ANTIBODY RESPONSE TO HOMOGRAFTS

VIII. Relation of Mouse Hemagglutinins and Cytotoxins*

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Rejection or acceptance by the host of transplated tissues is still the most sensitive means by which subtle differences between individuals of a species can be revealed. That available serological techniques lag behind the transplantation assay is witnessed by the fact that humoral antibodies can not readily be detected in combinations of certain strains of mice in which skin grafts are promptly rejected, e.g. A anti CBA or C3H and most of the combinations of mice within one H-2 type (1, 2). Further development of *in vitro* serologic methods for the characterization of individuality differentials may make possible the matching of at least major determinants between the graft and host for eventually successful homografting. Methods for demonstration of antibodies elicited by homografts are relatively limited in scope, since the antigens involved have not yet been isolated in soluble form. However, an extensive body of knowledge about red blood types of inbred strains of mice has been accumulated and recently comprehensively reviewed by Amos (3). Antigens revealed by hemagglutination were previously thought to be unrelated to transplantation antigens (2), but their importance in transplantation has become pre-eminent as a consequence of the recent work of Brent, Medawar, and Ruszkiewicz (4), who found that hemagglutinogens could not be separated from cell-free transplantation antigens. Hence, hemagglutinogens may be *the* antigens responsible for homograft immunity. Histocompatibility antigens could then be assayed by their capacity to absorb hemagglutinating activity (5, 6). Moreover, hemagglutination may demonstrate the same antigens revealed by lymphocyte cytotoxicity tests since Jensen and Stetson have maintained that in the mouse, both types of activities are produced by a single class of antibodies (7).

The evidence to be presented here suggests certain qualifications to the above conclusions, (a) not *all* transplantation antigens are hemagglutinogens, and (b) not *all* lymphocyte cytotoxinogens are hemagglutinogens. Therefore, hemag-

glutination does not reveal all transplantation antigens, and lymphocyte cyto-toxicity can expose more of the weaker histocompatibility antigens than hemagglutination. Minor antigenic differences between 2 closely related strains which had led to rejection of skin homografts was not exposed by hemagglutination but was detectable by an improved lymphocyte cytotoxicity reaction. Quantitative absorption tests also indicated that hemagglutination tests may fail to disclose heterogeneity between graft and host since erythrocytes are roughly 1000 times less antigenic than lymph node cells.

Methods

Animals.—Inbred mice of the following strains were employed: A/Cal, C57 Bl/6 Jax, CBA/Jax, and AKR/Jax.

Immunization.—Unless otherwise noted, the mice were immunized by 4 to 10 intraperito-neal injections of fresh minced spleen, thymus, or kidney tissue at 1 to 2 week intervals. 7 to 14 days after the last injection, the mice were bled from the retro-orbital sinus for antisera.

Hemagglutination.—These tests were performed with slight modifications of the methods of Gorer and Mikulska (1) and Stimpling (8). Stock 6 per cent dextran solution in saline of average 75,000 MW (Hyland Laboratories, Los Angeles) was diluted for use to 3 per cent in pH 7.0 phosphate-buffered saline (8). Antisera were diluted in 0.025 ml of dextran by two-fold steps using spiral loop tendrils in plastic trays with small wells of 0.15 ml capacity (9). To this was added 0.025 ml of 2 per cent red blood cell suspension (prepared after 2 washings in phosphate saline to 10 per cent, then diluted down to 2 per cent with equal volumes of phosphate saline and human serum previously heated at 56°C for 30 minutes and absorbed 3 times with mouse spleen and liver cells). To prevent non-specific clumping in employing these small quantities of fluid, it was found essential to reduce evaporation to a minimum. This was accomplished by addition of 2 drops of mineral oil to each well. Oil also retarded evapora-tion from the wells during the time the cells were being examined and permitted subsequent re-examination. Upon 1½ hours' incubation in a shallow water bath at 37°C, the cells were streaked on a slide and inspected under dark field illumination. For hemagglutination tests by the Stimpling method (8), polyvinyl pyrrolidone (PVP, type NP-K60, Antara Chemicals, New York) was employed at a 0.75 per cent concentration in phosphate saline containing 10 per cent distilled water. To antibody dilutions made by 0.05 ml volumes in PVP with spiral loop tendrils was added 0.025 ml of a 2 per cent suspension of erythrocytes in phosphate saline. Following 2 hours' incubation at room temperature the cells were examined micro-scopically as in the dextran test.

Lymphocyte Cytotoxicity.—All tests were executed in small disposable polyethylene centri-fuge tubes of 0.5 ml capacity (Beckman-Spinco, microcentrifuge tubes). To 0.2 ml volumes of antisera diluted in Hanks' balanced salt solution, was added 50,000 lymph node cells (0.01 ml of a 5 $\times$ 10^6/ml suspension). Lymph node cells were obtained by teasing out the cells with two sharp forceps in Hank's solution, washing once, and suspending in Hanks' solution with 10 per cent normal mouse serum. Desiccated guinea pig complement (C') obtained from Cappel Laboratories, West Chester, Pennsylvania, was reconstituted for use in distilled water (discarding the diluent containing preservatives supplied by the manufacturer). It was used at dilutions of 1:20 to 1:4 depending on the number of days that it was stored (at −50°C) and the C' activity as indicated when tested with a standard known antiserum. In tests re-quiring high concentrations of mouse antiserum (1:2 to about 1:25) the mouse serum was re-moved after 30 minutes' incubation at 37°C before the addition of C' to eliminate its anti-complementary effect. With higher antibody dilutions, 0.2 ml of C' was added directly to the

antibody and cells. Upon incubation for a total of 1 hour in a water bath shaker at 37°C the tubes were spun at 15,000 RPM in a microcentrifuge (Beckman-Spinco) for 5 seconds. The sedimented cells were removed with a fine-tipped pipette onto a dried eosin dot (0.002 ml of 1 per cent eosin Y), and 200 cells were counted immediately with phase contrast microscopy employing a 43 X objective. The percentage of viable cells was compared to that in the control tubes and expressed as the cytotoxic index:

$$\text{C.I.} = \frac{C - E}{C}$$

where C = per cent viable cells in control
E = per cent viable cells in experimental

