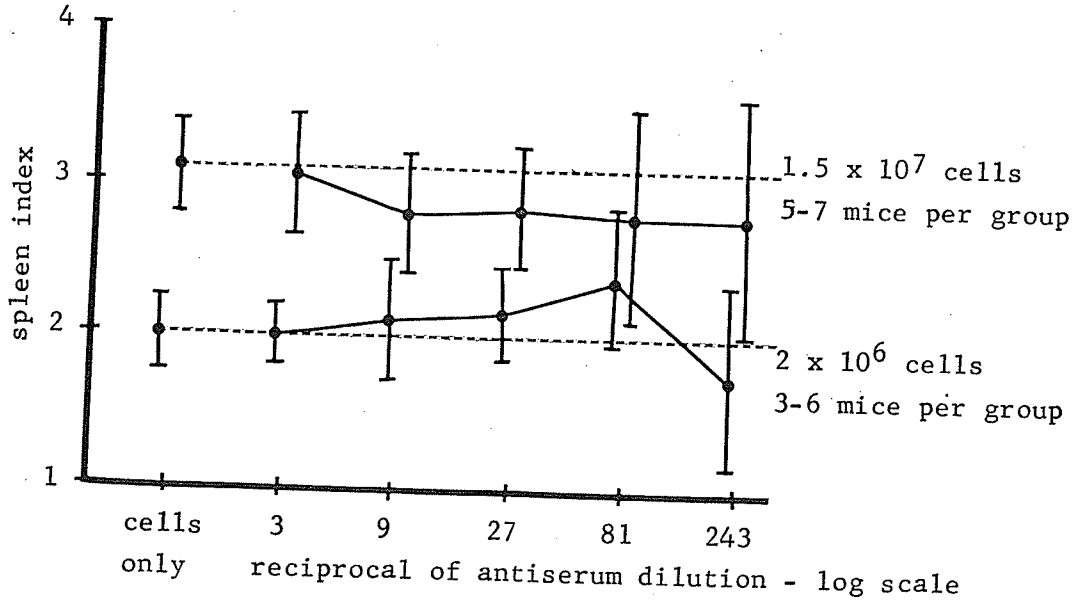
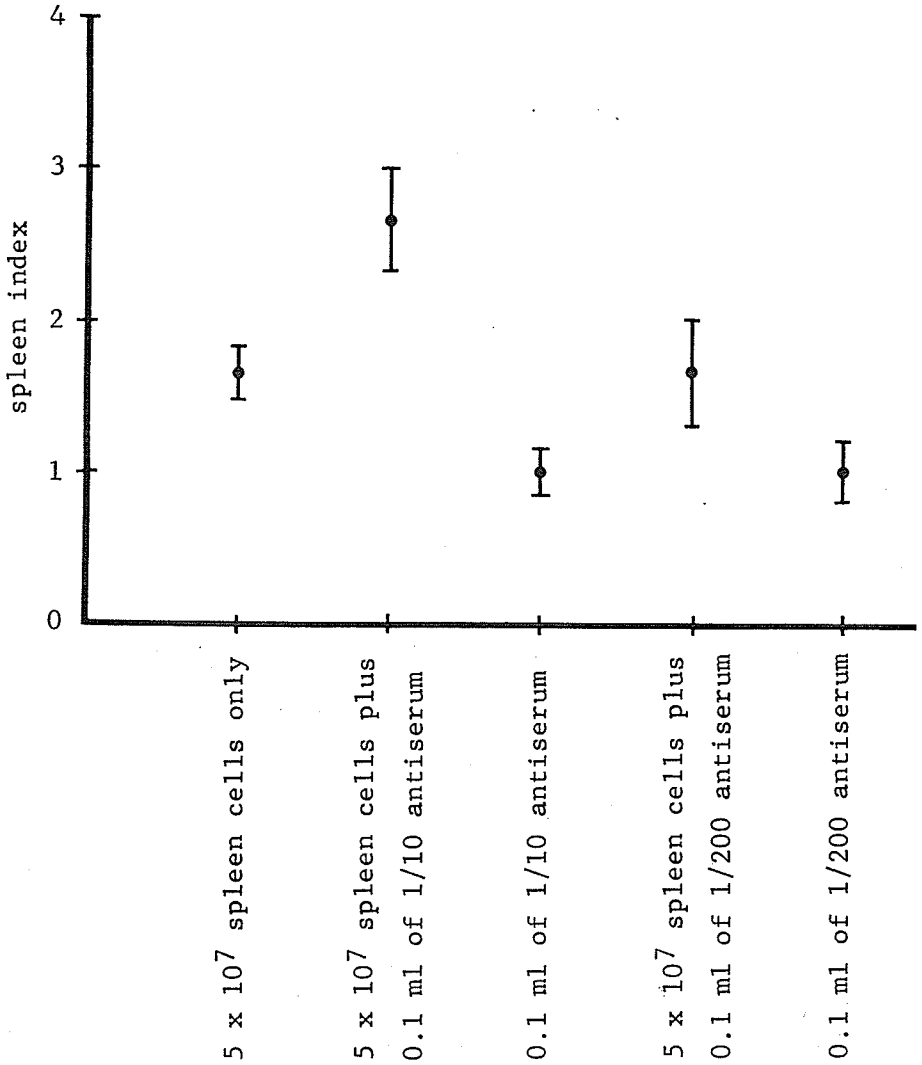


Figure 28

Spleen indices in adult (B10.D2 x B10.BR)F_1 mice injected with B10.BR spleen cells and B10.BR anti-B10.D2 (2) serum



Each group consisted of seven male and female mice, aged between twelve and eighteen weeks.

0.1 ml of HBSS containing parental spleen cells. Since the intravenous route may increase the sensitivity of the test, an attempt was made to inject the newborn mice intravenously, but most of them died; therefore, intraperitoneal injections had to be used. With both adult and newborn mice, cell injections followed the antiserum injections within four hours.

The only antiserum which altered any spleen index under the conditions used in these experiments was B10.BR anti-B10.D2 (2) (figure 25). This antiserum increased the spleen index produced by 5×10^7 viable B10.BR spleen cells in adult (B10.D2 x B10.BR)F_1 mice from 2.0 to 3.0. This increase was obtained using from 100 µl to 1.25 µl, which is approximately from 5 µl to 0.0625 µl per gram of body weight. No significant effect of this antiserum could be detected in newborn mice using either 1.5×10^7 or 2×10^6 viable B10.BR spleen cells (figure 27). The antiserum doses for newborn mice were from 33 µl to 0.4 µl, which is from 21.7 µl to 0.26 µl per gram of body weight. To establish if the effect of the B10.BR anti-B10.D2 (2) serum in adult recipients was reproducible, the experiment was repeated with two dilutions of antiserum, one expected to be active and the other expected to be inactive. Figure 28 confirms that a 1/10 dilution of this antiserum can increase the spleen index, but when diluted to 1/200 it is ineffective. It is also shown that this antiserum injected alone does not have any effect on the spleen size at either dilution.

