GRAFT-VS.-HOST REACTIONS IN RECIPROCAL HYBRID MICE

I. DISSOCIATION OF T-CELL ACTIVITIES IN THE MIXED LYMPHOCYTE REACTION AND TWO GRAFT-VS.-HOST ASSAYS

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The graft-vs.-host (GVH) reaction produced in F1 recipients of parental lymphocytes is a complex phenomenon determined by many interacting elements. The antigenic disparity between donor and host (1), the host reaction to attack by donor cells (2), modulating effects of the host on donor cell reactivity (3), and the proportional representation of subpopulations of thymus-derived cells (T cells) in the donor inoculum inducing the reaction (4) are but some of the relevant factors which have been elucidated. An additional component of the reaction has been revealed in the recent studies of Uphoff (reviewed in reference 5). She demonstrated that in lethally irradiated reciprocal hybrid F1 mice reconstituted with bone marrow from parental strains, a significant survival advantage was conferred on recipients when the marrow donor strain was syngeneic with the maternal strain of the hybrid cross. It was suggested that maternal modification in utero of the expression of paternal histocompatibility phenotype on F1 cells, resulting in reduced antigenicity, may be responsible for this phenomenon. She also proposed that this effect may serve as a partial explanation for the survival of semiallogeneic fetuses in the uterus of a mother potentially capable of mounting a homograft reaction against fetal histocompatibility antigens of paternal type.

In the current studies we have evaluated the reactivity of parental splenic lymphocytes against tissues from reciprocal hybrid mice in three systems: the mixed lymphocyte reaction (MLR); the Simonsen spleen weight GVH assay; and a GVH mortality assay. Our results reveal no difference in the capacity of parental cells to proliferate in the MLR on exposure to cells from either cross, but demonstrate marked discrepancies in the ability of cells from maternal

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Abbreviations used in this paper: cpm, counts per minute; GVH, graft-vs.-host; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; SEM, standard error of the mean.

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Materials and Methods

Mice.—6- to 8-wk old male BALB/cAnN (H-2<sup>d</sup>) and C57BL/6N (H-2<sup>b</sup>) mice and 1- to 6-day old reciprocal hybrid litters of these strains (BALB/c × C57BL/6) F<sub>1</sub> (H-2<sup>d</sup>/H-2<sup>b</sup>), (BALB/c × C57BL/6) F<sub>1</sub> (H-2<sup>d</sup>/H-2<sup>d</sup>), and (C57BL/6 × BALB/c) F<sub>1</sub> (H-2<sup>b</sup>/H-2<sup>d</sup>) were obtained from the Rodent and Rabbit Production Section, National Institutes of Health, Bethesda, Md. Mothers of all F<sub>1</sub> litters were primiparous.

Mixed Lymphocyte Reactions.—Spleen cell suspensions from adult mice were prepared and cultured as previously described (6). Briefly, 1 × 10<sup>6</sup> spleen cells from C57BL/6 or BALB/c mice were cultured with 1 × 10<sup>6</sup> spleen cells from (BALB/c × C57BL/6) F<sub>1</sub> or (C57BL/6 × BALB/c) F<sub>1</sub> mice in 0.2 ml RPMI 1640, supplemented with 5% fetal calf serum, in round-bottom microtiter wells. Cultures were maintained in a humidified 5% CO<sub>2</sub> atmosphere at 37°C for 72 h. 16 h after the addition of 1 μCi of <sup>3</sup>H-thymidine to each well, the cells were harvested with a microculture harvesting device (7), modified by one of us (M. R. H.)<sup>2</sup> and the amount of radioactivity incorporated into acid precipitable material measured by scintillation spectrometry. Results are expressed as the mean ± the standard error of the mean (SEM) of the counts per minute (cpm) incorporated in four replicate cultures. Stimulation was considered to have occurred when the cpm incorporated in the mixed cultures exceeded the cpm incorporated in control syngeneic mixtures of the stimulator and responder cells. Stimulator cells were not inactivated before addition to mixed cultures. Stimulated/control ratios (S/C) were calculated as

\[
S/C = \frac{2 \times (parent + F_1) \text{ cpm}}{(parent + parent) \text{ cpm} + (F_1 + F_1) \text{ cpm}}.
\]

GVH Reactions.—Cell suspensions from spleens of adult BALB/c and C57BL/6 mice were prepared in Hank's balanced salt solution by mincing with scalpels and filtration through triple-layered gauze.