RESULTS

Technical Improvement of Cytotoxicity Tests: Reduction in Number of Cells and Removal of the Anticomplementary Action of Mouse Serum.—Sensitivity of the cytotoxicity test (10) was increased by 2 simple modifications. First, with the use of microcentrifuge tubes it was possible to reduce the number of cells to the bare minimum necessary to read as one small drop on a microscope slide (50,000) (*c.f.* method of Boyse, Old, Thomas, 11).

Second, with weak antibodies, the mouse antiserum was centrifuged out before the addition of complement. Often with weak antisera it was noted that cytotoxicity was not proportional to the amount of antisera added, and a marked "prozone" existed. This effect in which apparently excess antibody may *protect* against cytotoxicity had made further analysis difficult. It was found that the prozone could be eliminated by the simple expedient of removing mouse antisera before the addition of guinea pig complement as has been mentioned by Amos and Wakefield (12). An experiment which demonstrates the effectiveness of this procedure is illustrated in Fig. 1. An antiserum of strong potency, C57 anti A, was diluted 1:1000, 1:400 and 1:100 in normal C57 mouse serum to create antisera of graded strengths. Dilutions of this artificial antisera were tested with A strain lymph node cells by simultaneous addition of guinea pig C' and incubation for 1 hour (solid line), or incubation for $\frac{1}{2}$ hour in the mouse antisera, centrifugation and replacement of antisera with C' followed by a further $\frac{1}{2}$ hour's incubation (dotted line). It can be seen that with an attenuated antibody (1:1000) if C' was added directly to the murine antisera no cytotoxicity was disclosed, whereas a linear cytotoxic effect of the antiserum was revealed if the antisera was removed before C' addition. But if the potency of the antibody was increased (1:400 to 1:100), a relatively weaker "prozone" effect was noted. It can thus be concluded that feeble antibodies at low dilutions can best be shown by removal of the antiserum before addition of C'. The explanation for the "prozone" effect is evident from the following experiment in which pre-sensitized cells (50,000 C57 lymph node cells incubated with 0.2 ml of 1:100 A anti C57 serum for $\frac{1}{2}$ hour and washed once) were added to dilutions of normal mouse serum plus C'. Murine serum in concentrations of 1:2 or 1:4 can be seen to protect the already sensitized cells from the action of complement (Fig. 2). Thus with C' at a 1:32 dilution, sensitized cells did not lyse if high concentrations of normal mouse serum were present. This anticomplementary effect of mouse serum however could be reversed by elevated levels of C' (1:16, 1:8).
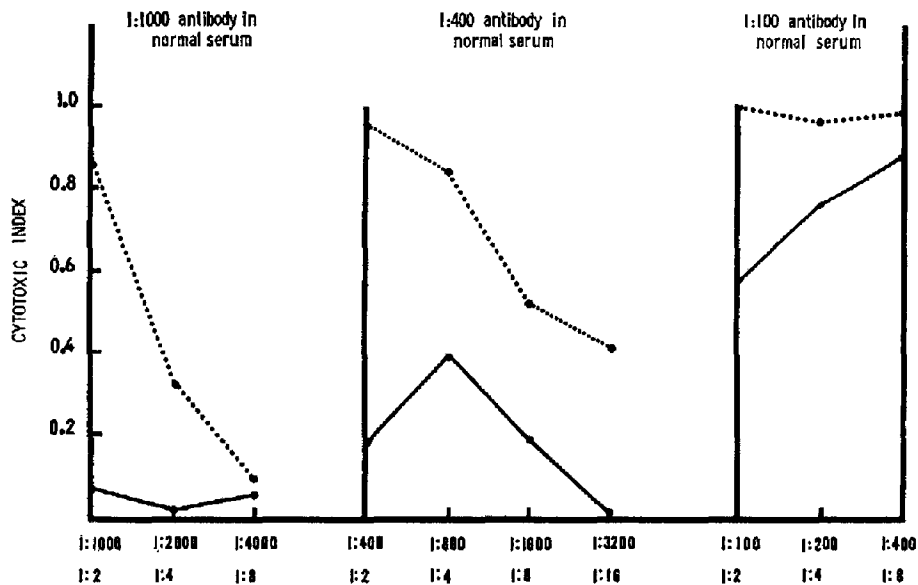
FIG. 1. Removal *versus* non-removal of mouse antibody. C57 anti A antibody was diluted to 1:1000, 1:400, or 1:100 in normal C57 mouse serum to produce sera of graded strengths. These 3 sera were then diluted from a 1:2 dilution, and incubated with A strain lymph node cells: (a) together with C' for 1 hour at 37°C (solid line), (b) without C' for ½ hour, then the mouse antisera was replaced with C' and incubated for a further ½ hour at 37°C (dotted line). Cytotoxic index was determined on this and the following graphs by eosin dye exclusion as described in the methods section.
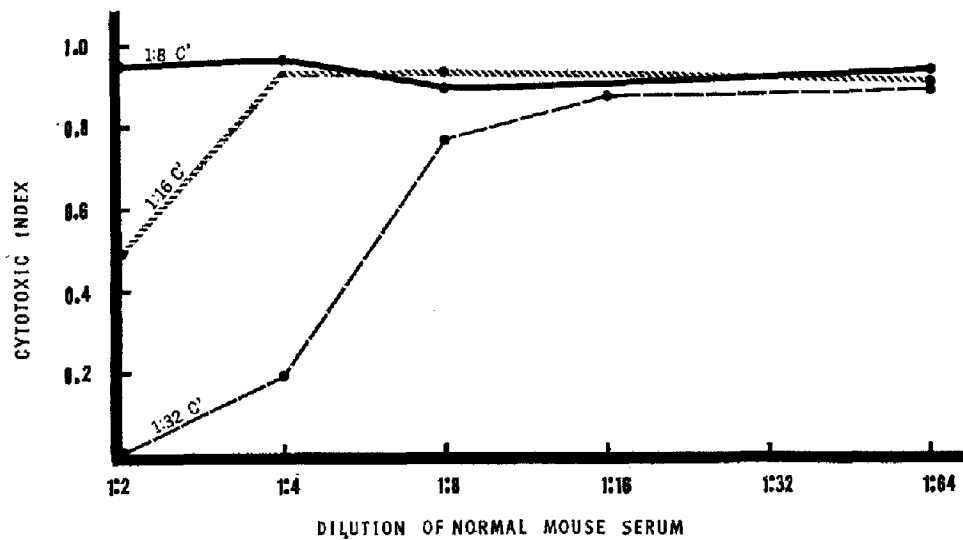

