ANALYSIS OF GRAFT-VERSUS-HOST DISEASE IN SYRIAN HAMSTERS

IV. THE REFRACTORY STATE AND IMMUNOLOGIC COMPETENCE

BY J. WAYNE STREILEIN*

(From the Department of Cell Biology, The University of Texas, Southwestern Medical School at Dallas, Dallas, Texas 75235)

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The tempo and severity of graft-versus-host (GVH) disease in Syrian hamsters is strongly influenced by the dose and route of parental strain lymphoid cell inoculation (1, 2). If upwards of 50 million MHA strain cells are injected intracutaneously into adult (CB × MHA)F1 hybrid hosts, death intervenes within 2-3 wk. Alternatively, 200 million MHA lymphoid cells delivered intravenously induce a more subacute process, one that extends through the ensuing 4-6 wk and from which many animals "recover," giving the outward appearance of well-being. However, their continued susceptibility over the following months to sudden death and a variety of wasting syndromes suggests that an ongoing, though subrosa, systemic GVH reactivity persists. Field and Gibbs (3), have reported that rats who have recovered from acute GVH disease were "refractory" to a subsequent challenge with lymphoid cells isologous to the original donors, i.e., a second inoculation of donor-type cells failed to evoke the same acute GVH syndrome as did the first. The findings to be reported will document that F1 hybrid hamsters that have survived the early phases of GVH disease are similarly refractory to a subsequent challenge with lymphoid cells of the original donor genotype. Evidence linking the pathogenesis of this phenomenon to alterations in immunologic competency of GVH-ravaged hosts as well as changes in their lymphatic tissue mass will be presented.

Materials and Methods

Experimental Animals.—Three isogenic strains of domestically maintained Syrian hamsters (MHA, CB, and PD4) and their appropriate F1 hybrid progeny were employed in these experiments. Since the median survival times of exchanged skin homografts among these strains is characteristically less than 12 days (4; S. Zakarian, personal communication), it is very likely that each possesses a unique allele at the major histocompatibility locus operant in this species. (CB × MHA)F1 hybrids between 3 and 6 months of age regularly served as hosts for the study of graft-versus-host disease incited by lymphoid cells of MHA strain origin.

Lymph Node Cell Suspensions.—Suspensions were prepared as previously described (5). In the induction of graft-versus-host disease, 200 × 10^6 MHA-anti-CB cells were delivered...
either as a single bolus of 0.5 cc through a 30 gauge needle into the long saphenous vein, or into multiple cutaneous sites where 0.1 cc portions of 10 million cells each served to initiate local GVH reactions, immune lymphocyte transfer reactions.

**Immune Lymphocyte Transfer Reaction.**—Reaction sites were evaluated and scored at 24 and 48 hr as previously described (6). An animal was considered as reactive in this regard if a mean value greater than one plus was obtained by averaging its reaction scores.

**Skin Grafting.**—Skin grafting was executed and scored according to the technique of Billingham and Silvers (7). When necessary, specific sensitivity was boosted by exposure to a second skin graft or by intracutaneous injection of lymphoid cell suspensions from the same donor strain. Lymphoid cells used to initiate graft-versus-host disease were obtained from donors sacrificed 1 wk after boosting.

**Tetanus Toxoid Immunization.**—Immunization was attempted by inoculating recipients intracutaneously with five 0.1 ml injections of alum-precipitated tetanus toxoid on days 1 and 29, followed by an intraperitoneal injection of 0.5 cc of aqueous tetanus toxoid on day 36. Sera from immunized animals were tested for circulating antibodies by gel diffusion (8) on days 26, 35, 47, and 55.

**Whole Body X-Irradiation.**—Whole body X-irradiation designed to deliver a total of 900 R was carried out with a 200 kv X-ray source as described elsewhere (9).

**Bone Marrow Cell Suspensions.**—Suspensions were obtained from appropriate donors by flushing femurs, tibiae, and humeri with Hanks' balanced salt solution. To reconstitute irradiated animals, one donor equivalent of bone marrow cells was injected intravenously 24 hr after X-ray exposure.

**EXPERIMENTS AND RESULTS**

Previous studies have shown that the lymphoid apparatus of F1 hybrid hosts is the primary immunologic focus of attacking donor cells in hamster graft-versus-host disease. On the basis of these findings, it was suggested that alterations within this tissue represent the only specific lesions in the GVH process, whereas destruction of nonlymphoid tissues, though often severe, can be accounted for by nonspecific pathogenetic mechanisms (10).

The initial response of F1 hybrid lymphoid tissues to lymphocytes from specifically sensitized parental strain donors was hypertrophy of nodes and spleen. These organs attained maximum size between 10 and 14 days, and then gradually involuted so that by day 35 after inoculation only atrophic remnants remained. In concert with these fluctuations in lymphoid mass, the concentration of lymphocytes in the peripheral blood initially rose and then fell to lymphopenic levels by 35 days (11). One might reasonably expect that the immunologic competence of animals so affected would be considerably blunted, and as a prelude to a study of the refractory state it seemed appropriate to determine their immunologic status, especially after the disease had run its acute course. In these studies of both antibody and cell-mediated immunity, adult (CB X MHA)F1 hybrids were employed in which GVH disease was

2 Tetanus toxoid aluminum phosphate adsorbed ultrafined, diluted 1:10 with phosphate-buffered saline; Wyeth Laboratories, Philadelphia, Pa.

3 Tetanus toxoid fluid, diluted 1:10 with phosphate-buffered saline; Eli Lilly and Company, Indianapolis, Ind.
incited by MHA-anti-CB lymphoid cells injected intravenously ($200 \times 10^6$ cells) or intracutaneously ($30 \times 10^6$ cells).

