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).

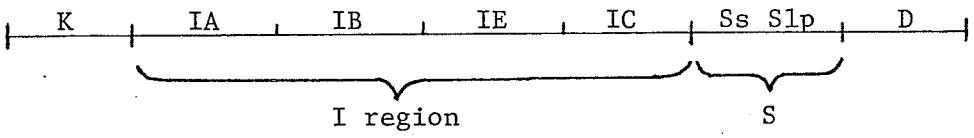
Genetics of the graft versus host reaction

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.