A modification of the Simonsen spleen-weight assay (8) was performed by administration of varying doses of parental spleen cells to litters of 1-4-day old F<sub>1</sub> recipients. 9 days later, inoculated mice and their littermate controls were sacrificed and the spleen index for each litter determined. The mean spleen index for each cell dose was computed by averaging the mean indices obtained from each litter inoculated with that cell dose. Spleen indices greater than 1.3 are considered to represent significant GVH activity (9).

A GVH mortality assay was performed as described by Tigelaar and Asofsky (10). Briefly, 1-6-day old F<sub>1</sub> mice were sublethally irradiated with 400 R 1-3 h before intraperitoneal injection with varying doses of parental spleen cells. Inoculated mice and unoinoculated controls were observed for 50 days. Since the majority of deaths in control animals (averaging 10%, and due primarily to cannibalism) occurred during the first week, analysis of mortality between days 7 and 50 was used to measure GVH activity. Cumulative mortality was calculated as the percentage of dead mice at day 50 relative to the number alive on day 7. Only male mice were used as donor animals in both GVH assays.

RESULTS

MLR’s.—To determine if spleen cells from either C57BL/6 or BALB/c mice could discriminate between (BALB/c × C57BL/6) F<sub>1</sub> and (C57BL/6 ×
BALB/c F1 target cells in vitro, MLR’s were performed with all combinations of these cells. The results presented in Table I indicate that C57BL/6 and BALB/c spleen cells responded to (BALB/c X C57BL/6) F1 and (C57BL/6 X BALB/c) F1 cells equally. In addition, there is no significant difference between (BALB/c X C57BL/6) F1 and (C57BL/6 X BALB/c) F1 cells in their capacity to stimulate cells of either parental strain in the MLR.

Spleen-Weight GVH Assays.—The in vivo reactivity of C57BL/6 and BALB/c spleen cells in (C57BL/6 X BALB/c) F1 and (BALB/c X C57BL/6) F1 recipients as measured by the Simonsen spleen-weight GVH assay is shown in Fig. 1. The two top panels indicate the dose-response curves obtained on injection of parental cells from both strains into either (C57BL/6 X BALB/c) F1 (Fig. 1, a) or (BALB/c X C57BL/6) (Fig. 1, b) recipients. In agreement with the MLR studies, the reactivity of spleen cells from each parental line in either hybrid cross was essentially equivalent. In the two lower panels, this data has been replotted to show comparisons of the activity of cells from one parental, C57BL/6 (Fig. 1, c) or BALB/c (Fig. 1, d), with both F1 recipients. Contrary to the predictions of the MLR’s, greater splenomegaly was observed at all cell doses tested in (BALB/c X C57BL/6) F1 than (C57BL/6 X BALB/c) F1, regardless of the grafted parental strain.

Mortality GVH Assays.—The GVH reactivity of parental spleen cells in the reciprocal hybrids was also assessed in the mortality assay (Fig. 2). The top three panels of this figure indicate the cumulative mortality over time resulting from inoculation of (BALB/c X C57BL/6) F1 or (C57BL/6 X BALB/c) F1 recipients with 2.5 X 10^6, 5 X 10^6, or 10 X 10^6 BALB/c spleen cells. The lower three panels present the cumulative mortality over time observed following in-

### TABLE I

| Spleen cell mixture*                                      | [H]Thymidine Incorporation (cpm Mean ± SEM) | S/C†       |
|-----------------------------------------------------------|---------------------------------------------|-----------|
| C57BL/6 + C57BL/6                                         | 3,582 ± 450                                 | 3.57      |
| C57BL/6 + (C57BL/6 X BALB/c) F1                           | 8,994 ± 368                                 | 3.05      |
| C57BL/6 + (BALB/c X C57BL/6) F1                           | 9,056 ± 524                                 | 3.06      |
| BALB/c + BALB/c                                           | 6,449 ± 407                                 | 3.05      |
| BALB/c + (C57BL/6 X BALB/c) F1                            | 12,059 ± 658                                | 3.06      |
| BALB/c + (BALB/c X C57BL/6) F1                            | 11,982 ± 1072                               | 3.06      |
| (C57BL/6 X BALB/c) F1 + (C57BL/6 X BALB/c) F1             | 1,449 ± 89                                  | 3.06      |
| (BALB/c X C57BL/6) F1 + (BALB/c X C57BL/6) F1             | 1,394 ± 191                                 | 3.06      |