In figure 20(b) it appears that C57BL/6 anti-B6AF_1 may lower the spleen index slightly, but figure 20(c) shows that this could not be repeated.

The effect of absorbtions of B10.BR anti-B10.D2 (2) on its activity.

To study the nature of the antibodies in the B10.BR anti-B10.D2 (2) serum responsible for the enhanced spleen index reported in the previous section, this antiserum was absorbed with several types of cells. For each experiment a 1/10 dilution of each antiserum sample was prepared, and each mouse was injected intravenously with 0.1 ml of one of the diluted antisera. The use of this amount of antisera allowed the limited volumes of some of the antiserum samples to be used for many mice, while being well within the range in which the unabsorbed antiserum was effective. Immediately after the injection of the antisera, a preparation of B10.BR spleen cells was made and 5×10^7 viable cells were injected intravenously into each mouse. Since a large number of experimental groups were used, at least in the initial experiments, a series of experiments were performed, each with one mouse per group.

The following problems were encountered. The F_1 recipients were not available in large numbers of the same age because they had to be bred in the Animal Care Centre of this University, therefore, there were age differences between the mice used in different experiments. Moreover, the age range in each experiment was also too large. This was partly due to the range of ages of the available mice, but was made worse by using mice of the same sex in each experiment, and by using four dilutions of each antiserum on several occasions, which produced large experiments. The use of the three higher antiserum dilutions was stopped after three experiments when it was found that too many mice were required. Since the results of only three experiments were not conclusive, they are not reported here. Although the spleen cell

suspensions for each experiment were prepared in exactly the same way, there may have been some variations in the activity of the cell preparations from experiment to experiment.

There was a large variation in the spleen indices obtained in the different experiments and the resulting standard errors were very large (table 2 and figure 29). This high variability in the results was probably due to the lack of homogeneity in the animals. Therefore, the results may appear to be statistically insignificant if analysed by conventional methods. However, it was possible to take into account the differences between the experiments with the use of the appropriate statistical methods. A two way analysis of variance test was done as shown in the statistics section (tables 3, 4 and 5). Only five experiments were used for this analysis because only complete and identical experiments can be used in this test (table 3). The results show that there were significant differences between both experimental groups and different experiments.

This conclusion was not sufficient since the objective of these experiments was to examine the effect of the absorbtion procedures on the capacity of the antiserum to increase the spleen index. To find out what group(s) were significantly different from the controls, paired t tests were used to compare the spleen indices treated with each absorbed antiserum sample to those produced using cells alone, and to those produced using the unabsorbed antiserum. In order to use a paired t test to compare two experimental groups, their variances must be equal. These comparisons were made using an F test.

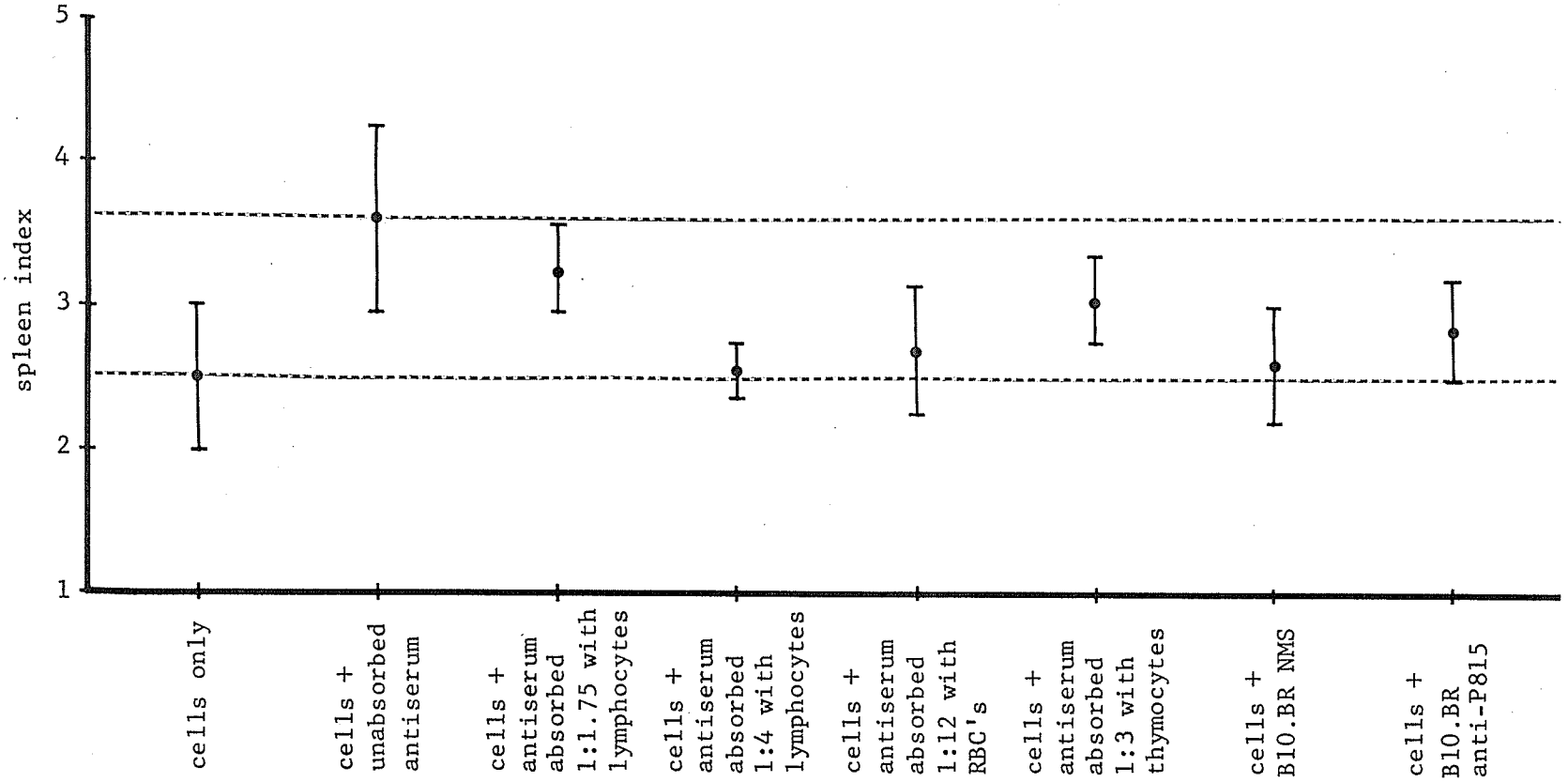
Table 2

Effect of 0.1 ml of a 1/10 dilution of absorbed or unabsorbed B10.BR anti-B10.D2 (2) serum on the spleen index in adult (B10.D2 x B10.BR)F_1 mice injected with 5 x 10^7 B10.BR spleen cells.