FIG. 2. Anti-C' activity of mouse serum. C57 lymph node cells were sensitized with A anti C57 antisera, washed, and added to dilutions of normal mouse serum together with 3 different dosage levels of C'. Cytotoxicity was determined following 1 hour's incubation at 37°C.
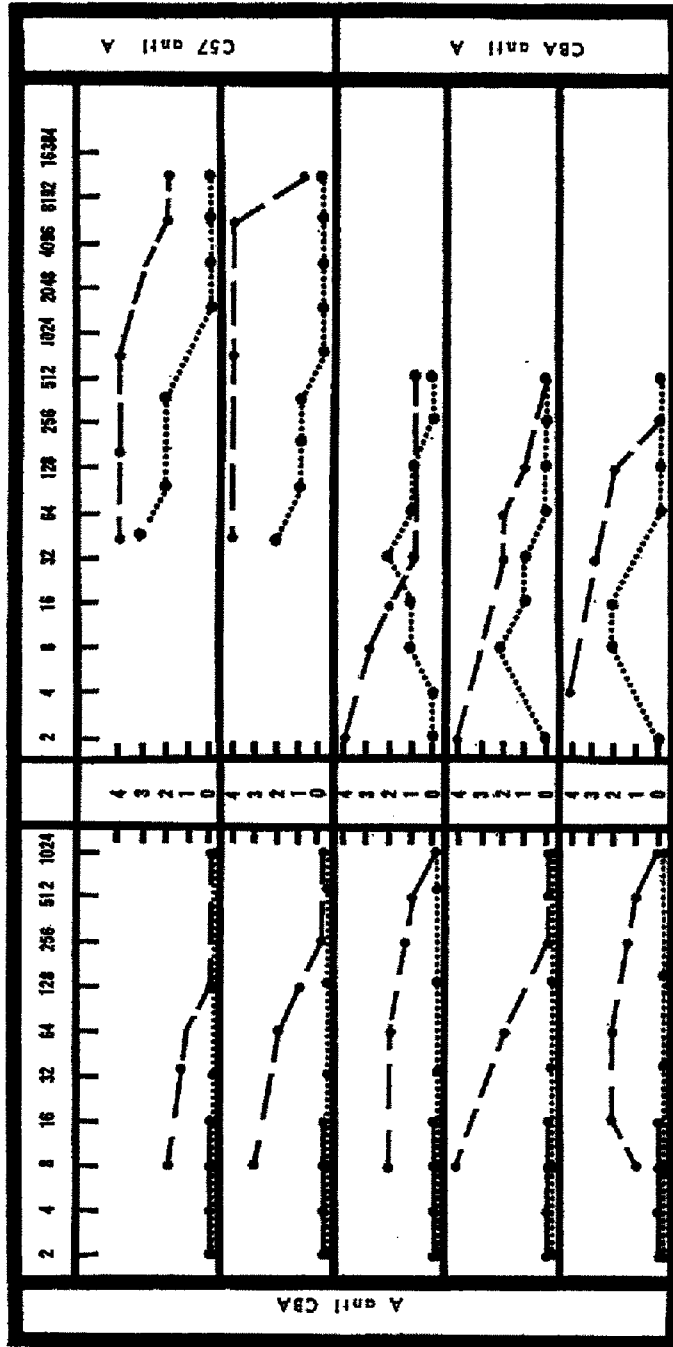
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Fig. 3. Effect of allogenic mouse anti spleen sera. Separate pools of antisera were tested concurrently with erythrocytes and lymph node cells of a donor strain mouse. On the ordinate is given the degree of agglutination or death of all cells = 4; three-quarters cells = 3; one-half cells = 2; one-quarter cells = 1. The reciprocal of serum dilution is given on the axis. Comparison of cytotoxic activity — — — with hemagglutinating activity using PVP ———— or dextran-human serum ....

Since high concentrations of guinea pig serum are often toxic and C' levels are trouble-
some to standardize accurately, the expedience of removing anticomplementary
mouse serum before addition of C' was taken whenever dealing with low dilutions of
antisera (1:2 to 1:32).

