

THE FACTOR OF IMMUNIZATION IN THE RAT  
THE EFFECT OF ALLOGENEIC IMMUNIZATION ON GRAFT-VERSUS-HOST ACTIVITY

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Graft-versus-host (GVH)<sup>1</sup> assay is one of many methods of measuring the immunological activity of lymphoid cells against histocompatibility antigens. It is the most suitable in vivo method when a quantitative comparison of cell populations is required. After immunization with allogeneic antigen the GVH activity of an animal's lymphocytes is usually increased. However, in strain combinations of mice involving a difference at the H-2 locus, the augmentation of GVH activity after immunization (the "factor of immunization") is very slight; the augmentation increases with the weakness of the antigenic barrier (1, 2).

In the rat, GVH activity can be satisfactorily measured by a lymph node weight assay (3, 4). Using this assay it has been found that to produce an arbitrarily chosen lymph node enlargement of 10.0 mg requires 50–100 times as many cells in weak as compared with strong strain combinations. In the present experiments the effect of specific allogeneic immunization on the GVH activity of lymphoid cells has been investigated using several weak and strong combinations of the inbred rat. The paradoxical inverse relationship between antigenic strength and the factor of immunization has been confirmed.

In further experiments the possible effects of other variables on the factor of immunization have been tested, namely: (a) the schedule of immunization used; (b) the interval elapsing between immunization and assay; and (c) the population of cells used for assay. Lastly, an attempt has been made to test a suggestion (5) that the specific inhibitory effect of humoral alloantibody might be responsible for the low factor of immunization in strong strain combinations.

*Methods*

*Rats.*—The GVH activities of lymphocytes from immune and nonimmune donors matched for age and sex were compared. The following strain combinations were used (where X → Y

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<sup>1</sup> Abbreviations used in this paper: F.I., factor of immunization; GVH, graft-versus-host; PBS, phosphate-buffered saline; TDL, thoracic duct lymphocytes.

denotes the assay of lymphocytes from inbred strain X in (X × Y) F<sub>1</sub> hybrids). AS → AS<sub>2</sub>, AS<sub>2</sub> → AS, AS → BN, and F → BN (all AgB different); AS → F and F → AS (AgB identical). In further experiments, cells from (AS × BN) F<sub>2</sub> hybrids selected to be AgB heterozygotes after serological screening were assayed in (AS × BN) F<sub>1</sub> hybrids. In this case the GVH reaction was against half the minor loci, on average, at which the AS and BN strains differ.

*GVH Assay.*—The popliteal lymph node assay has previously been described in detail (4). In the present experiments at least four footpads were injected with each of three graded doses of lymphocytes. Thus each estimate of GVH activity is based on the reaction of at least 12 lymph nodes. In the AgB-identical strain combinations, doubling doses were used, usually starting at  $20 \times 10^6$ ; in the AgB-different combinations, tripling doses were used, usually starting at  $0.33 \times 10^6$ .

Preparation of thoracic duct lymphocytes (TDL) and spleen cells were as previously described (4). Lymph node cells were prepared as were spleen cells.

*X-Irradiation.*—In some experiments donors were subjected to 300 R of whole body X-irradiation 24 hr before immunization. Rats were irradiated singly in Perspex cages from a 225 kv source delivering 17 R/min.

*Alloantibody Titration and AgB Classification.*—Serum was obtained from the irradiated donors before immunization, 6 days after immunization, and at the time of assay of their lymphocytes. Nonirradiated, immunized control rats were bled at the same time. Hemagglutinating alloantibody was titrated using 3-fold serum dilutions in phosphate-buffered saline (PBS). To 2 drops of serum dilution was added 1 drop of a 2% erythrocyte suspension in 9 parts of 6% dextran (Glaxo Laboratories, Ltd., Greenford, England) and 1 part of normal F<sub>1</sub> rat serum. Macroscopic reading was after a 2 hr incubation at 37°C. The screening of (AS × BN) F<sub>2</sub> rats for the identification of AgB heterozygotes was performed using a similar technique. Erythrocytes from the unknown rats were titrated against an AS anti-BN serum and a BN anti-AS serum. With the aid of appropriate control erythrocytes (BN, AS, and F<sub>1</sub>) double-reacting erythrocytes could be easily identified.

