NEGATIVE SELECTION OF T CELLS CAUSING
LETHAL GRAFT-VERSUS-HOST DISEASE
ACROSS MINOR HISTOCOMPATIBILITY BARRIERS
Role of the H-2 Complex*

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Transfer of unprimed H-2-compatible lymphoid cells into heavily irradiated allogeneic mice causes a high incidence of lethal graft-versus-host disease (GVHD) in certain strain combinations (1). GVHD reflects alloaggression by specifically reactive T (Thy-1-positive) lymphocytes that respond to the multiple minor histocompatibility antigens (HA) of the host.

The mechanism by which T cells elicit lethal GVHD to minor HA is obscure. A question of particular interest is whether the response is restricted by the major histocompatibility complex (MHC), i.e., as for cytotoxic T cells (2–5), delayed-type hypersensitivity (6), and T-B collaboration (7). In the case of cytotoxic T cells generated against minor HA in vitro, both the effector phase (4, 5) and the induction phase (8) show marked H-2 restriction. Because cytotoxic T cells for minor HA are difficult to demonstrate in vivo, however, the relevance of these findings to the induction of GVHD is questionable. Perhaps of more direct relevance is the finding that rejection of minor HA-bearing skin grafts fails to show H-2 restriction (9–12). Hence, one might be tempted to dismiss the known examples of H-2 restriction to minor HA in vitro as being nonphysiological and having little to do with the response in vivo.

To examine this question we investigated whether the T cells that cause lethal GVHD to minor HA in H-2-compatible combinations exhibit H-2 restriction in the early induction phase. From studies on the GVHD reactivity of unprimed T cells filtered from blood to lymph for 1 d through minor HA-different irradiated mice of various H-2 haplotypes, we conclude that precursor T cells fail to respond to minor HA presented on H-2-different cells in vivo. These findings imply that at least one component of GVHD to minor HA is H-2 restricted.

Materials and Methods

MICE. CBA/J (CBA), B10.BR/SgSn (both H-2k), C57BL/10J (B10) (H-2b), B10.D2 (H-2d), and B10.A (H-2a) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

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Abbreviations used in this paper: BM, bone marrow; GVHD, graft-versus-host disease; HA, histocompatibility antigens; LN, lymph node; MHC, major histocompatibility complex; MST, median survival time(s); TDL, thoracic duct lymphocytes.
B10.K (H-2\text{k}) and B10.AQR (H-2\text{k}) (fourth backcross generation) mice were a gift from W. L. Elkins, University of Pennsylvania, Philadelphia, Pa. B10.OL (H-2\text{k}) and B10.TL (H-2\text{k}) mice were kindly provided by C. David, Mayo Clinic, Rochester, Minn. B10.A(4R) (H-2\text{k}4), B10.HTT (H-2\text{k}), and B10.S (H-2\text{k}) mice were donated by P. Doherty and B. Knowles, both at The Wistar Institute, Philadelphia, Pa. (B10.A × B10.OL)F\text{1} and (B10 × CBA)F\text{1} mice were bred in our own colony (University of Pennsylvania). (C57BL/6 [B6, H-2\text{b}] × CBA)F\text{1} mice were obtained from Cumberland View Farms, Clinton, Tenn. Male mice were used as cell donors, filtration hosts, and recipients.

Medium. RPMI-1640 (Microbiological Associates, Walkersville, Md.) supplemented with 2% fetal calf serum was used.

Injections. Cell suspensions were given intravenously via the tail vein in vol of 0.5-1.0 ml.

Preparations of Cells. Suspensions of bone marrow (BM) cells and lymph node (LN) cells (pooled from mesenteric, axillary, inguinal, and cervical nodes) were prepared as described previously (1, 13). BM cells were depleted of mature T cells by treatment with anti-Thy-1.2 serum and complement (guinea pig serum) (1).

Antisera. B10 anti-B10.D2 (anti-H-2\text{d}) antiserum was provided by H. R. Snodgrass, University of Pennsylvania. C57BL/6 anti-CBA (anti-H-2\text{k}) and anti-Thy-1.2 antisera were prepared as described previously (1).