Correlation of Hemagglutinin Levels with Cytotoxin Levels.—As shown in
Fig. 3, 5 separate pools of A anti CBA spleen sera were cytotoxic in dilutions
up to 1:512 without being hemagglutinating. This lack of hemagglutinating
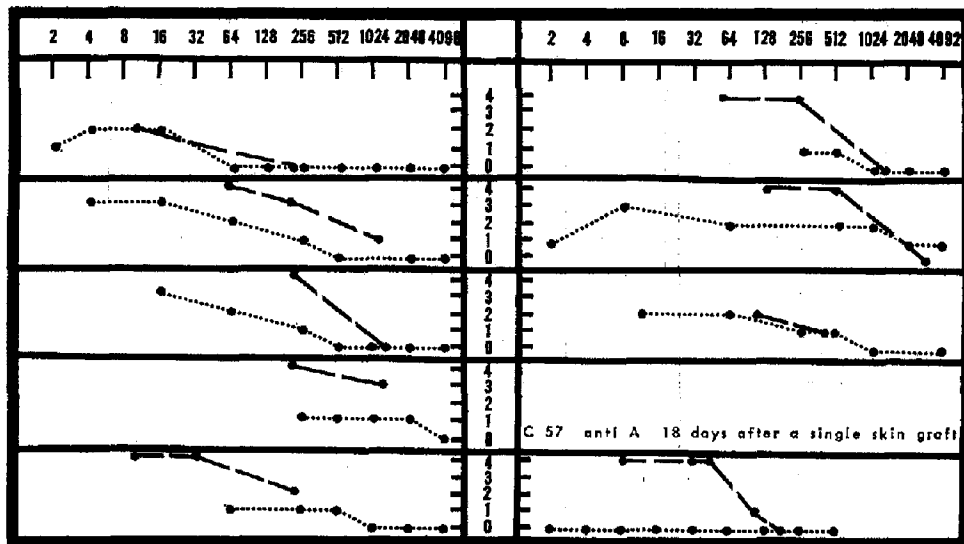

Fig. 4. Effect of allogenic anti-kidney and anti-skin graft sera. A anti C57 kidney sera and
C57 anti A skin graft serum were concurrently tested with red blood cells and lymphocytes.
Scale for coordinates same as in Fig. 3.

Comparison of hemagglutinating (dextran) . . . . and cytotoxic __ __ __ activity.

activity was confirmed by both the dextran and the PVP methods. It can be
seen, too, that a definite, though weaker, discrepancy between the 2 types of
antibody action was found in the C57 anti A spleen system. Thus at high dilu-
tions of serum almost all lymph node cells were killed, whereas erythrocytes
were not agglutinated. Although such a difference may only express divergence
in the sensitivity of the 2 techniques (the cytotoxic test employed here used 8
times the volume of antibody used for hemagglutination), with the same
methods, the antisera produced in CBA mice against A spleen were equally
active upon erythrocytes and lymphocytes (Fig. 3). Antibodies elicited in A
strain mice to C57 kidney cells fall into the same category of being roughly
equally effective in the cytotoxic and hemagglutinating tests (Fig. 4). On the
other hand, serum from C57 mice grafted with A strain skin was markedly
cytotoxic without being hemagglutinating (Fig. 4).

Antibodies to thymic cells were again both hemagglutinating and cytotoxic in roughly similar titers in the CBA anti A system but were cytotoxic and not hemagglutinating in the reverse A anti CBA combination (Fig. 5). Thus the unshared determinants present in CBA cells but absent in A strain mice are revealed by the cytotoxicity tests but not by the hemagglutinating methods. Anti-thymus antibodies were found to increase with immunization, and remain at about the same levels after 4 injections (Fig. 5).
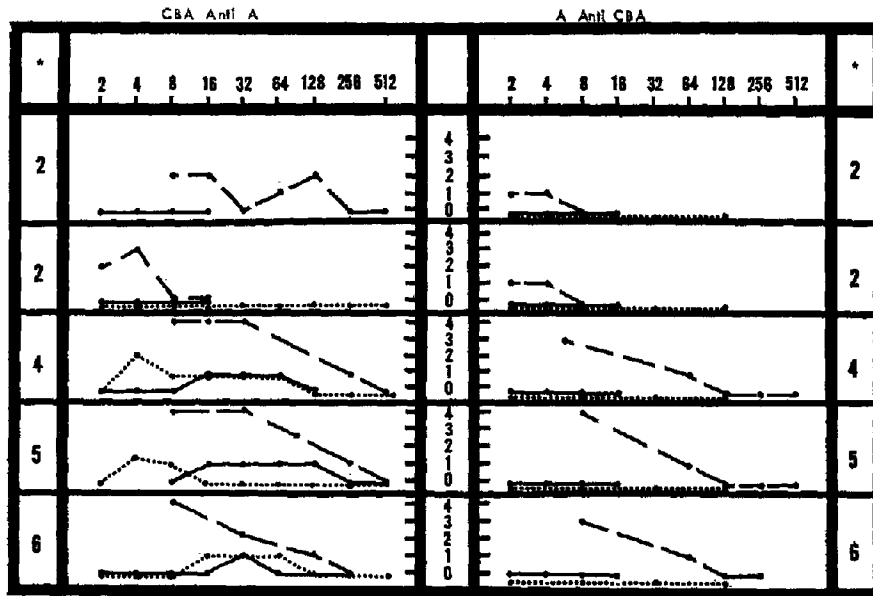

FIG. 5. Effect of allogenic anti thymus antisera. A anti CBA and CBA anti A sera were tested for hemagglutination and lymphocyte cytotoxicity. Scales for coordinates same as Fig. 3.

Comparison of cytotoxic activity _ _ _ with hemagglutinating activity using PVP ————— or dextran-human serum ....