* See Materials and Methods for details of conditions.
† Stimulated/control ratio (S/C) = \(\frac{2 \times (\text{parent} + \text{F1}) \text{ cpm}}{\text{(Parent + parent) cpm + (F1 + F1) cpm}}\)
Fig. 1. Simonsen spleen-weight assay of the graft-vs.-host reactivity of spleen cells from BALB/c and C57BL/6 donors in (C57BL/6 X BALB/c) F1 and (BALB/c X C57BL/6) F1 hosts. Each point represents the mean spleen index obtained from 6-22 recipient litters. Vertical bars indicate the limits of 1 SEM. (See text for further details.)

oculation of these F1 mice with the same doses of C57BL/6 spleen cells. These results demonstrate that at spleen cell doses of 5 X 10^6 and 10 X 10^6, inocula of BALB/c spleen cells produced greater mortality in (C57BL/6 X BALB/c) F1 than (BALB/c X C57BL/6) F1 mice (Fig. 1, a-c). By comparison, mortality produced in the reciprocal hybrids by administration of C57BL/6 spleen cells is greater at each cell dose tested in (BALB/c X C57BL/6) F1 than (C57BL/6 X BALB/c) F1 recipients, although the differences are great only at a dose of 2.5 X 10^6 cells. Both male and female mice were survivors in all assay combinations.

To further evaluate the differing reactivities observed in these combinations, the percentages of 50 day mortalities (from Fig. 2) were plotted on a probit scale as a function of numbers of cells injected, plotted on a logarithmic scale (Fig. 3). The left hand panel demonstrates the mortality observed in
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Fig. 2. Mortality assay of graft-vs.-host reactivity of spleen cells from BALB/c and C57BL/6 donors in irradiated (C57BL/6 × BALB/c) F1 and (BALB/c × C57BL/6) F1 hosts. Each line represents the cumulative mortality observed in 25–43 recipient F1 mice in two experiments over a 50 day observation period.

Fig. 3. Comparisons of cumulative percent mortality among irradiated F1 recipients of graded numbers of BALB/c and C57BL/6 spleen cells over a 50 day observation period. The 50 day percent mortalities are plotted on a probit scale as a function of the logarithm of the cell inoculum.

(C57BL/6 × BALB/c) F1 recipients of BALB/c or C57BL/6 spleen cells while the right panel presents the data for (BALB/c × C57BL/6) F1 recipients of parental cells. In contrast to the findings of the spleen-weight GVH assay which demonstrated differential reactivity dependent on the hybrid cross (BALB/c × C57BL/6) F1's giving greater splenomegaly than (C57BL/6 × BALB/c) F1's, C57BL/6 spleen cells are more lethal than BALB/c cells regardless of the F1 host employed.
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The results presented in this paper can be summarized as indicated in Table II. The top two lines present the relative reactivities in the MLR, spleen-weight GVH assay, and mortality GVH assay of (BALB/c X C57BL/6) F1, as compared with (C57BL/6 X BALB/c) F1 mice ([BALB/c X C57BL/6] F1/ [C57BL/6 X BALB/c] F1), on exposure to spleen cells from either parental strain. The two lower lines compare the relative reactivities of parental spleen cells (BALB/c/C57BL/6) when presented with either (C57BL/6 X BALB/c) F1 or (BALB/c X C57BL/6) F1 cells. Because reactivity curves did not form parallel lines in either the spleen-weight (Fig. 1) or mortality GVH assays, a more quantitative comparison of activities in these assays cannot be made. However, this simplified analysis does permit a useful qualitative evaluation of the relative reactivities observed.

**DISCUSSION**

It has been generally accepted that genetically determined phenotypic differences between mouse strains detected by (a) elicitation of alloantibodies on cross immunization, (b) stimulation in one-way MLR's, (c) rapid skin graft rejection, or (d) development of GVH reactions in mice incapable of responding to donor cells were identical and coded for by the H-2 region of the major histocompatibility complex (MHC). The laws of Mendelian genetics dictate that reciprocal hybrid mice should have identical genotypes and phenotypes and would not predict differential capacity for cells from these crosses to stimulate parental cells in the MLR or for parental cells to show differential reactivity with these hybrids in GVH assays. In keeping with this view, our studies with reciprocal hybrids, (BALB/c X C57BL/6) F1's and (C57BL/6 X BALB/c) F1's, revealed no differences between these strains in their capacity to stimulate parental BALB/c or C57BL/6 cells in the MLR (Table I). However, the demonstration (Figs. 1-3, Table II) of marked discrepancies between these hybrids in their responses to parental cells in the spleen-weight and mortality GVH assays directly challenges this dogma.
Several possible explanations for these heretical observations of variable reactivity between reciprocal hybrids should be considered: (a) maternal modification of histocompatibility antigens; (b) antireceptor antibodies; (c) suppressor cells; and (d) enhancing antibodies.