*Immunization.*—Two skin allografts with an interval of at least 2 wk between them was the standard immunization procedure. The second set tempo of rejection of the second graft showed that some degree of immunization had already been produced by the first graft. Some groups were “boosted” after skin grafting by the subcutaneous injection of  $100 \times 10^6$  allogeneic spleen and lymph node cells. Three injections were given at weekly intervals and each dose was distributed between the four footpads and the angles of the mouth. Further groups of rats were immunized solely by one injection of  $100 \times 10^6$  spleen and lymph node cells distributed equally between the four footpads. Unless otherwise stated, the lymphoid cells for GVH assay were removed from the immune donors between 10 days and 3 wk after the last immunization.

*Validity of Comparing Immune and Nonimmune Cells by GVH Assay.*—Quantitative comparison of the GVH activity of immune and nonimmune populations depends (a) on the slopes of the dose-response lines being similar and (b) the tempos of the lymph node responses being similar. Condition (a) could be seen to be fulfilled with each acceptable assay. The time course of the popliteal lymph node enlargement was studied in preliminary experiments in both weak and strong combinations. Graded doses of TDL were given to a large number of recipients. Groups of four at each dose level were killed at days 3, 5, and 7 after injection. In the strong combination there was no difference in the tempo of the responses produced by the immune and nonimmune populations. In weak strain combinations immune cells did not produce a more rapid response but with high doses the lymph nodes ceased enlarging by day 5. In fact, with these immune populations dose saturation corresponded to a lower lymph node response than in nonimmune populations or in strong strain combinations. However, acceptable assays could be performed by using smaller doses of immune cells which gave the same gradation of response as did nonimmune cells.

In another local GVH system (that after the injection of lymphocytes into guinea pig skin) immune and nonimmune cells have also been found to produce reactions which develop at a similar tempo (6).

#### RESULTS

The GVH activity of lymphoid cells from putatively immune donors was assayed in comparison with cells from matched, nonimmune rats. The result of such an assay is expressed as a potency ratio: the ratio of the number of nonimmune cells to the number of immune cells required to produce equal lymph node enlargement. In the case of immune vs. nonimmune assays this potency ratio is called the factor of immunization (F.I.).

##### *Series A: AgB-Identical Strain Combinations* (See Table I)

*Group 1: Immunization with Two Skin Grafts (Assay of TDL).*—Four assays were performed in each of the reciprocal strain combinations AS  $\rightarrow$  F and F  $\rightarrow$  AS. In all eight assays the GVH activity of immune TDL was increased above that of nonimmune, but the mean activity was only doubled by immunization.

*Group 2: Immunization with Two Skin Grafts plus Boosting with Allogeneic Lymphoid Cells (Assay of TDL).*—Four assays were performed in the combination AS  $\rightarrow$  F. The mean F.I. was higher than without boosting and there was no overlap in the results of individual assays.

*Group 3: Immunization with Two Skin Grafts (Assay of Spleen Cells).*—Five assays were performed in the combinations F  $\rightarrow$  AS. The consequence of assaying spleen cells rather than TDL was higher F.I., which again did not overlap with group 1.

*Group 4: Immunization with Two Skin Grafts plus Boosting with Allogeneic Lymphoid Cells (Assay of Spleen Cells).*—In this situation the factors by which group 2 and group 3 differed from group 1 were imposed together. Four assays in the combination AS  $\rightarrow$  F gave very high F.I. with a mean of 52. These F.I. were clearly much greater than in any of the other groups.

Taken as a whole the results of groups 1–4 indicate that the F.I. in weak strain combinations depends on the immunization procedure (even two skin grafts are not optimal) and also on the population of cells used for assay. When the results of groups 2 and 4 are analyzed, it can be estimated that before immunization spleen cells have slightly less GVH activity than TDL (0.5–1.0 times the activity) but after immunization they are 7–10 times more active.

*Group 5: AgB-Heterozygous (AS  $\times$  BN) F<sub>2</sub> Donors Immunized with Two Skin Allografts (F<sub>1</sub>) plus Boosting with Allogeneic (F<sub>1</sub>) Lymphoid Cells (Assay of TDL).*—In this situation the GVH activity is against the antigens present in the F<sub>1</sub> recipients but not the F<sub>2</sub> donor, i.e., antigens determined by the minor