Selection to Minor HA. The filtration procedure was essentially similar to that reported previously for inducing selection to H-2 determinants (13). In brief, 1-2 × 10\text{9} CBA LN cells were injected intravenously into irradiated (850 rad) syngeneic or minor HA-different allogeneic hosts. Thoracic duct cannulae were inserted in the recipients \(\approx\) 15 h later, and thoracic duct lymphocytes (TDL) were collected between 18 and 40 h after the LN cell injection; TDL were pooled from two to four mice per group. In H-2-compatible situations, testing with appropriate alloantisera and complement (13) showed that \(\geq 95\%\) of the lymph-borne cells were T (Thy-1-positive) cells of donor-strain origin. For positive selection, the TDL were collected on day 5 postinjection, the mice being cannulated on day 4.

Irradiation. Mice were exposed to \(^{137}\text{Cs}\)-\(\gamma\)-irradiation at a dose of \(\approx\) 100 rad/min.

Mortality Assay for GVHD. As described previously (1), 2- to 4-mo-old B10.BR mice were exposed to a midlethal dose of irradiation (750 rad) and then, \(\approx\) 6 h later, were injected intravenously with a mixture of T cell-depleted CBA BM (10\text{7} viable cells treated with anti-Thy-1.2 serum and complement) together with the test population of CBA T cells. The use of donor rather than host marrow limited the possibility that GVHD was simply a reflection of hematopoietic failure after marrow destruction by the donor T cells. Recipients of marrow cells alone served as controls. Groups of mice were placed in separate cages in a laminar flow room and were checked for mortality three to five times per week for 80 d postirradiation. Neomycin (50 \(\mu\text{g/liter}\) (American Pharmaceutical Co., Passaic, N. J.) and Polymyxin B (50 \(\mu\text{g/ml}\)) (Burroughs Wellcome Co., Research Triangle Park, N. C.) were added to the drinking water for the first 3 wk postirradiation.

Statistical Analysis. Median survival times (MST) were calculated according to Litchfield (14), and the Wilcoxon-Mann-Whitney two-sample rank test (15, 16) was used to compare median survival times between individual experimental groups.

Results

Experimental Design. In the case of MHC differences, transfer of T cells into irradiated MHC-incompatible rats (17) or mice (13) leads to a sequence of negative and positive selection of the donor T cell response to the alloantigens of the host. At 1–2 d posttransfer, the reactive T cells become sequestered in the lymphoid tissues where they proliferate extensively. During this stage of negative selection the thoracic duct lymph is specifically devoid of host-reactive T cells. After day 3, the reactive cells recenter the circulation in large numbers as blast cells, the stage of positive selection.

The aim of this study was first to demonstrate that analogous selection could be induced to minor HA, and then to investigate whether selection was restricted by the H-2 determinants of the minor HA-different hosts used for selection. The approach
was to determine whether acute recirculation of CBA (H-2^b) T cells through irradiated H-2-different mice of the B10 congenic lines interfered with the capacity of the donor cells to cause lethal GVHD after transfer to irradiated H-2-compatible B10.BR mice. (As discussed elsewhere [1], GVHD in the CBA → B10.BR combination appears to be directed predominantly to the multiple [six or more] minor HA differences of the host rather than to the other antigenic differences separating the two strains, e.g., Ly, Qa, and Mls determinants.)

Doses of 1-2 × 10^6 unprimed CBA LN cells were transferred intravenously into heavily irradiated (850 rad) syngeneic mice or allogeneic mice of the B10 congenic lines and recovered from thoracic duct lymph of the recipients at 18-40 h postinjection. The filtered CBA T cells (>95% Thy-1.2 positive) were then transferred with anti-Thy-1.2-treated CBA marrow cells into irradiated (750 rad) B10.BR mice (Materials and Methods). Mortality of the recipients was studied over 80 d.

**Negative Selection to Minor HA in Irradiated Mice of the B10 Congenic Lines.** The data illustrated in Figs. 1-3 and summarized in Table I show the incidence of lethal GVHD (mortality) in irradiated B10.BR mice given small doses (≤2 × 10^6) of CBA T cells recirculated through irradiated mice of various strains.