In studies of several pairs of reciprocal hybrid mice (5, 11-13) lethally irradiated and inoculated with bone marrow from both parental strains, Uphoff has demonstrated a significant reduction in mortality when marrow donors were syngeneic with the female of the hybrid cross. As late mortality in this system is indicative of GVH disease, her findings are completely predictive of the mortality assay data presented in Figs. 2 and 3; less mortality was observed in \((C57BL/6 \times BALB/c)\) F1 than \((BALB/c \times C57BL/6)\) F1 recipients of C57BL/6 cells and BALB/c spleen cells were more lethal for \((C57BL/6 \times BALB/c)\) F1 than \((BALB/c \times C57BL/6)\) F1 hosts. The mortality assay employed in the present study is more “purely immunologic” than the marrow transplantation system used by Uphoff. In her studies marrow cells serve two functions; protection of the irradiated host and production of GVH disease. In our system, cell inoculation is not required for survival of irradiated mice and essentially all deaths are due to GVH disease (10). While these findings stand in sharp contrast to the predictions of the MLR’s, it should be noted that MLR’s were performed with mixtures of adult parental and adult F1 cells whereas both the spleen-weight and mortality GVH assays involve exposure of neonatal F1 tissues to adult parental cells. By comparison with adult tissues, neonatal cells have been shown to have reduced stimulatory capacity in the MLR (14). However, neonatal tissues of these crosses should not differ and the differences seen between MLR and GVH mortality are still unexpected.

One explanation for these findings has been offered by Uphoff (11). She suggests that reduced antigenicity of the parental phenotype on F1 cells as a result of maternal modifications is responsible for the differential susceptibility of F1 mice to GVH mortality. She has also presented data to indicate that similar “antigenic defects” can be inherited in that ova transplant strain RIIIeB (RIII strain fetuses transferred to pregnant C57BL mice with the RIIIeB line derived from the progeny) is less susceptible to mortality induced by C57BL cells than is the ancestral RIII strain (13). Although a mechanism for this modification has not been described, confirmation of its heritable nature would be extremely important, for none of the other alterations to be discussed should be reflected in the genetic makeup of host animals.

Ramseier and Lindenmann have shown that adult F1 mice and rats multiply injected with lymph node cells from one parental strain produce antibodies reactive with the recognition structures by means of which these cells react with alloantigens of the other parental line (15, 16). These “alotypic” antibodies are capable of reducing the capacity of lymphoid cells from the immunizing strain to react with F1 tissues. Moreover, exposure of adult cells to such sera reduced the GVH mortality produced in recipient mice as compared with that
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resulting from inoculation of untreated cells (17)—an assay system quite similar to that employed in the present study. Although in our investigations neonatal F₁ mice were exposed to but one injection of parental cells, such antibodies could have been formed, reducing the effector capacity of the donor cell inoculum. If this were the case, it would require that (BALB/c × C57BL/6) F₁'s produce more antibody than (C57BL/6 × BALB/c) F₁'s on exposure to BALB/c spleen cells which were considerably more lethal in the latter strain (Figs. 2 and 3; Table II). Against this possibility are the data obtained in the Simonsen assay (Fig. 1) which demonstrates that greater splenomegaly was produced in (BALB/c × C57BL/6) F₁ than (C57BL/6 × BALB/c) F₁ recipients of BALB/c spleen cells. Since this antibody should be produced only after some period of time, the observation of discrepancies between spleen-weight and mortality assays might also be explained.

Under certain conditions, F₁ cells injected with parental lymphocytes reduce the splenomegaly produced in sublethally irradiated F₁ hosts from that effected by parental cells alone (3). Sublethally irradiated recipients were also employed in our mortality assay, but to obtain the observed effects (Fig. 1) one would have to postulate a differential capacity of the reciprocal hybrids to suppress the activity of parental effector cells. The data from the spleen-weight assay would suggest that (BALB/c × C57BL/6) F₁'s were less effective suppressors of parental cells than (C57BL/6 × BALB/c) F₁'s in that consistently greater splenomegaly is obtained with the former hybrid cross as hosts (Fig. 1), but this prediction is not supported by mortality assay data (Figs. 2 and 3).