TABLE I  
Factor of Immunization in AgB-Identical Strain Combinations

Group	Strain combination	Cells used for assay	GVH activity of nonimmune*	Immunization procedure	Results of individual assays (F.I.)	Geometric mean of F.I.	Significance
A-1	AS → F	TDL	$32 \times 10^6$	2 skin grafts	2.7, 1.6, 2.6, 1.2,	1.9 } overall mean 2.1 } = 2.0,	Higher than group B-1 (Table II) ( $P < 0.01$ )
A-1	F → AS	TDL	$24 \times 10^6$	2 skin grafts	1.7, 2.8, 2.9, 1.5		
A-2	AS → F	TDL	$32 \times 10^6$	2 skin grafts + lymphoid cells†	3.2, 4.0, 4.4, 2.9	3.6	Higher than group A-1 ( $P < 0.01$ )
A-3	F → AS	Spleen	$24 \times 10^6$	2 skin grafts	3.9, 3.2, 11.0, 4.2, 3.5	4.6	Higher than group A-1 ( $P < 0.01$ )
A-4	AS → F	Spleen	$64 \times 10^6$	2 skin grafts + lymphoid cells†	79, 60, 27, 59	52	Obviously higher than groups A-1, A-2, and A-3
A-5	AgB heterozygous (AS × BN)/F <sub>2</sub> → (AS × BN)/F <sub>1</sub>	TDL	$49 \times 10^6$	2 F <sub>1</sub> skin grafts + lymphoid cells†	Data of 4 assays pooled (See Fig. 1)	7.7	

\* No. of cells required to produce lymph node enlargement to  $10.0 \text{ mg}$  inversely related to strength of combination.

† Three injections of  $100 \times 10^6$  allogeneic lymph node and spleen cells given subcutaneously at weekly intervals.

loci for which the donor happens to be homozygous. Although the donors will on the average be homozygous at half the minor loci the proportion will vary from rat to rat. For this reason it might have been misleading to work out the F.I. on each (arbitrary) immune and nonimmune pair. The results of four assays were combined (Fig. 1) and the overall mean dose-response lines were drawn. By this method the overall F.I. was 7.7, which was twice as high as

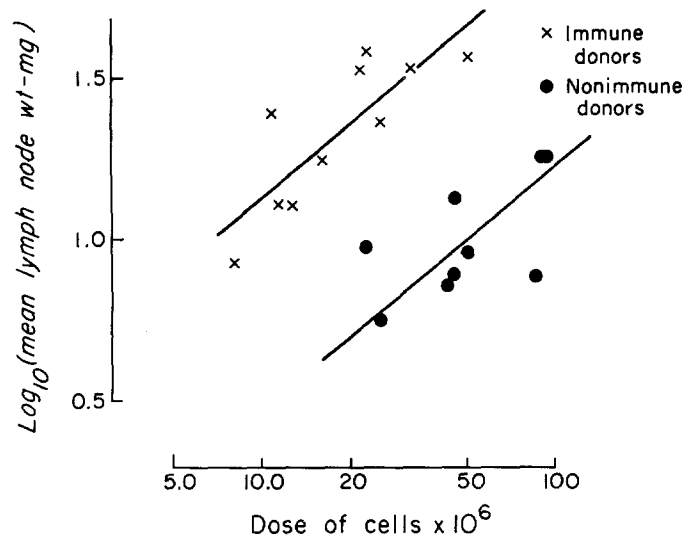


FIG. 1. TDL from AgB heterozygous (AS  $\times$  BN)  $F_2$  donors injected into feet of (AS  $\times$  BN)  $F_1$  recipients. Each point is geometric mean response of four lymph node weights. ●—TDL from four nonimmune donors. X—TDL from four immune donors. Responses not significantly greater than those produced by same dose of syngeneic  $F_1$  TDL have been omitted. Assay lines have been drawn through mean dose/mean response point of each group with a slope equal to the mean slope of the lines of best fit for each group. Immune cells were 7–8 times more potent than nonimmune cells.

the F.I. obtained by assay of TDL in the “ordinary” weak combinations (group 2) and also the F.I. in strong strain combinations (*vide infra*).

#### Series B: AgB-Different Strain Combinations

*Group 1: Immunization with Two Skin Grafts (Assay of TDL).*—Three assays were performed in each of three strain combinations (AS  $\rightarrow$  AS2, AS2  $\rightarrow$  AS, AS  $\rightarrow$  BN). In all assays the GVH activity of immune TDL was greater or the same as nonimmune TDL (Table II). The mean F.I. of 1.4 was very low and was probably lower than the F.I. of corresponding assays in the weak combinations ( $P < 0.01$ ).