Age of recipient in weeks	Sex	Cells only	Unabsorbed antiserum	Cells + B10.BR anti-B10.D2 absorbed with:				Other sera:	
				Lymphocytes (1:1.75	Lymphocytes (1:4)	RBC's (1:12)	Thymocytes (1:3)	B10.BR NMS	Anti-P815
18-30	m	1.85	3.51	2.92		2.71	3.17	2.99	3.07
26-32	f	1.55	3.30	3.46		3.13	2.88	1.79	2.34
8-20	f	3.78	4.67	2.79	2.56	2.68	3.11	3.44	3.63
9-11	m	2.51	3.40	3.24	2.31	2.00	2.92	2.95	2.91
	f	2.06	4.98	3.53	2.50	4.09	3.93	2.75	3.31
6-15	f	2.37	2.74		2.46	2.47	3.06	2.31	2.26
7-14	m	2.46	2.71		2.49	2.51	2.26	1.96	2.23
9-11	f	3.39		3.74	3.10	2.07	3.00	2.81	2.90
Mean		2.50	3.62	3.28	2.57	2.71	3.04	2.63	2.83
Standard error		0.27	0.34	0.15	0.11	0.23	0.16	0.20	0.18

Figure 29

The effect of 0.01 ml of absorbed samples of B10.BR anti-B10.D2 (2) serum on the spleen index produced by 5 x 10^7 B10.BR spleen cells in (B10.D2 x B10.BR)F_1 mice.



The spleen indices are listed in table 2, and figure 29 shows the means of each experimental group with two standard errors either side of the mean. The results indicate that only the unabsorbed antiserum produced an increased GVH reaction.

One sample of the B10.BR anti-B10.D2 (2) serum was absorbed with B10.D2 spleen and lymph node cells. Since these are the cells against which the antiserum was produced, they would be expected to remove all the activity. Although no cytotoxic activity was detected in the sample absorbed with lymphocytes using a serum to packed cell volume ratio of 1:1.75, some in vivo activity remained although it was significantly reduced. This suggests that absorbtion was not complete, so a further sample was absorbed with spleen and lymph node cells using a serum to packed cell volume ratio of 1:4. As expected, this sample showed neither in vitro cytotoxicity nor an in vivo effect on the spleen index significantly different from the controls.

Red blood cells carry D and K histocompatibility antigens, but Ia antigens are believed to be absent (25), therefore, one sample was absorbed with red blood cells to find the effect of the anti-Ia component of the antiserum. When red blood cell absorbtion was complete, the cytotoxic activity which remained was presumably due to anti-Ia antibodies. However, the effect on the spleen index was completely removed, so that the spleen indices were not significantly different from those produced by cells injected alone. From this result it appears that the anti-D and anti-K antibodies and not the anti-Ia antibodies were responsible for the increase in the spleen index. When the possibility of contamination of the red blood cells is considered, it

can be calculated from the ratios of serum volumes to packed cell volumes used, that as much as 15% by volume of lymphocytes contaminating the red blood cells would not have been sufficient to remove completely the effect on the spleen index. Secondly, the lymphocytes removed all detectable cytotoxic activity before the effect on the spleen index was completely removed, whereas the antiserum absorbed by red blood cells retained considerable cytotoxic activity even when the effect on the GVH reaction had been removed.

Absorbtion with thymocytes was used because Ia antigens on thymocytes are difficult to detect and were thought to be present in such low concentrations that anti-D and anti-K antibodies could probably be removed without affecting very much the anti-Ia antibodies (31). However, the results contradicted this expectation, in that the cytotoxic activity was completely removed, which suggests that thymocytes do carry Ia antigens. If anti-D and anti-K antibodies only, had been removed from the antiserum, it would be expected that some cytotoxic activity due to anti-Ia antibodies could remain, as was the case with the red blood cell absorbed sample. The spleen indices produced using this thymocyte absorbed sample were significantly lower than those produced by the unabsorbed sample, but remained significantly higher than those produced by cells alone. This result is similar to that obtained from the antiserum when absorbed 1:1.75 with lymphocytes, therefore the failure to remove completely all the activity is probably due to incomplete absorbtion. Further absorbtion was not attempted. Since the thymocytes appear to have absorbed out all known specificities of antibody, no information can be obtained

about the specificity of the antibody which produces the increase in the spleen index.

A normal B10.BR mouse serum control was used to rule out any possibility of a non-specific effect of B10.BR serum. Figure 28 shows that normal mouse serum produces no effect on the spleen index.

B10.BR anti-P815 was used as an anti-H-2^d antiserum, which should not contain anti-Ia antibodies, because P815 is believed not to carry Ia antigens. This antiserum did not have any significant effect on the spleen indices. Nothing can be learned from this result because, of the eight other antisera raised against H-2 complex determinants, seven did not affect the GVH reaction.