* Number of times immunized.

Between 2 mouse strains of the same H-2 type, definite cytotoxicity (C.I. = 0.5) of AKR anti CBA sera were obtained with 2 serum pools at a 1:256 dilution. Simultaneous hemagglutination tests with the dextran-human method were completely negative from 1:2 to 1:1024 dilutions after 1½ and 8 hours of incubation.

Quantitative Absorption of Antibody.—To demonstrate any differential absorption of cytotoxic or hemagglutinating antibodies, an antiserum which yielded comparable hemagglutinin and cytotoxic titers was absorbed with graded numbers of erythrocytes or lymph node cells. CBA anti A spleen antibody at a concentration of 1:16 in 3 per cent dextran-Hanks (6 per cent dex-

tran plus Hanks' balanced salt solution in equal volumes) was added in 0.25 ml volumes to varying numbers of sedimented A line cells. After 1 hour's absorption at 37°C, the supernatant fluid was tested for hemagglutinating and cytotoxic activity. As evident in Fig. 6, both types of antibody activity were removed proportionally by erythrocytes and lymphocytes, indicating no preferential absorption. It is noteworthy that lymphocytes were about 100
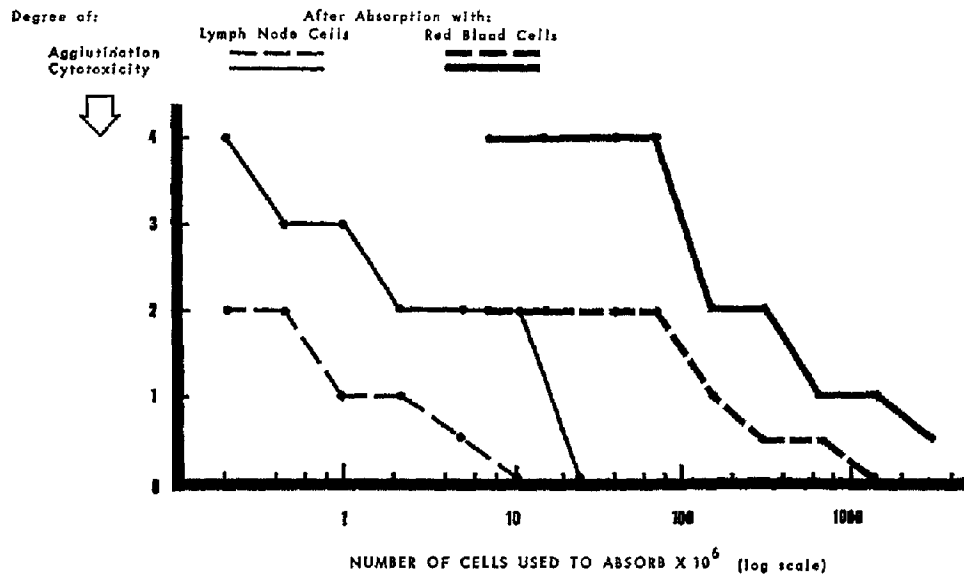

FIG. 6. Concurrent absorption of hemagglutinins and cytotoxins. CBA anti A spleen antiserum diluted 1:16 in 3 per cent dextran-Hanks was absorbed in 0.25 ml volumes with varying numbers of sedimented A strain cells. The supernatant fluid was assayed after one hour absorption at 37°C for cytotoxicity and hemagglutination by the scale given in Fig. 3.

times as effective as erythrocytes in removing *both* cytotoxic and hemagglutinating activity.