*Group 2: Immunization with Two Skin Grafts plus Boosting with Allogeneic*

*Lymphoid Cells (Assay of Spleen Cells).*—Four assays were performed in the combination AS2 → AS. In every case “immune” cells had less GVH activity than nonimmune, giving a mean F.I. of 0.60 (Table II). The F.I. was significantly lower than in the results of the assays in group 1. The finding that assaying spleen cells instead of TDL and boosting with lymphoid cells after grafting decreases the F.I. in strong combinations is in striking contrast to the situation in weak strain combinations where imposition of these two factors increases the F.I. by a factor of about 25.

*Group 3: Assay of Lymphoid Cells at Varying Intervals after a Single Immunizing Injection (Table III):*—(a) In three experiments F donors were immunized by a single intraperitoneal injection of  $100 \times 10^6$  BN spleen and lymph node cells. When spleen cells were taken 4 days after immunization and assayed against normal F spleen cells the F.I. were variable but certainly did

TABLE II  
Factor of Immunization in AgB-Different Strain Combinations

Group	Strain combinations	Cells used for assay	GVH activity of non-immune*	Immunization procedure	Results of individual assays (F.I.)	Geometric mean of F.I.	Significance
B 1	AS → AS2	TDL	$0.63 \times 10^6$	2 skin grafts	1.6, 1.2, 1.0	1.2 } Overall 1.4 } mean 1.5 } = 1.4	F.I. is less than B-1 ( $P < 0.001$ )
B 1	AS2 → AS	TDL	$1.1 \times 10^6$	2 skin grafts	1.3, 1.5, 1.5		
B 1	AS → BN	TDL	$0.42 \times 10^6$	2 skin grafts	1.6, 2.0, 1.0		
B 2	AS2 → AS	Spleen	$1.4 \times 10^6$	2 skin grafts + lymphoid cells†	0.42, 0.94, 0.39, 0.86		

\* No. of cells required to produce lymph node enlargement to 10.0 mg; inversely related to strength of combination.

† Three injections of  $100 \times 10^6$  allogeneic lymph node and spleen cells given subcutaneously at weekly intervals.

not indicate any exceptional increase in GVH activity at this stage. Comparison of methyl green pyronin-stained films of the immune and nonimmune spleen cells showed an obvious increase in the number of large pyroninophilic cells in the immune suspensions.

(b) In three experiments donors were immunized by the injection into the four footpads of a total of  $100 \times 10^6$  BN spleen and lymph node cells. After 4 days the draining lymph nodes (popliteal and brachial) were removed and cell suspensions assayed against cells from the same lymph nodes of non-immune rats. As in the previous subgroup little or no increase in GVH activity was found in these lymph node cells despite the fact that the lymph nodes were enlarged in reaction to the immunization and contained a high proportion of large pyroninophilic cells.

(c) In four experiments F donors were immunized by injection into the four footpads of a total of  $100 \times 10^6$  (F × BN) F<sub>1</sub> hybrid spleen and lymph node cells. After between 3 and 4 wk, when the reaction in the draining nodes had

subsided, cells prepared from the draining nodes were assayed against pooled lymph node cells from nonimmune donors. At the same time nondraining lymph node cells from the immune donors were assayed against nonimmune pooled lymph node cells. There was no significant difference between the GVH activity of the draining and nondraining lymph node cells, and although the F.I. was very variable, the mean value of less than unity suggested a tendency for the GVH activity of lymph node cells to be depressed below normal, as in group 2.

The experiments of series B fully achieved their object, which was to show that the very low F.I. found in the standard experimental situation (group 1) was not increased by altering the time between immunization and assay, the schedule of immunization, or the population of lymphoid cells assayed.

TABLE III  
*Factor of Immunization in AgB-Different Strain Combinations*

Group	Strain combinations	Cells used for assay	Immunization procedure	Interval between immunization and assay	Results of individual assays (F.I.)	Geometric mean
B-3 (a)	F → BN	Spleen	Allogeneic (BN) lymphoid cells i.p.	4 days	0.93, 1.6, 0.73	1.0
B-3 (b)	F → BN	Lymph node	Allogeneic (BN) lymphoid cells into footpads	4 days	1.5, 0.72, 2.8	1.4*
B-3 (c)	F → BN	Lymph node	(BN × F) F <sub>1</sub> hybrid lymphoid cells into footpads	3-4 wk	0.69, 0.58, 1.3, 0.77, 0.47, 0.68, 1.1, 0.62	0.80* 0.68‡

Immunized donor given single injection of  $100 \times 10^6$  spleen and lymph node cells.

\* Draining lymph node cells assayed against nonimmune lymph node cells.

‡ Nondraining lymph node cells assayed against nonimmune lymph node cells.