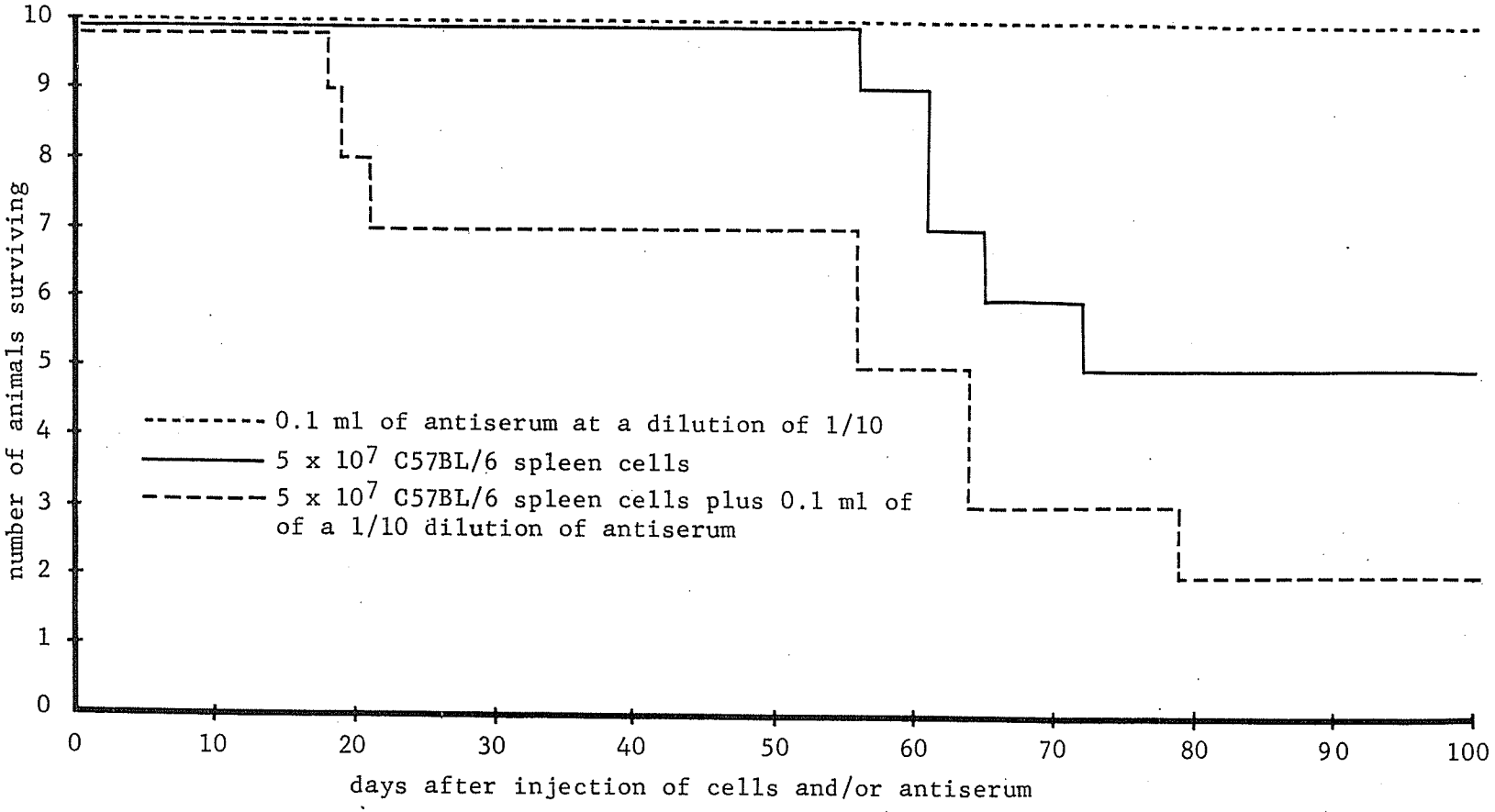
Mortality Assay

A small mortality assay was carried out to investigate the possibility that an antiserum which had no detectable effect on the spleen index might have an effect on the mortality. Ideally, the B10.BR to (B10.D2 x B10.BR)F_1 strain combination should have been used to show whether the effect of B10.BR anti-B10.D2(2) on the spleen index was paralleled by an effect on mortality, but insufficient numbers of (B10.D2 x B10.BR)F_1 mice were available. The C57BL/6 to B6AF_1 strain combination was used because some of these mice were left over from previous experiments.

Three groups of 10 B6AF_1 mice were injected as follows. Group 1 was injected with 0.1 ml of a 1/10 dilution of C57BL/6 anti-B6AF_1 serum. Group 2 was given 5 x 10^7 C57BL/6 spleen cells. Group 3 was given both antiserum and cells. All the mice in group 1 survived

Figure 30

Mortality of $B6AF_1$ mice following the injection of C57BL/6 spleen cells and C57BL/6 anti-$B6AF_1$ serum



150 days. Of group 2, five survived, and the median survival time of those which died was 63 days. Of group 3, two survived, and the median survival time of those which died was 47 days (figure 30).

C57BL/6 anti-$B6AF_1$ serum appeared to increase mortality in $B6AF_1$ mice injected with C57BL/6 spleen cells, but had no effect on the spleen index under the conditions used in these experiments. This mortality assay should be repeated using more mice before concluding that the observed effect is genuine and reproducible. If it was established that this antiserum can definitely alter the mortality but not the spleen index, then it might be concluded that the two effects may be mediated by different antiserum components.

STATISTICS

Analysis of variance

Analysis of variance is a statistical test used to compare the means of several experimental groups. In this case it was necessary to test for significant differences between the experimental groups, but it was also suspected that there might be differences between the experiments carried out on different days. Therefore, two way analysis of variance was used to test for significant differences between the experiments and between the experimental groups. This test has the advantage that, while examining either one of these variables, the effect of the other is eliminated. Only complete experiments can be used in this test, therefore, only the five experiments shown in table 3 could be used.

This test makes use of the fact that, if the means of the experimental groups are different, the variance of the combined groups will be larger than the variances of the separate groups. If, on the other hand, all the experimental groups have the same mean (i.e. belong to the same population), the variance of the combined groups should be similar to the variances of the separate groups, since both are estimates of the variance of the same population. Table 4 shows the procedure for the calculation, and page 75 shows the results of these calculations using the spleen indices in table 3.

Table 3

Spleen indices used in the two way analysis of variance test

Experiment number	Cells only	Unabsorbed antiserum	Cells + B10.BR anti-B10.D2 absorbed with:			Other sera:	
			Lymphocytes (1:4)	RBC's (1:12)	Thymocytes (1:4)	B10.BR NMS	Anti-P815
Exp.1	3.78	4.67	2.56	2.68	3.11	3.44	3.63
Exp.2	2.51	3.40	2.31	2.00	2.92	2.95	2.91
Exp.3	2.06	4.98	2.50	4.09	3.93	2.75	3.31
Exp.4	2.37	2.74	2.46	2.47	3.06	2.31	2.26
Exp.5	2.46	2.71	2.49	2.51	2.26	1.96	2.23

Table 4. Formulae for calculation of two-way analysis of variance.