An even greater difference in antigenicity between erythrocytes and lymphocytes was apparent with A anti CBA and C57 anti CBA sera. The A anti CBA serum was diluted 1:8 in 3 per cent dextran-Hanks for absorption and the C57 anti CBA serum was diluted 1:200 in Hank's salt solution. Fig. 7 and 8 clearly show that with both combinations of sera, approximately 1000 times as many red blood cells were required to reduce the toxicity of the serum to a level comparable to that produced by lymph node cells. Since the magnitude of the difference was so great, certain precautions were taken to minimize the possibility of contamination of the red blood cell suspensions with leucocytes. For absorption of A anti CBA serum, heparinized CBA blood from 6 donors
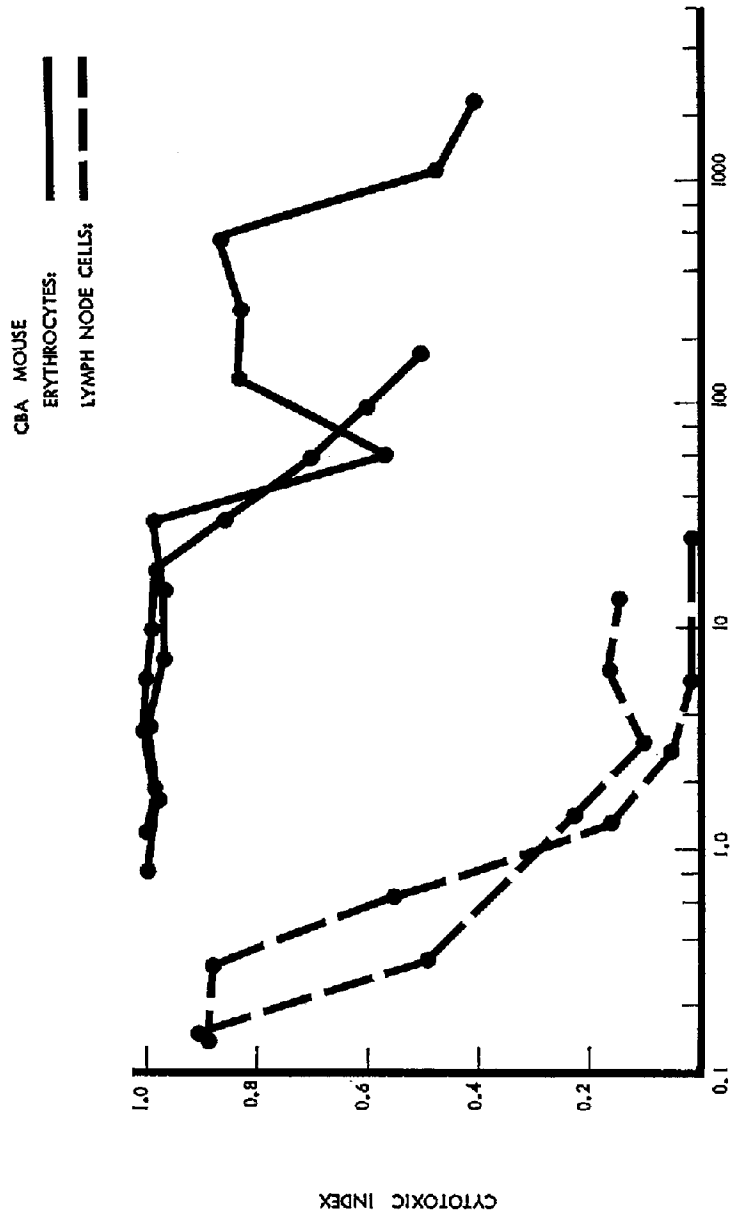
Fig. 7. Absorption of lymphotoxins. In 2 separate experiments, 0.2 ml aliquots of C57 anti CBA spleen antisera diluted 1:200 in Hank's solution were incubated for 1 hour at 37°C with varying numbers of sedimented CBA cells. The supernatant fluid was then tested for cytotoxicity against CBA lymph node cells.
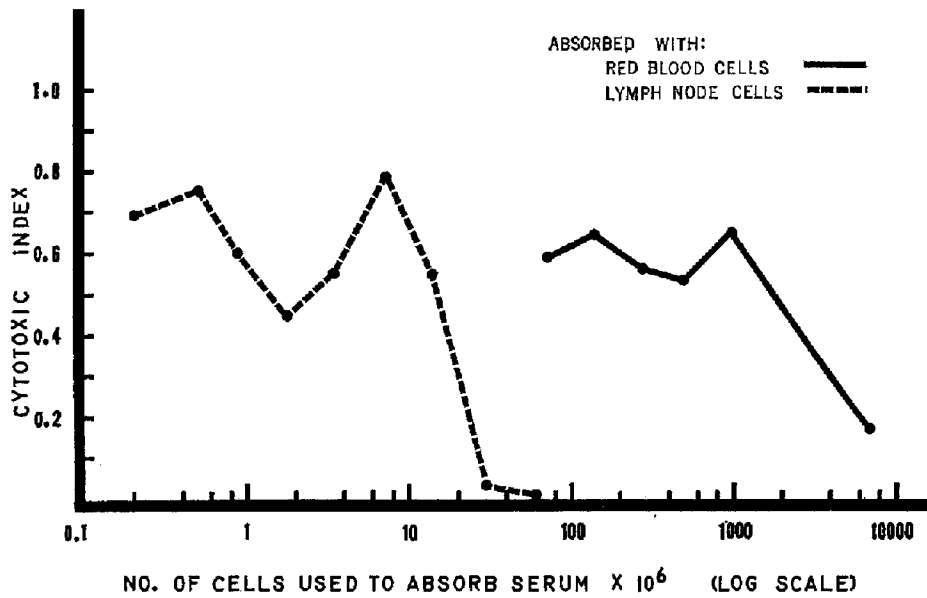
Fig. 8. Absorption of lymphotoxins. Aliquots of 0.2 ml of 1:8 A anti CBA spleen antiserum in 3 per cent dextran-Hanks were absorbed with sedimented CBA cells. Upon one and $\frac{1}{2}$ hour's absorption at 37°C, the supernatant fluid was tested for cytotoxicity with CBA lymph node cells.
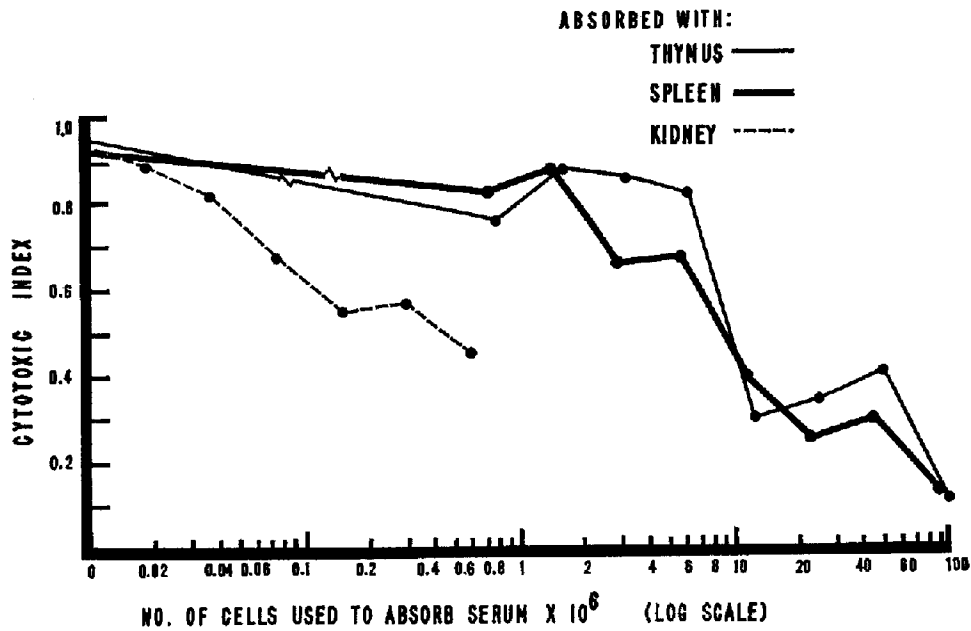

Fig. 9. Absorption of allogenic antisera to kidney tissue. An A anti C57 kidney antiserum was diluted 1:50 and added in 0.2 ml aliquots to sedimented C57 cells. Following $\frac{1}{2}$ hour's incubation at 37°C the antiserum was tested for cytotoxic activity with C57 lymph node cells.