*Series C: Factor of Immunization in Rats Incapable of an Alloantibody Response*

The low F.I. in strong strain combinations may be a consequence of the high frequency of antigen-sensitive cells in nonimmune animals specifically, directly against a particular complement of strong histocompatibility antigens (1, 2). An alternative explanation is that the humoral alloantibody response, which is readily detectable against strong histocompatibility antigens, is responsible for partly inhibiting the development of the cellular immune response, a process which might be described as partial immunological enhancement. This latter possibility is not, of course, at all inconsistent with the first; both mechanisms may operate. However, if inhibition by alloantibody were the sole factor responsible for the low F.I. in strong strain combinations, then it would be predicted that immunization of an animal which had been manipulated so as to be capable of a cellular but not a humoral response would result in a raised F.I.

A critical dose of whole body X-irradiation inhibits the humoral alloantibody response of mice without detectably prolonging skin allograft survival (7). It was found empirically that a suitable dose of X-irradiation for this purpose in AS and F rats was 300 rads. The survival of skin allografts applied the day after irradiation was not detectably prolonged and the alloantibody response to (BN  $\times$  AS) F<sub>1</sub> hybrid lymphoid cells injected into AS rats was completely suppressed. Seven nonirradiated immunized control rats all produced hemagglutinating alloantibody at titers of 1/27 or 1/81 on day 6 and at 3 wk after immunization, whereas none of seven irradiated, immunized rats had detectable antibody on either day.

In the first three experiments (Table IV, C-1) F rats were immunized with a BN skin graft 1 day after 300 rads of whole body X-irradiation and their TDL were assayed between 10 days and 3 wk later. This assay was done by

TABLE IV  
*Effect on F.I. of 300 Rads of Whole Body X-Irradiation 24 hr before Immunization*

Group	Strain combination	GVH activity of normal TDL*	Immunization procedure	F.I. i.e. irradiated immune	F.I. in non-irradiated controls	Potency ratio of irradiated immune
				irradiated, nonimmune		normal (nonimmune)
C-1	F $\rightarrow$ BN	$0.50 \times 10^6$	Single skin allograft	1.0, 1.6, 0.44	—	0.77, 0.31, 0.23
C-2	AS $\rightarrow$ BN	$0.42 \times 10^6$	$100 \times 10^6$ (AS $\times$ BN)F <sub>1</sub> lymphoid cells s.c.	0.84, 1.8	1.0, 1.5	0.33, 0.40

TDL used for assay.

\* No. of TDL required to produce lymph node enlargement to 10.0 mg inversely related to strength of combination.

comparison with irradiated, nonimmune F donors and also untreated F donors. The thoracic duct outputs of the two irradiated groups were depressed to about 10% of normal and the residual TDL had lower GVH activity than normal. The F.I. was variable but since the highest value was 1.6 there was no suggestion that irradiation before immunization had produced a higher F.I. than in the experiments of series B.

In other experiments (Table IV, C-2) AS rats were immunized 1 day after 300 rads of whole body X-irradiation by injection into the footpads of  $100 \times 10^6$  (AS  $\times$  BN) F<sub>1</sub> spleen and lymph node cells. The GVH activity of their TDL obtained 3 wk after irradiation was compared to that of TDL from irradiated, nonimmune donors. TDL from two other control groups were included, i.e. (a) nonirradiated and immunized and (b) untreated. The results of these assays were very similar to those of the first group. Whole body X-irradiation is followed by a low output of TDL of diminished GVH activity. However, the F.I. remains low as in the experiments of series B. It was remarkable that although irradiated, immunized rats rejected skin allografts



at the usual tempo, the GVH activity of their TDL was in all cases less than that of TDL from normal (nonimmune) donors.

#### DISCUSSION

The object of the present experiments was to measure the change in GVH activity after specific alloimmunization (expressed as the factor of immunization) in a number of experimental situations. In the mouse (1) and in the chicken (8) the F.I. is greater when the antigenic disparity is less; when a strong histocompatibility difference is involved it is near unity. The present results fully confirm earlier results in less satisfactory assay systems (9, 10) which hinted that the dependence of F.I. on antigenic strength is valid in the rat also.