	Sum of squares	Degrees of freedom	Mean square
Column means	$\sum \frac{T_{i+}^2}{r} - \frac{T_{++}^2}{rc} = S_c$	$c - 1$	$\frac{S_c}{c-1}$
Row means	$\sum \frac{T_{+j}^2}{c} - \frac{T_{++}^2}{rc} = S_r$	$r - 1$	$\frac{S_r}{r-1}$
Remainder	$S_T - S_c - S_r = S_R$	$(c - 1)(r - 1)$	$\frac{S_R}{(c-1)(r-1)}$
Total	$\sum\sum X_{ij}^2 - \frac{T_{++}^2}{rc} = S_T$	$rc - 1$	

T_{i+}'s = totals of columns (experimental groups)

T_{+j}'s = totals of rows (experiments)

T_{++} = sum of all observations

c = number of columns (number of experimental groups)

r = number of rows (number of experiments)

F ratio to test for difference between experiments

$$= \frac{S_c}{c-1} \div \frac{S_R}{(c-1)(r-1)}$$

If this value is larger than the value for $F_{0.95}[(c-1),(c-1)(r-1)]$ given by the F distribution table it can be concluded that there is a difference between experiments.

F ratio to test for difference between experimental groups

$$= \frac{S_r}{r-1} \div \frac{S_R}{(c-1)(r-1)}$$

If this value is larger than the value for $F_{0.95}[(r-1),(c-1)(r-1)]$ given by the F distribution table it can be concluded that there is a difference between experimental groups.

Table 5. Calculation of two way analysis of variance for the data in table 3.

	Sum of squares	Degrees of freedom	Mean square
Column means	4.9611	6	0.8269
Row means	6.5477	4	1.6362
Remainder	6.6024	24	0.2751
Total	18.1082	34	

Test for a significant difference between experiments done on different occasions.

$F = \frac{1.6362}{0.2751} = 5.9477$ $\qquad F_{0.99}(4,24) = 4.22$

There is a significant difference between experiments done on different occasions. $P > 0.99$

Test for a significant difference between different experimental groups.

$F = \frac{0.8269}{0.2751} = 3.0058$ $\qquad F_{0.95}(6,24) = 2.51$

$F_{0.99}(6,24) = 3.67$

There is a significant difference between the different experimental groups. $P > 0.95$

t tests

The object of the experiment was to show whether any of the absorbed B10.BR anti-B10.D2 (2) antiserum samples retained the ability to produce an increase in the spleen index, and also whether B10.BR NMS or B10.BR anti-P815 had any such effect. Therefore, it was necessary to compare each experimental group given cells plus an antiserum sample to the group given cells only. The effect of each of the absorbed B10.BR anti-B10.D2 (2) antiserum samples was also compared to that of the unabsorbed sample, to show whether the absorbtion had significantly reduced the spleen index augmenting effect.

Having established that there are significant differences between different experiments, a paired t test was chosen to make these comparisons. This test uses the difference between a given spleen index and the control in the same experiment, so that the difference between experiments is not involved. All available pairs of spleen indices were used for each comparison (table 4)

The paired t test requires that the variances of the groups being compared are equal

$$\text{Variance} = s^2 = \frac{\Sigma(x - \bar{x})^2}{N - 1}$$

Where x = spleen index
$\bar{x}$ = mean spleen index
N = number of mice in the group

To compare two variances, their ratio is calculated. This ratio is known as F.

$$F = \frac{s_1^2}{s_2^2}$$

A theoretical value for F, for the existing number of degrees of

freedom and the chosen probability value, can be obtained from F distribution tables. It was decided to accept that $s_1^2 = s_2^2$ if $F = s_1^2/s_2^2$ lay between $F_{0.025}$ and $F_{0.975}$. This gives a 5% level of significance. $F_{0.025}(N_1-1)(N_2-1)$ and $F_{0.975}(N_1-1)(N_2-1)$ were read from the F distribution table for each pair of groups being compared. Table 6 gives the probability values between which the calculated F values lie.

Table 6 Results of the F tests for the comparison of variances

Group	Compared to:	
	Cells only	Cells + unabsorbed antiserum
Cells only	—	0.25 - 0.75
Cells + unabsorbed antiserum	0.25 - 0.75	—
Cells + lymphocyte absorbed antiserum (1:4)	0.025 - 0.975	0.0005 - 0.9995
Cells + RBC absorbed antiserum	0.25 - 0.75	0.10 - 0.90
Cells + thymocyte absorbed antiserum	0.10 - 0.90	0.05 - 0.95
Cells + NMS	0.10 - 0.90	0.05 - 0.95
Cells + B10.BR anti-P815	0.10 - 0.90	0.10 - 0.90

The only pair of groups which were shown by this test to have unequal variances, were the group injected with cells plus unabsorbed antiserum, and the group injected with cells plus antiserum absorbed 1:4 with lymphocytes.

The t value was calculated as follows: $t = \frac{\bar{d}}{s_d/\sqrt{N}}$

where d = difference between a pair of spleen indices

$\bar{d}$ = mean difference between pairs of readings

s_d = standard deviation of d's

N = number of pairs

These values were compared to the values of $t_{0.95}(N-1)$ or $t_{0.975}(N-1)$ taken from a t distribution table. Table 7 shows the significance of the differences between the groups.

Table 7 Results of the t tests

Group	Compared to:	
	Cells only	Cells + unabsorbed antiserum
Cells only	—	0.05
Cells + unabsorbed antiserum	0.05	—
Cells + lymphocyte absorbed antiserum (1:4)	NS	*
Cells + RBC absorbed antiserum	NS	0.05
Cells + thymocyte absorbed antiserum	0.05	0.05
Cells + NMS	NS	0.025
Cells + B10.BR anti-P815	NS	0.025

* Not calculated because variances were different. See table 6.

The group given antiserum absorbed with lymphocytes could not be compared to the group given unabsorbed antiserum, because of the differences between their variances. In this case a paired t test indicated no significant difference, although it can be seen from figure 29 and table 3 that the absorbtion completely removed all the activity from the antiserum.

DISCUSSION

The objectives of these experiments were, firstly, to reproduce the work of several authors who have reported an inhibition and/or stimulation of the GVH reaction by alloantisera (33,34,35,36,37) and, secondly to investigate the role (if any) of anti-Ia antibodies in producing these effects. The only authors who have previously used antibodies of restricted specificity were Jose et al. (37) who obtained a reduction in the spleen index by injecting antiserum against the whole spectrum of H-2 antigens.

Antisera against Ia antigens can be obtained by immunizing one congenic-recombinant strain of mouse with lymphocytes from another strain which differs only in the H-2I region. Suitable strains for the production of such antisera were not available and therefore anti-Ia sera could not be made directly. The two congenic strains, B10.D2 ($H\text{-}2^d$) and B10.BR ($H\text{-}2^k$) were chosen because they differed only in the H-2 region and therefore it was possible to produce an antiserum containing antibodies only against H-2 specificities. It was intended that, after investigating the activity of these anti-H-2 sera, the anti-D and anti-K antibodies should be removed by absorption with tumour cells or red blood cells (31,58,60), making it possible to separate the effects of the anti-D plus anti-K antibodies and the anti-Ia antibodies contained in the same antiserum. The data in the literature indicates that antisera produced in different strain combinations may have completely opposite results (33,34,35,36,37) even if the same immunization procedure is used (34), therefore, no

valid comparison can be made between two antisera produced in two different strains. When the first two antisera produced in the B10.BR and B10.D2 strain combination failed to have any effect on the GVH reaction, the C57BL/6 and A/J strain combination was used in the hope that these strains might produce an effective antiserum.