CBA T cells filtered through irradiated syngeneic mice caused 100% mortality within 50 d. GVHD failed to occur, however, when the cells were filtered through irradiated B10.BR mice or through mice of the closely related B10.K strain (Fig. 1; Table I). Unresponsiveness (negative selection) was specific because CBA cells filtered...
Fig. 2. Features of selection to minor HA. The data show mortality in irradiated (750 rad) B10.BR mice given CBA (H-2k) T cells plus T cell-depleted CBA marrow as for Fig. 1. CBA and B10.BR: 10⁶ CBA T cells filtered for 1 d through irradiated CBA or B10.BR mice, respectively; CBA + B10.BR: a mixture (10⁶ of each) of CBA T cells filtered through CBA or B10.BR mice, respectively; B10.D2 (α-H-2k): 10⁶ viable CBA T cells treated with anti-H-2k serum plus complement after filtration for 1 d through irradiated B10.D2 (H-2k) mice; B10.BR (day 5): 10⁶ CBA T cells derived from irradiated B10.BR mice given CBA LN 5 d before. The data were from a single experiment that involved six mice per group, except for the B10.D2 (α-H-2k) group, which was from a separate experiment. The latter group (MST = 32.9 ± 1.2 d) was run in parallel with a separate group of 10⁶ syngeneic-filtered CBA T cells (not illustrated) that gave an MST of 34.6 ± 1.2 d.

Fig. 3. H-2 restriction of selection to minor HA with limited doses of T cells. The data show mortality in irradiated (750 rad) B10.BR mice given CBA T cells plus T cell-depleted CBA marrow as in Fig. 1. The CBA T cells were transferred in doses of 10⁵ or 10⁶ after recirculation for 1 d through irradiated CBA (CBA 10⁵ and CBA 10⁶), B10 (B10 10⁵ and B10 10⁶) or (B10 × CBA)F₁ mice [(B10 × CBA)F₁ 10⁶]. The data were from a single experiment that involved six mice per group.

through irradiated B10.BR mice (CBA → B10.BR T cells) caused 100% mortality when transferred to H-2-different (B6 [H-2b] × CBA)F₁ mice (Table I, footnote). Suppression did not appear to be involved because a mixture of CBA → CBA and CBA → B10.BR T cells caused rapid mortality (Fig. 2). Negative selection was followed by positive
# Table I

*Mortality in Irradiated B10.BR Mice Given H-2-Compatible Minor HA-Different T Cells after Filtration through Heavily Irradiated Mice of Various Strains*

| Irradiated selection hosts used for recirculation of CBA LN cells* | H-2 haplotype of selection hosts | Dose of T cells transferred to irradiated B10.BR mice (× 10⁶) | 80-d mortality of irradiated B10.BR mice |
|---|---|---|---|
| | | | Dead/total | Number of experiments | MST ± SD |
| CBA | kkkkkkkk | 2.0 | 18/18 (3) | | 24.9 ± 1.7¶ |
| B10.BR | kkkkkkkk | 1.0 | 22/22 (4) | | 29.3 ± 1.5_parse_t Reference_1 |
| | | 0.1 | 6/6 (1) | | 39.0 ± 1.6 |
| B10 | bbbbbbb | 2.0 | 18/18 (3) | | 23.3 ± 1.7 ¶ |
| | | 1.0 | 5/5 (1) | | 26.2 ± 1.2² |
| | | 0.1 | 5/6 (1) | | 34.3 ± 1.8³ |
| B10.D2 | dddddddd | 2.0 | 6/6 (1) | | 27.6 ± 1.3⁴ |
| | | 1.0 | 6/6 (1) | | 32.9 ± 1.2 |
| B10.K | kkkkkkkk | 2.0 | 0/6 (1) | | | |
| B10.S | sssssss | 1.0 | 8/8 (1) | | 30.4 ± 1.3⁷ |
| (B10 × CBA)F₁ | bbbbbbb | 1.0 | 0/6 (1) | | | |
| B10.A(4R) | kbbbbb | 1.0 | 10/12 (2) | | 36.2 ± 1.7⁸ |
| B10.A | kkkkkkdd | 1.0 | 11/12 (2) | | 36.0 ± 2.3⁹ |
| B10.OI | ddddddd | 1.0 | 12/12 (2) | | 21.5 ± 1.5¹⁰ |
| B10.AQR† | kkkkkkdd | 1.0 | 19/20 (3) | | 32.8 ± 2.2¹¹ |
| B10.HTT | ssskkkkk | 1.0 | 8/8 (1) | | 28.4 ± 1.7¹² |
| B10.TL | skkkkkkk | 1.0 | 7/8 (1) | | 31.4 ± 1.4¹³ |
| (B10.A × B10.OI)F₁ | kkkkkkdd | 1.0 | 3/12 (2) | | | |
| CBA BM only (no T cells) | | | 1/48 (8) | | | |
| Irradiation only (no BM, no T cells) | | | 14/16 (4) | | 15.8 ± 1.2 |