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was first spun in an inverted centrifuge tube with a rubber stopper. Red blood cells obtained from the bottom with a hypodermic needle attached to a syringe were then spun at 1,500 g for 20 minutes in the cold in polyethylene tubes (P.E. 100 to 200) which were then cut 5 to 10 mm below the buffy coat layer.

Immunization of mice with allogenic kidney cells resulted in antibodies that were cytotoxic to lymph node cells as illustrated by the 7 pools shown in

ABSORBED WITH:
------- A STRAIN KIDNEY CELLS
———— C 57 STRAIN KIDNEY CELLS

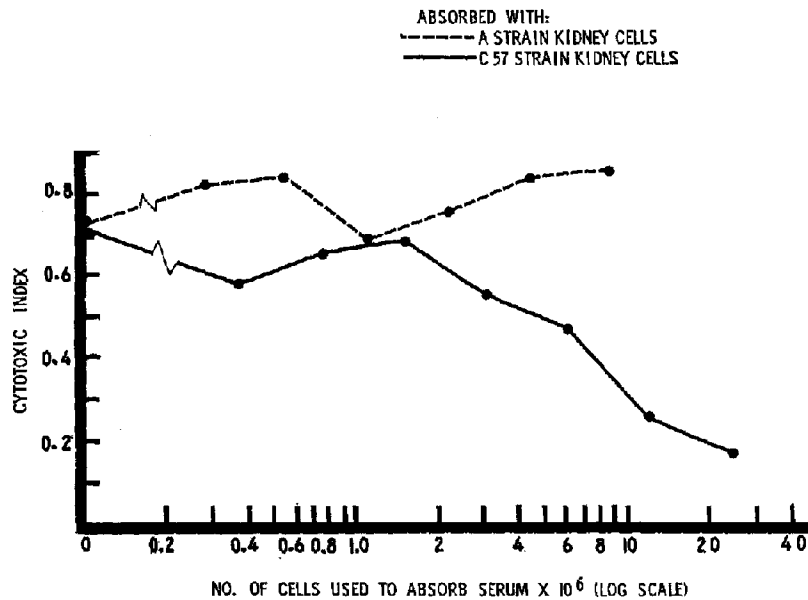NO. OF CELLS USED TO ABSORB SERUM X 10^6 (LOG SCALE)

Fig. 10. Absorption of anti-kidney serum with kidney cells. A anti C57 kidney serum at 1:100 dilutions was absorbed with trypsinized kidney cells for ½ hour at 37°C, then tested with C57 lymph node cells for cytotoxic activity.

Fig. 4. This lymphotoxic activity could be absorbed by kidney, spleen, and thymic cells (Fig. 9). On a per cell basis, trypsinized kidney cells removed lymphotoxic antibodies more readily than spleen or thymic cells. Absorption was strain-specific in that isologous kidney cells did not remove the antibody (Fig. 10).

Attempts to dissociate the hemagglutinating and cytotoxic activities by heat at 56°C for different time intervals was unsuccessful. Both activities were heat-stable from 5 to 50 minutes at 56°C.

DISCUSSION

The technique of hemagglutination in its present form (1, 8) apparently does not measure *all* the transplantation antigens. The most conspicuous

example of the inadequacy of the technique occurs in the commonly used A
and CBA strains of mice. Although reciprocal skin homografts between these
strains are sloughed rapidly in about 11 days (13), hemagglutinins are found
only in the CBA mouse against A strain, but not in the reverse combination (2).
The present work confirmed the finding that A anti CBA serum is non-hemag-
glutinating with either the dextran-human serum method or the PVP method.
Thus hemagglutination assays, though they measure the more important H-2
antigens, are incapable of detecting all the antigens against which a homograft
reaction is directed. On the other hand, it has been possible to show that a
serologic response of A strain mice against CBA homografts can be demon-
strated if lymphocytes are used as the target cell. With an improved cyto-
toxicity test, A anti CBA sera were shown here to be cytotoxic to a 1:512 dilu-
tion without being hemagglutinating (Figs. 3, 5). Parallel results were obtained
with two mouse strains of the same H-2 K type. AKR anti CBA sera were
cytotoxic at a 1:256 dilution, but completely non-hemagglutinating. Evidence
that leucocytes possess more allogenic antigens than erythrocytes is also evi-
dent in the earlier work of Amos (14) and Amos and associates (15).

With regard to the question of identity of cytotoxinogens with transplanta-
tion antigens conclusive evidence is lacking, though cytotoxins can be absorbed
by various presumably antigenic subcellular fractions (16). The assumption
that cytotoxinogens are identical or similar to hemagglutinogens in the mouse
is suggested by Jenson and Stetson's finding that one type of antibody is re-
sponsible for both the hemagglutinating and cytotoxic activity of allogenic
mouse antisera (7). Three lines of evidence were presented: (a) with 60 samples
of sera, the titers of hemagglutination and cytotoxicity were consistently cor-
related, (b) cytotoxic activity could be absorbed by erythrocytes, and (c),
the 2 activities could not be separated electrophoretically. This general con-
clusion was further supported by Möller and Möller's finding that cytotoxic
sensitivity of tumor cells closely paralleled their ability to absorb hemag-
glutinins (17).