Of the eight antisera made and tested in four different parent to F_1 GVH reactions, seven had no effect on the spleen index under the conditions used in these experiments, in spite of the fact that a wide range of antiserum doses and in some cases more than one parental spleen cell dose were used. Only the B10.BR anti-B10.D2 (2) serum had any effect on the spleen index. Volumes of 0.1 ml to 0.00125 ml of this antiserum were capable of raising the spleen index produced by 5×10^7 B10.BR spleen cells in adult (B10.D2 x B10.BR)F_1 mice from 2.0 to 3.0, and this effect was reproducible (figures 25 and 28). The same effect could not be reproduced in newborn mice with this same antiserum (figure 27). The C57BL/6 anti-B6AF_1 antiserum, which had no effect on the spleen index, did appear to have some effect on the mortality in B6AF_1 mice injected with 5×10^7 C57BL/6 spleen cells.

It is difficult to explain why one antiserum should have such a marked effect on the spleen index, while seven others had no effect under similar experimental conditions. The same immunization procedure was used for both the B10.BR anti-B10.D2 (2) and the B10.D2 anti-B10.BR (2) sera and yet the B10.D2 anti-B10.BR (2) serum was ineffective. Therefore, the immunization procedure alone cannot be responsible for the difference. The B10.BR anti-B10.D2 (1) serum was produced by a slightly different immunization procedure, but did

not have any effect on the spleen index. This observation, if taken in isolation, would suggest that the immunization procedure is important. The procedure used by Jose et al. (37) to produce their CBA anti-DBA/2 spleen index reducing antisera, was followed exactly in the production of antisera in A/J, C57BL/6 and $B6AF_1$ mice, but none of these four antisera had any effect on the spleen index.

It is possible that the immunoglobulin class of an antibody may influence its effectiveness in altering the GVH reaction. Jose et al. (37) found that only the IgG_2 and IgG_3 fractions of their antiserum were effective, but they were the only authors to investigate the Ig class. If the effect of alloantisera on the GVH reaction are related to the enhancement of allografts, it may be relevant to note that enhancing activity has been found consistently in the IgG_2 sub-class (52,47,62,46). However, all these examples involve the inhibition of an immune response and not an augmentation as was seen with the B10.BR anti-B10.D2 (2) serum.

The cytotoxicity titre of the B10/BR anti-B10.D2 (2) serum was one of the two highest of the eight produced, but this antiserum was effective in volumes from 0.1 ml down to 0.00125 ml, while the others had no effect when using 0.1 ml, therefore, the activity cannot be related to the titre of antibodies cytotoxic for lymph node cells.

It seems that factors such as strain combination, immunization procedure, Ig class and perhaps other unknown factors may be important in determining what effect an antiserum will have on the GVH reaction. It is not impossible that the antisera which had no effect on the spleen index may have had an effect if the cell dose, serum dose,

route or timing of the injection, or method of evaluating the GVH reaction had been different. For instance, the C57BL/6 anti-B6AF$_1$ serum decreased the survival rate of B6AF$_1$ mice undergoing a GVH reaction, although it did not affect the spleen index. The only previous study which examined the effects of one antiserum on both spleen index and mortality, found that both were increased (36). The mechanism of an increase in mortality due to antiserum is not known. It is unlikely that the antiserum would persist for a sufficient time and be of sufficient quantity (0.01 ml) to have a cytotoxic effect on the host cells. Therefore, it may have the effect of modifying the response of the donor cells.

The effect of B10.BR anti-B10.D2 (2) serum could not be reproduced in newborn mice. There are three possible explanations for this: (i) The right spleen cell and antiserum dose were not found. This is unlikely to be the correct explanation because two spleen cell doses and a wide range of antiserum doses were used. (ii) The route of injections for the newborn mice was intraperitoneal, whereas in the adult it was intravenous. However, in previous reports, injections of antiserum (34,36,37) and cells (36,37) have been made intraperitoneally without losing the effectiveness of the antisera. (iii) It may be necessary for the recipient to be immunologically mature for the antiserum to have any effect. However, this cannot be the case with every antiserum because spleen enlargement has been obtained in (A/He x CBA)F$_1$ newborn mice after injection with A/He cells and A/He anti-CBA serum (36).

Having obtained an antiserum (B10.BR anti-B10.D2) which increased the spleen index, this was then absorbed with B10.D2 lymphocytes, red blood cells and thymocytes. All these absorptions removed or lowered the activity of the antiserum.

Complete absorption by lymphocytes would be expected because the antiserum was raised against lymphocytes and lymphocytes express all H-2 antigens (28). The first absorption, using an antiserum to packed cell volume ratio of 1:1.75, removed all cytotoxicity from the antiserum at a dilution of 1/10, but the GVH effect was not completely removed. The second sample was absorbed at a ratio of 1:4, which removed both the cytotoxic activity and the effect on the spleen index. Therefore it was concluded that the first sample was incompletely absorbed, and that the component responsible for the GVH effect was either not cytotoxic antibody, or was active in vivo in small amounts which were not detectable in the cytotoxicity test. Since the unabsorbed antiserum was active in vivo in very small amounts, it is not surprising that a partial absorption, even if sufficient to remove all antibody detectable in the cytotoxicity test, may have left enough of such antibody for the retention of some in vivo activity.