* Selection hosts were given 850 rad plus 1-2 × 10⁶ CBA LN intravenously. The donor T cells were collected from thoracic duct lymph of the recipients 18-40 h later and transferred intravenously into irradiated (750 rad) B10.BR mice plus 10⁷ anti-Thy-1.2 serum-treated CBA BM.

† Although only of the fourth backcross generation, these mice carry ~94% of the B10 genome.

‡ Transfer of 2 × 10⁶ CBA-H-2b T cells plus T cell-depleted CBA BM into six irradiated (750 rad) (CBA × B6)F₁ mice caused 100% mortality (MST = 9.5 ± 1.2 d); no mortality occurred in controls given CBA BM alone.

¶ Treated with anti-H-2³ antiserum and complement before transfer.

* P values: P > 0.05 for 1 vs. 2, 1 vs. 6, 2 vs. 4, 2 vs. 8, 2 vs. 9, 2 vs. 11, 2 vs. 12, 2 vs. 13, 3 vs. 5, 7 vs. 12, 7 vs. 13, and 9 vs. 10; P = 0.05 for 2 vs. 3, P = 0.02 for 2 vs. 10 and 8 vs. 10.

In marked contrast to filtration through H-2-compatible B10.BR or B10.K mice, CBA (H-2b) T cells recirculated through irradiated H-2-different B10 (H-2³) or
B10.D2 (H-2d) mice caused rapid mortality in B10.BR mice (Fig. 1; Table I). Studies with limited doses of T cells (10⁵ and 10⁶) indicated that the potency of CBA-cBA and CBA-B10 T cells was quite similar (Fig. 3; Table I). To exclude the remote possibility that the minor (=5%) component of radioresistant host cells was the cause of GVHD after filtration through H-2-different mice, the filtered T cells were treated with anti-host alloantiserum and complement before transfer. As shown in Fig. 2, CBA-B10.D2 T cells treated with anti-H-2d serum after filtration retained the capacity to cause rapid mortality in B10.BR mice.

It could be objected that the failure to cause negative selection to minor HA in H-2-different mice was the result of antigenic competition as a result of the strong donor response to the host H-2 determinants. To counter this argument, negative selection was also studied in H-2-semiallogeneic mice. As shown in Fig. 3, CBA T cells caused no mortality in B10.BR mice after filtration through (B10 × CBA)F₁ mice.

**Negative Selection to Minor HA in Irradiated B10 Recombinant Mice.** The preceding data indicated that negative selection of CBA T cells to the minor HA of the B10 background required a sharing of H-2 determinants between the donor T cells and the hosts used for filtration. To attempt to define which part of the H-2 complex controlled selection, B10 congenic lines with H-2-recombinant haplotypes were used for filtration.