The results described here indicate, however, that although allogenic mouse
antibodies may be both cytotoxic and hemagglutinating, certain antibodies
are cytotoxic without being hemagglutinating. Contrary to the close corre-
spondence in the hemagglutinin and cytotoxin titers found with the Balb/c
and C57/B1 mice (7), many instances in which the titer of cytotoxins were
more than 100 to 500 times greater than the titers of hemagglutinins were noted
with other mouse strains. Apparently the strain involved is a more critical
factor than the particular tissue used to immunize. A anti CBA sera prepared
against splenic cells or thymic cells (relatively free of erythrocytes) were both
lymphotoxic, without being hemagglutinating, whereas CBA anti A spleen or
thymus produced both effects in about equal titers (Figs. 3, 5). These results

also argue against the possibility that relative sensitivity of the 2 tests accounts for the difference in titers since close correspondence of titers were shown in certain strain combinations, but not in others.

Although the findings of Jensen and Stetson (7) that erythrocytes were able to absorb cytotoxins was confirmed, it was also shown that an extremely disproportionate number of erythrocytes were needed. Despite the use of dextran in the absorbing media (7), 1000 erythrocytes were needed to absorb the quantity of antibody absorbed by one lymphocyte in the A anti CBA combination—similarly, 100 erythrocytes to one lymphocyte in the CBA anti A system (Figs. 8, 6). Using another antibody, C57 anti CBA, again approximately 1000 times as many erythrocytes were needed when absorption was carried out in Hank's solution (Fig. 7). Thus variability can be shown with different strains and different media, but the magnitude of variance in antigenic content of the 2 types of cells is obvious. Contamination of the erythrocyte suspension with leucocytes may account for some of the absorptive power of high numbers of erythrocytes, though smears have revealed contamination of less than one per 10,000 cells. Considerations of the surface area available, as have been emphasized by the work of Möller and Möller (17) do not apply here, for surface area difference of the 2 cell types is inappreciable. In addition, correlation of cytotoxic sensitivity of cells to their capacity to absorb hemagglutinins apparently also are not applicable to their capacity to absorb lymphotoxins since thymic and splenic cells were found to absorb lymphotoxic antibodies as well as lymph node cells on a per cell basis (Fig. 9), despite the fact that thymic cells are presumably less susceptible to the action of cytotoxic antibodies (18). Dissociated kidney cells also were capable of absorbing antibodies in relatively small numbers (Fig. 9, 10), though preliminary results indicate they are less sensitive than lymphocytes to the toxic effect of antibody and C'.

In summary, the present results suggest that allogenic antigens are present in immensely greater quantities on lymphocytes than erythrocytes, and that this may explain the ease with which cytotoxic activity with certain sera can be demonstrated in the absence of hemagglutinating activity. Some indication that there are no qualitative difference in antigenic content between lymphocytes and red blood cells is suggested by the absorption of cytotoxins by erythrocytes from the critical A anti CBA antisera which itself was non-hemagglutinating (Fig. 8).

Although it appears from the foregoing that all transplantation antigens are not hemagglutinogens, it is also of considerable practical importance to determine if the converse is true, that is, whether all hemagglutinogens function as histocompatibility determinants. In the mouse, there is strong evidence that hemagglutinogens shown by allogenic antibody can be used to group mice into

histocompatibility types within which tumors of various kinds can be freely transplanted (3, 19, 20). From such extensive experiments and the findings of Brent, Medawar, and Ruskiewicz (4), the inference that mouse isohemagglutinogens are actually transplantation antigens would seem justifiable. That the blood group antigens of man may play a similar role has been generally discounted since close matching of blood groups for as many as 12 factors (see Woodruff and Allan, 21) has been of no avail in successful skin homografting. Though it would appear from this that hemagglutinogens in man are not strong histocompatibility determinants, it may be of importance to note that the homograft reaction can act with equal rapidity upon "weak" as well as "strong" antigens, and a matching of the major determinants does not necessarily produce a readily detectable prolongation in graft survival. Thus, many mice of the same blood group (H-2 type) rapidly reject reciprocal grafts: the intensity of the reaction being determined by other "weak" antigens. Whether some of the known blood groups of man are the major determinants as are the mouse hemagglutinogens remain to be determined. Some red blood cell factors certainly may be inconsequential to histocompatibility, and in the rabbit, apparently certain known hemagglutinogens are weak transplantation antigens whereas cytotoxinogens may function as the major histocompatibility determinants (Kapitchnikov, Ballantyne, and Stetson, 22).

SUMMARY

Antigenic differences between certain inbred strains of mice which could not be revealed by hemagglutination techniques were readily disclosed by lymphocyte cytotoxicity. With an improved cytotoxicity test lymphotoxic titers were as high as 1:512 with non-hemagglutinating A anti CBA antisera. In other mouse strain combinations, a close parallel of both types of antibody activity was obtained. Though both activities were absorbed from antisera proportionally by erythrocytes and lymph node cells, 100 to 1000 times as many erythrocytes as lymphocytes were necessary to produce an equivalent reduction in antibody activity These findings suggest that erythrocytes may possess only subthreshold quantities of certain antigens which are present in readily detectable levels on lymphocytes. Lymphocyte cytotoxicity therefore may assay a wider range of allogenic antigens than hemagglutination.

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