Absorption with red blood cells was carried out to remove anti-D and anti-K antibodies, leaving an anti-Ia serum (25,58). Since an I region difference alone was sufficient to produce splenomegaly in the GVH reaction, but D and K region differences alone were not able to induce a GVH reaction (13), Ia antigens were thought to stimulate the GVH response. Therefore, the

anti-Ia antibodies were expected to alter the GVH reaction. The results of the cytotoxicity test supported the belief that absorption with red blood cells had produced an anti-Ia serum. When absorption was complete (as judged by no further reduction in the cytotoxicity titre being possible after repeated absorptions), there was still a population of cytotoxic antibodies which gave 80% lysis at a dilution of 1/10 and 50% lysis at a dilution of 1/40. Such cytotoxicity remaining after red blood cell absorption has previously been shown to be due to anti-Ia antibodies (58). On this basis the red blood cell absorbed antiserum was believed to have still strong anti-Ia activity, while the anti-D and anti-K activities appeared to be sufficiently reduced to obtain indirect information about the respective role of these two groups of antibody in the augmentation of the GVH reaction. This antiserum had no effect on the spleen index. The paired t tests showed that the spleen indices in mice injected with cells plus red blood cell absorbed antiserum were not significantly different from the spleen indices in mice injected with cells alone, but were significantly different from the spleen indices in mice injected with cell plus unabsorbed antiserum. Since anti-D and anti-K antibodies are believed to be the only ones which are removed by red blood cell absorption (25), this observation suggests that these antibodies are the ones which produced the spleen index augmenting effect, and not the anti-Ia antibodies. Absorption of anti-Ia antibodies by contaminating lymphocytes can be ruled out for the following reason. The absorption with lymph node cells showed that a number of lymphocytes sufficient

to remove the GVH reaction augmenting activity would have been more than sufficient to remove all cytotoxic activity from the antiserum, whereas the red blood cells absorption removed the GVH activity without removing all the cytotoxicity.

Thymocytes have previously been used to prepare an anti-Ia serum from an anti-H-2 serum (31), but recent evidence has shown that certain Ia specificities are probably present on at least a sub-population of thymocytes although in lower concentration than on B cells (28,30,42,53,65). The t tests showed that absorption with thymocytes significantly reduced the spleen indices produced by antiserum and cells, although these indices remained significantly higher than those of mice given cells alone. Unlike the red blood cell absorbed sample, the thymocyte absorbed sample showed no remaining cytotoxicity at a dilution of 1/10, which would suggest that all cytotoxic antibodies had been removed, including anti-Ia cytotoxic antibodies. The comparison of the cytotoxicity tests for antiserum absorbed with red blood cells and that absorbed with thymocytes indicate that thymocytes do in fact express Ia antigens. The absorption with red blood cells has shown that the activity of the antiserum is removed when the antibodies against D and K specificities are removed, therefore it would be expected that thymocytes would also be able to remove all the activity. Since the activity was only partially removed, the most probable explanation is that the absorption was not complete. The absorption with lymph node cells has already shown that it is possible to remove all detectable cytotoxicity (at the 1/10 dilution used) while still leaving some GVH reaction activity.

The B10.BR normal serum and the B10.BR anti-P815 did not have any effect on the spleen index. Although the B10.BR anti-P815 was expected to contain anti-D and anti-K antibodies, no conclusions can be drawn from the fact that it had no effect, because seven other antisera expected to contain such antibodies also had no effect.

From the comparison of the effects of the absorbtion with lymphocytes, thymocytes and red blood cells, it may be concluded that the antibodies responsible for augmenting the GVH reaction were directed against antigens represented on all these cells. Therefore, these have the same tissue distribution as the so called 'serologically detectable' antigens. The term 'serologically detectable' was originally used to refer to D and K antigens at a time when I region genes were only known by their ability to induce mixed leucocyte reactions, GVH reactions and to control certain immune responses. It is no longer accurate to designate D and K specificities as 'serologically detectable' since it is now known that antibodies can also be made against Ia antigens, therefore in this discussion D and K antigens and Ia antigens have been refered to specifically. However, it can not be ruled out that there may be other antigen(s) controlled by loci linked to the H-2 complex, which have a similar tissue distribution to D and K antigens. There is no evidence either for or against the possibility that antibody against such an H-2 linked antigen might be responsible for the effect of the antiserum on the GVH reaction. Antibody against non-H-2 antigens can be ruled out in this case because the strains used to produce the antiserum differed only at the H-2 complex.

There are several other possibilities which should also be considered. The B10.D2 lymphoid cells injected into B10.BR mice to produce the antiserum might have carried, in addition to H-2^d antigens, recognition sites for the H-2^k antigens of B10.BR mice. The B10.BR mice could have made anti-idiotypic antibody against these recognition sites (44). These could have been absorbed out by B10.D2 lymphoid cells, but should not have been removed by B10.D2 red blood cells. It is difficult to imagine how they could effect the GVH reaction since they should only react against B10.D2 cells and not F_1 or B10.BR cells.

Another important possibility is the presence of specific or non-specific factors other than antibody. One example of a non-specific factor is the allogeneic effect factor previously described (16,62). This would act on B10.BR or F_1 B cells, but since it is not antigen specific, it is unlikely that it could be absorbed out by B10.D2 cells, especially red cells. The presence of other, unknown, non-specific factors cannot be ruled out.

The helper factor described by Munro and Taussig (32) would co-operate with B10.BR or F_1 lymphocytes and have specificity for B10.D2 antigens, therefore, it should be absorbed out by an B10.D2 cells. This factor would be able to co-operate with the donor B10.BR cells in the GVH reaction against the B10.D2 antigens of the host, and might augment the reaction in this way. It might have been advantageous to have used as a control, an antiserum sample absorbed with B10.BR lymphocytes, since B cells co-operating with helper factor are able to absorb it (32).

Whatever component of the antiserum is responsible for the increased spleen index, it is very difficult to speculate about the mechanism of its action, since the mechanism of the spleen enlargement in GVH reactions is itself obscure. It is known that initially the donor cells proliferate in response to host antigens and take part in an immune reaction which is dependent on donor T cells. At the time of maximum spleen enlargement most of the proliferating cells are thought to be host cells. Since the host cannot react against the donor antigens, the proliferation of host cells must be brought about by a non-specific stimulus, which presumably originates from the donor cells and is of a totally unknown nature. It can be stated however, that since antiserum alone does not alter the spleen size in the absence of B10.BR cells, the effect is not a summation of separate effects of antiserum and B10.BR spleen cells, but an augmentation of the effect of B10.BR cells by the antiserum.