Finally, Hellström et al. (18) have shown that "enhancing antibodies" from mice immune to paternal alloantigens protect F₁ cells from destruction by immune maternal lymphocytes. This antibody could be formed during pregnancy as a result of maternal exposure to paternal antigens with subsequent transplacental passage, or could be produced by occasional maternal cells gaining access to the F₁ fetus in utero (19). Again, such antibodies might affect the results seen in the mortality but not the spleen-weight GVH assays.

The possibility should also be considered that the modifying substances mentioned above, be they antibodies or the products of suppressor cells, might have their effects only early or late in the GVH reaction. For instance, maternally transferred antibody would have a relatively short half-life, might affect the spleen weight assay which is terminated 9 days after inoculation of donor cells while having no effect on the 50 day mortality assay in which data accumulation is initiated at day 7. By comparison, maternally transferred cells may require a longer period of time to produce alloantibodies with this effect becoming evident only in the long-term mortality assay.

Recent studies have demonstrated that in both splenomegaly (4, 20, 21) and mortality assays (4, 10) of GVH activity, two populations of T cells interact synergistically to produce increased spleen weight or death in F₁ recipients of parental cells. Data from this work also suggested that the effector cells pro-
ducing splenomegaly or death may be different (10). The latter study (10) was performed with (C57BL/6 × BALB/c) F1 recipients of BALB/c cells and demonstrated that by comparison with reactivity curves for thymocytes in spleen-weight and mortality assays, spleen and peripheral lymph node cells had different relative capacities to produce splenomegaly or death. The data in the present studies show a similar dissociation of splenomegaly and mortality. BALB/c spleen cells are quite effective in producing splenomegaly in (BALB/c × C57BL/6) F1 mice (Fig. 1) but have minimal activity in the mortality assay with this host line (Figs. 2 and 3).

The studies of Livnat et al. (22) in strains of mice serologically indistinguishable and known to differ only at intra-MHC but non-H-2 loci [BIO-A (2R) and BIO-A (4R)] are also relevant to our investigations. They demonstrated that while it was possible to elicit GVH splenomegaly by inoculation of 2R neonates with 4R cells and that 2R cells serve as good stimulators for 4R cells in the MLR, skin grafts exchanged between these strains survive indefinitely, thus dissociating GVH effector cells from those responsible for skin graft rejection. Taken together, these studies suggest that predictions for activity of lymphocytes in one T-cell system, e.g. spleen-weight GVH, based on their reactivity in another assay, e.g. mortality, may be unjustified in that these systems may reflect the activity of different T-cell subpopulations. However, caution is indicated in using these data to indicate heterogeneity if any of the above mentioned speculations on alteration of GVH reactivity with time are true.

It should be emphasized that the latter studies (22) were performed in mice with known genetic differences, this being predictive of phenotypic variation as well as the possibility of detecting these differences with appropriate assay systems. The central paradox of the current investigations is that (BALB/c × C57BL/6) F1 and (C57BL/6 × BALB/c) F1 mice which should be genotypically and phenotypically identical vary so greatly on exposure to parental cells. While it is clear that no unifying explanation is currently available for our findings, it is also evident that should the basis for reduced mortality of (BALB/c × C57BL/6) F1 recipients of BALB/c cells be defined, the opportunities for routine clinical marrow transplantation could be greatly expanded.

SUMMARY

Spleen cells from BALB/c and C57BL/6 mice were tested for their reactivity against reciprocal hybrid tissues ((BALB/c × C57BL/6) F1 and (C57BL/6 × BALB/c) F1) in three assay systems: the mixed lymphocyte reaction (MLR); the Simonsen spleen-weight graft-vs.-host (GVH) assay; and a GVH mortality assay. It was shown that both F1's serve as equally effective stimulators of parental cells in the MLR. In the spleen-weight assay, BALB/c and C57BL/6 cells were equally active in a given host, but greater splenomegaly was observed in (BALB/c × C57BL/6) F1 hosts regardless of the donor strain. By contrast, BALB/c cells were much less lethal than C57BL/6 cells in (BALB/c ×
C57BL/6) F1 hosts than in (C57BL/6 × BALB/c) F1 hosts, and to a lesser
degree C57BL/6 cells were less lethal than BALB/c cells in (C57BL/6 × 
BALB/c) F1 hosts. The possibility that modifying substances may differen-
tially alter reactivity of parental lymphocytes and that considerations other
than genotype determine the outcome of a GVH reaction are discussed in detail.

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