The data in Fig. 4 (and Table I) show the effects of filtering CBA (R^k I-A^k I-B^k I-J^k I-E^k I-C^k S^k D^k) (kkkkkkkk) T cells through irradiated B10.A (kkkkkkkk), B10.A(4R) (kkhhhhhh), B10.OL (dddddtkk), B10a.AQR (gkkkkkdd), or (B10.A × B10.OL)F₁ mice. T cells recirculated through H-2S/D-compatible hosts (B10.OL) caused rapid mortality after transfer to B10.BR mice (curiously with a shorter MST than with the control CBA-cBA cells [Table I]). Likewise, T cells filtered through H-2K/I-matched
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Fig. 5. Failure to induce negative selection to minor HA in H-2 recombinant mice matched with the responder only in the I region. The data show mortality in irradiated B10.BR mice given 10^6 CBA T cells plus T cell-depleted CBA marrow as in Fig. 1. The responder cells were compatible with the filtration host either in the entire I region from I-A through S (B10.TL skkkkkkd) or only in the IE/C and S regions (B10.HTT ssskkkkk); H-2-compatible B10.BR (kkkkkkkd) and totally H-2-different B10.S (ssssssss) mice were used as controls; additional controls with CBA cells filtered through CBA mice (not shown for simplicity) caused 100% mortality with an MST of 31.2 ± 1.2 d (compared with MST of 28.4 ± 1.7, 31.4 ± 1.4, and 30.4 ± 1.3 d for cells filtered through B10.HTT, B10.TL, and B10.S, respectively). The data are from a single experiment that involved eight mice per group.

Discussion

The main finding in this paper is that negative selection of T cells that cause lethal GVHD to minor HA in H-2-compatible mice required a full sharing of H-2 determinants between the donor T cells and the irradiated minor HA-different intermediate hosts used for selection. In the case of both H-2-compatible and H-2-semiallogeneic combinations, negative selection was marked. Thus, whereas syngeneic-passaged CBA T cells caused 100% mortality in B10.BR mice with doses as low as 10^5 cells, 20-fold higher doses of T cells filtered through H-2-compatible B10 congenic hosts (B10.BR and B10.K) failed to elicit GVHD. In direct contrast, T cell filtration through totally
H-2-different B10, B10.D2, or B10.S hosts failed to cause detectable selection. In this situation, within the limits of the assay system the potency of the filtered T cells was equivalent to that of the control T cells. These data imply that, at least in the induction phase, GVHD is not directed to minor HA but to minor HA associated with self H-2 determinants. Whether analogous restriction applies during the effector phase remains to be established. Because virtually nothing is known of the properties and mechanism of action of the effector cells involved in GVHD, answering this question will clearly be difficult.

The use of H-2 recombinant mice as selection hosts indicated that selection depended upon the donor and host being matched at both the K- and D-ends of the H-2 complex, e.g., as with filtration through (B10.A × B10.OL)F1 (KkDd × KdDk) mice. Matching only at the H-2K/I, H-2S/D, H-2I, or H-2I/S regions failed to cause demonstrable selection, i.e., in these situations the GVHD-inducing capacity of the filtered T cells was not discernably less than that of cells passaged through syngenic hosts. How can these data be explained? The simplest explanation is that as for cytotoxic T cells (24), the cells controlling GVHD to minor HA comprise two discrete subgroups of T cells restricted to H-2K and H-2D determinants, respectively. Filtration through B10 minor HA-bearing hosts matched with the donor at only one end of the H-2 complex (e.g., at H-2D) would thus be expected to remove (select) the subgroup restricted to Dk plus minor HA but not affect the Kk-restricted subgroup; the reverse would apply to matching only at H-2K. Assuming that the T cell subgroups are of equal potency and do not cross-react, the reactivity of the filtered T cells would thus be reduced by a factor of only twofold. Here it should be noted that the assay system is not sufficiently sensitive to detect such a difference, even with large group sizes (Table I, line 1 vs. line 2).

This interpretation admittedly rests on a number of unsubstantiated assumptions (and fails to explain the paradoxical increased potency of CBA T cells filtered through B10.OL mice [Table I]). Nevertheless it is difficult to think of an alternative explanation for the data. Proving the above interpretation would require the technically difficult procedure of double negative selection. For example, the capacity of CBA T cells to kill B10.BR mice after filtration through B10.OL (KdDk) mice should disappear after subsequent filtration through B10.A (KkDd) mice but not after a second passage through B10.OL mice. Such studies are in progress.