REFERENCES

1. Gowans, J.L. Ann, N.Y. Acad. Sci., 99:432, 1962.
2. Billingham, R.E., Brent, L. Proc. Roy. Soc. B., 242:439, 1959.
3. Kaplan, H.S. and Rosston, B.H. Stanford Med. Bull. 17:77, 1959.
4. Trentin, J.J. Proc. Exp. Biol. N.Y. 96:139, 1957.
5. Simonsen, M. Acta. Path. Microbiol. Scand. 39:21, 1957.
6. Billingham, R.E. and Brent, L. Trans, Bull. 4:67, 1957.
7. Van Bekkum, D.W. and Weyzen, W.W.H. Xe Congress International de Biologie Cellulaire, Paris, 1960.
8. Fox, M. Immunology 5:489, 1962.
9. Zeiss, I. and Fox, M. Nature 197:673, 1963.
10. Hilgard, H.R., Martinez, C., Good, R.A. J. Exp. Med. 122:1017, 1965.
11. Elkins, W.L. Transplanation 11:551, 1971.
12. Singh, J.N. Sabbadini, E. and Sehon, A.H. J. Exp. Med. 136:39, 1972.
13. Klein, J., Park, J.M., J. Exp. Med. 237:1213, 1973.
14. Argyris, B.F. Transplantation 17:387, 1974.
15. Ptak, W., Hanczakowska, M. and Showron-Cendrzak, A. Transplantation 19:12, 1975.
16. Osborne, D.P. Jr. and Katz, D.H. J. Exp. Med. 136:439, 1972.
17. Lindholm, L., Rydberg, L. and Strannegard, O. Eur. J. Immunol. 3:511, 1973.
18. Streilen, J.W., Stone, M.J. and Duncan, W.R. J. Immunol. 114:255, 1975.
19. Simonsen, M. Prog. in Allergy, 6:349, 1962.
20. Simonsen, M. Acta. Path. Microbiol. Scan. 40:480, 1957.
21. Billingham, R.E. and Brent, L. Phil, Trans. B. 242:439, 1959.

22. Billingham, R.E. The Harvey Lectures, Series 62. 1966-67.

23. Colombani, J., Colombani, M., Shreffler, D.C. and David, C. Tissue Antigens, 7:74, 1976.

24. Bach, F.H., Widmer, M.B., Segall, M., Bach, M.L. and Klein, J. Science 176:1024, 1972.

25. David, C.S., Shreffler, D.C., Frelinger, J.A. Proce. Nat. Acad. Sci. U.S.A. 70:2509, 1973.

26. Klein, J., Bach, F.H., Festenstein, F., McDevitt, H., Shreffler, D., Snell, G. and Stimpfling, J. Immunogenetics 1:184, 1974.

27. Shreffler, D., David C., Götze, D., Klein, J., McDevitt, H. and Sachs, D. Immunogenetics 1:189, 1974.

28. Sachs, D., David, C.S., Shreffler, D.C., Nathenson, S.G. and McDevitt, H.O. Immunogenetics 2:301, 1975.

29. Sachs, D.H. and Cone, J.L. J. Exp. Med. 138:1289, 1973.

30. Hauptfeld, V., Klein, K. and Lein, J. Science 181:167, 1973.

31. Hauptfeld, M., Hauptfeld, V. and Klein, J. J. Immunol. 115:351, 1975.

32. Munro, A.J. and Taussig, M.J. Nature 256:103, 1975.

33. Voisin, G.A. and Kinsky, R. Transplantation. Ciba Foundation Symposium, Churchill, London, 1962.

34. Batchelor, J.R. and Howard, J.G. Transplantation 3:161, 1965.

35. Voisin, G.A., Kinsky, R. and Maillard, J. Transplantation 6:187, 1968.

36. Humphrey, D.M., Fitch, F.M. and Coppleson, L.W. Transplantation 13:77, 1972.

37. Jose, D.G., Stutman, O. and Good, R.A. Transplantation 17:211, 1974.

38. Safford, J.W. and Tokuda, S. Proc . Soc. Exp. Biol. Med. 133:651, 1970.

39. Hammerling, G.J., Deak, B.D., Mauve, G., Hammerling, U., McDevitt, H.O. Immunogenetics, 1:68, 1974.

40. Dalmasso, A.P., Martinez, C. and Good, R.A. Proc. Soc. Exp. Biol. Med. 110:205, 1962.

41. Cantor, H. Cell Immunol. 3:461, 1972.

42. Shreffler, D.C. and David, C.S. Advances in Immunol. 20:125, 1975.

43. Salaman, M.H., Wedderburn, N., Festernstein, H. and Huber, B. Transplantation 16:29, 1973.

44. Ramseier, H. and Lindenmann, J. Eur. J. Immunol. 1:348, 1971.

45. McKearn, T.J., Hamada, Y., Stuart, F.P., Fitch, F.W. Nature, 251:648, 1974.

46. Cruse, J.M., Lewis, G.K., Whitten, H.D., Watson, E.S., Fields, J.F., Adams, S.T. Jr., Harvey, G.F., Paslay, J.W. and Porter, M. Progr. Exp. Tumor Res. 19:110, 1974

47. Takasugi, M. and Klein, E. Immunology 21:675, 1971.

48. Takasugi, M. and Hildemann, W.H. J. Nat. Cancer Inst. 43:857, 1969.

49. David, C., Meo, T., McCormick, S.O. Shreffler, D. J. Exp. Med. 143:218, 1976.

50. French, M.E. and Batchelor, J.R. Transplant. Rev. 13:115, 1972.

51. Bloom, B.R. Adv. Immunol. 13:102, 1971.

52. Sjögren, H.O., Hellström, I., Bansal, S.C. and Hellström, K.E. Proc. Nat. Acad. Sci. 68:1372, 1971.

53. Fathman, G.C.G., Cone, J.L., Sharrow, S.O., Tyrer, H., Sachs, D.H. J. Immunol. 115:584, 1975.

54. Cohen, A., Schlesinger, M. Transplantation, 10:130, 1970.

55. Wong, A.K.C. and Sabbadini, E. J. Immunol. 112:2251, 1974.

56. Howard, J.G. Reticuloendothelial Soc. 1:29, 1964.

57. Thoenes, G.H., White, E. and Hildenmann, W.H. J. Immunol. 104:1447, 1970.

58. Jansen, J.L.J., Koene, R.A.P., Kamp, G.J., Hagemann, J.F.H.M. and Wijdeveld, P.G.A.B. J. Immunol. 115:392, 1975.

59. Duc, H.T., Kinsky, R.G. and Voisin, G.A. Ann. Immunol. (Inst. Pasteur) 124C:567, 1973.

60. Davies, D.A.L. Transplantation Proceedings 8:37, 1976.

61. Tada, T., Taniguchi, M., Takemori, T. Transplant. Rev. 26:106, 1975.

62. Armerding, D., Sachs, D.H. Katz, D.H. J. Exp. Med. 140:1717, 1974.

63. Rubinstein, R., Decary, F., Streun, E.W. J. Exp. Med. 140:591, 1974.

64. Hauptfeld, V., Hauptfeld, M. and Klein, J. J. Immunol. 113:181, 1974.

65. Frelinger, J.A., Niederbuber, J.E., David, C.S. and Shreffler, D.C., J. Exp. Med. 140:1273, 1974.