The most dramatic difference in F.I. between weak and strong strain combinations was found when spleen cells were assayed after immunization with two skin allografts followed by boosting injections of allogeneic lymphoid cells. In the weak combination the F.I. of about 50 is very similar to the values from comparable experiments in the mouse (1). In the strong combination the F.I. of less than unity has also a precedent in mice (11, 12). These workers attributed the depression of GVH activity to immunological enhancement. In the present experiments there are no grounds for favoring enhancement over the alternative explanation of partial tolerance. However, preliminary experiments (Simonsen, M., unpublished data) designed to test for the enhancing effect of AgB-hemagglutinating antisera in both directions of the AS-AS2 combination have been performed. Normal spleen cells were mixed with equal volumes of undiluted antisera against the recipient antigen or, for control, with normal sera before injection into the hind feet. No difference in the popliteal lymph node enlargement was observed between test and control groups.

After two skin allografts had been rejected the F.I. in a weak combination could be further increased by boosting with allogeneic lymphoid cells, especially if spleen cells were assayed. The particular effectiveness of living peripheral lymphoid cells in allogeneic immunization as compared with skin grafts may reflect their high content of histocompatibility antigen (13, 14) and their widespread distribution in the spleen and lymph nodes before their destruction by a host-vs.-graft reaction (15).

Spleen cells, as a source of donor cells for the GVH assay, gave a higher F.I. than did TDL in weak combinations. About half of the small lymphocytes in the spleen belong to the same recirculating pool as do the great majority of TDL (16). Under the conditions of these experiments the greatly increased GVH activity of spleen cells after immunization can be attributed mainly to the nonrecirculating population in the spleen. This conclusion can be compared to recent data on immunological memory to conventional antigens (17, 18). Although it was confirmed that immunological memory is carried by recircu-

lating cells (19) these studies showed that the antigenically stimulated lymph node responds to a second injection of antigen more quickly and more strongly than does the contralateral node. Apparently immunological memory is not confined to recirculating lymphocytes but is also strongly represented in the nonrecirculating population of a lymph node.

In AgB-different (strong) strain combinations the several factors which influenced the F.I. in weak combinations were completely ineffective in raising the F.I. It was particularly remarkable that when draining lymph nodes were assayed 4 days after subcutaneous immunization at the height of the large pyroninophilic cell response the F.I. was near unity. By contrast, a large increment of stimulation in mixed-lymphocyte culture was found when spleen cells were removed 4 days after intraperitoneal immunization with allogeneic spleen cells (20) but in a very similar experiment (B-3[a]) no increment was found in GVH assay.

The failure of irradiation applied before immunization to bring about a higher F.I. suggested that the inhibitory effect of alloantibody is not solely responsible for the low F.I. in strong strain combinations. Of course it cannot be certain that partial inhibition of the cellular response did not result from concentrations of enhancing antibody which were too low to be detected. However this result is especially convincing of the intrinsic limitation of the F.I. in strong combinations, since irradiated animals provide a superior environment for immune responses mediated by transferred cells (21); it is reasonable to suppose that the surviving cells reacting to the antigen would be favored in the same way by the extra space available in the lymphoid tissue.

An alternative way to immunize animals incapable of a humoral alloantibody response is to use bursectomized chickens. Experiments are at present being performed in this laboratory to measure the F.I. of such animals. So far the F.I. has not shown any tendency to increase in bursectomized chickens in spite of the absence of a detectable antibody response.

The conclusion that the limitation of the F.I. in strong combinations is a consequence of a very high proportion of antigen-sensitive cells in the non-immune animal (1, 2) has been reinforced by the present experiments. This is quite consistent with the possibility that the cells reactive to strong antigens generate predominantly effector cells (which may not count in GVH assay), whereas the cells reactive to weak antigens generate both effector cells and more of themselves, thus producing high F.I. (22).

#### SUMMARY

Using a popliteal lymph node weight assay the graft-*versus*-host activity of lymphocytes from donors immunized with allogeneic tissue has been assayed by comparison with that of lymphocytes from nonimmune donors. When the donors were immunized against weak histocompatibility antigens (non-AgB)

the specific GVH activity of its lymphocytes was increased. This increase was greater if spleen cells rather than thoracic duct lymphocytes were the source of the donor cells used for assay. The increase in GVH activity was also greater if the standard immunization procedure of two successive skin allografts was followed by three boosting injections of allogeneic lymphoid cells.

When donors were immunized against strong histocompatibility antigens the specific GVH activity of the donors' lymphocytes was slightly increased, was unchanged, or was actually decreased depending on the experimental situation. In donors rendered incapable of a humoral alloantibody response by whole body X-irradiation, immunization across a strong barrier was followed by little or no increase in the specific GVH activity of TDL. In the rat, as in other species, the increase in GVH activity after immunization is inversely proportional to the strength of the antigenic barrier involved.

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