Because I-region-restricted T helper cells appear to control the generation of cytotoxic responses to minor HA in vitro (21–23), it is of interest that filtration through H-2I-matched hosts (B10.A, B10.A[4R], B10.AQR, and B10.TL) did not discernably influence the severity of GVHD. At face value one might conclude from these data that I-region-restricted T cells play no role in the induction of GVHD to minor HA. However, such a conclusion has to be viewed with caution for a number of reasons. First, although the GVHD-inducing potency of CBA-B10.AQR and CBA-B10.TL T cells compared with the control CBA T cells was remarkably similar (Table I), more comprehensive experiments with larger groups of mice and limiting doses of T cells might well reveal a difference. Second, one could argue that the putative I-region-restricted cells failed to undergo negative selection. Without an assay for detecting these cells this question is obviously difficult to test (although it is noteworthy that I-region-restricted T helper cells involved in T-B collaboration are fully susceptible to negative selection [25]). Because, unlike K/D-restricted T cells, I-
region-restricted T cells express the Ly-1 antigen (26), establishing the Ly phenotype of the T cells that cause GVHD might be useful for assessing these two possibilities. However, even if purified populations of Ly-1\(^{2,3}\) (i.e., K/D-restricted) T cells proved to be potent inducers of GVHD, this would not constitute prima facie evidence against the involvement of I-restricted cells. Thus, one could still argue that T helper cells arise \textit{de novo} from the donor marrow inoculum or even that the host itself provides autoreactive T helper cells.

In addition to acting as T helper cells, it is also conceivable that I-region-restricted T cells per se could mediate GVHD to minor HA. To obtain direct information on this question would require testing the reactivity of T cells filtered through H-2K/D-matched, H-2I-mismatched mice, i.e., \textit{kxxxxkk} mice in the case of CBA responder cells. Unfortunately, mice with this haplotype have not been described. Approaching this question will thus necessitate changing the \textit{H}-2 haplotype of the responders and GVHD hosts. Such experiments are in progress.

Although the experiments in this paper establish that GVHD-inducing T cells exhibit H-2 restriction, the precise mechanism of antigen presentation to these T cells is far from clear. A priori, one might expect any cell that expresses the requisite association of minor HA plus self K/D determinants to be able to present antigen. Alternatively, some form of antigen processing, e.g., by macrophages, might be required (8, 27). According to this latter possibility, exposure of T cells to foreign minor HA in H-2-different mice should lead to selection provided that the donor T cells were supplemented with appropriate syngeneic antigen-presenting cells, e.g., macrophage-enriched populations. To date, experiments in which large doses of syngeneic spleen cells or peritoneal exudate cells were added to the donor T cells during selection have failed to confirm this prediction. Whether this signifies that processing of antigen is not involved or that the injected antigen-presenting cells failed to home effectively remains to be established.

Summary

With a model in which CBA T cells cause lethal graft-versus-host disease (GVHD) in irradiated B10.BR mice (H-2-compatible mice that express multiple minor histocompatibility antigen [HA] differences), information was sought on whether the induction phase of GVHD to minor HA is H-2 restricted. When unprimed CBA (H-2\(^b\)) T cells were recirculated from blood to lymph for 1 d through irradiated H-2-compatible B10.BR or B10.K mice, the T cells underwent specific negative selection to the minor HA of the host, i.e., the filtered T cells failed to cause GVHD after transfer to B10.BR mice. With filtration through totally H-2-different B10 (H-2\(^b\)), B10.D2 (H-2\(^a\)), or B10.S (H-2\(^s\)) mice, by contrast, no selection occurred, i.e., the filtered cells were unimpaired in their capacity to kill B10.BR mice. Selection was marked after filtration through H-2-semiallogeneic (B10 × CBA)F\(_1\) mice. These data, together with the results of filtering T cells through various \textit{H}-2 recombinant strains, indicated that selection depended upon the donor and filtration host sharing determinants encoded by both the \textit{K}- and \textit{D}-ends of the \textit{H}-2 complex. Compatibility only in the \textit{I} region failed to cause demonstrable selection.

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