**Immunologic Competence of GVH-Affected Hamsters.**

*Antibody-forming capacity:* The ability of GVH-diseased animals to make circulating antibody in response to an antigenic stimulus, tetanus toxoid (TT), was assessed. Two groups of experimental animals were available: (a) recipients of 200 million donor cells injected intravenously 70–200 days postinoculation, and (b) hosts that had received sublethal intracutaneous injections of attacking cells 60–150 days previously (see Fig. 1). After a standard TT immunizing regimen (see Materials and Methods), none of these animals had demonstrable serum antibody by day 26, and only 27% ever did develop precipitins for TT as revealed by agar gel diffusion. The three hamsters with positive serum were all members of group (a); none had received its inciting dose of parental cells intracutaneously. For comparison's sake, the responsiveness of normal F1 hybrid hamsters to the same immunizing regimen was determined and is also presented in Fig. 1; while sera from a few normal animals contained TT-precipitating antibody on day 26, by day 35 virtually all were positive. It was concluded that GVH-affected hamsters have a reduced capacity to form antibody in response to a conventional antigenic stimulus.

*Cell-mediated immune response:* The cell-mediated aspect of immunologic responsiveness was assessed in these animals by determining their capacity to reject skin grafts from homologous (PD4 hamster) and heterologous (DA rat) donors. The isogenic PD4 strain differs from both parental strains (CB and MHA) at the major hamster histocompatibility locus; thus the median survival
time (MST) of orthotopically grafted PD4 skin on normal (CB × MHA)F1 hybrid recipients was short: 11.9 ± 0.6 days. Of the test animals challenged with PD4 skin, group 1 (Table I) comprised 18 adult (CB × MHA)F1 recipients of 200 × 10^6 MHA-anti-CB lymphoid cells injected intravenously 17–140 days previously. 10 animals utterly failed to reject the homologous skin and displayed perfect grafts with abundant white fur 100 days later. Grafts that ultimately were rejected survived for considerably longer periods than the normal MST. This evidence seemed sufficient to implicate deficient primary cellular hypersensitivity in animals suffering from GVH disease.

**TABLE I**

<table>
<thead>
<tr>
<th>Group</th>
<th>Recipients (CB × MHA)F1 hybrids that:</th>
<th>No.</th>
<th>Survival times* days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Received 200 million MHA-anti-CB cells i.v. 17–60 days previously</td>
<td>18</td>
<td>13-14-15-16-18-23- &gt;100 × 10</td>
</tr>
<tr>
<td>2</td>
<td>Were presensitized to PD4 antigens, then received 200 million MHA-anti-CB cells i.v. 17–30 days previously</td>
<td>7</td>
<td>11-17-19-28-31- &gt;100 × 2</td>
</tr>
<tr>
<td>3</td>
<td>Received 200 million MHA-anti-CB and anti-PD4 cells i.v. 17 days previously</td>
<td>9</td>
<td>(11-13-15)-15-(18)-(18)- &gt;50 × 3</td>
</tr>
</tbody>
</table>

* Median survival time of PD4 skin on normal (CB × MHA)F1 hybrid recipients = 11.9 ± 0.6 days.
† Numbers in parentheses represent animals that died with epidermal necrolysis before rejecting their grafts.

A second panel of hybrids (group 2, Table I) were specifically sensitized to PD4 tissue isoantigens by exposure to first- and second-set skin homografts, after which GVH reactivity was initiated by the intravenous inoculation of 200 × 10^6 MHA-anti-CB lymphoid cells. When challenged orthotopically with PD4 skin homografts 14–30 days thereafter, none mounted an accelerated rejection process and only one rejected its graft by 11 days. Six grafts survived beyond 16 days, and two remained healthy as long as 100 days. Clearly, not even pre-existent cellular immunity in these animals could weather the rigors of a systemic GVH reaction.

GVH disease was then induced in a third group of F1 hosts by the intravenous injection of lymphoid cells derived from MHA donors specifically sensitized to CB and PD4 transplantation antigens. This panel was subsequently challenged with PD4 skin grafts, and, as the results of Table I indicate, the pattern of graft survival times did not differ appreciably from the previous two
groups. Once again defective cell-mediated immunity was demonstrated even though the GVH-inducing lymphoid cells had been obtained from animals specifically immunized to the antigens on the test skin grafts.

It is worth pointing out that several animals in group 3 died during the course of the grafting experiment (numbers in parentheses, Table I). For reasons which are obscure, all of these animals developed toxic epidermal necrolysis 5–7 days after the application of PD4 grafts. In no previous experimental situation had intravenous recipients of parental lymphoid cells acquired the TEN syndrome (2), and it was therefore surprising to discover it in these animals. Whether its development was in any way related to the specific sensitivity of donor cells to PD4 graft antigens is conjectural.

In additional studies bearing on immunologic competence, hamsters with GVH disease rejected rat skin heterografts quite promptly, within 6 days, an interval quite similar to that of normal hamsters. The preservation of this modality of immune capacity in the face of obvious incompetency with respect to other antigenic systems is not surprising in light of the potency of this heterologous stimulus, and thus underscores the fact that the immunologic deficiency in these animals was incomplete.

In summary, graft-versus-host disease significantly impaired certain primary and secondary cellular immune capabilities in adult hamsters; more impressively, it prevented the expression of preexistent, donor-based, cellular hypersensitivity to a third or unrelated set of tissue isoantigens, suggesting that a defect in the central or efferent limb of the immunologic reflex was involved.

Studies on Refractoriness in GVH-Affected Hamsters.—Epidermolysis stands alone as the single most reliable clinical indicator of graft-versus-host disease in hamsters, namely, normal adult F1 hybrid recipients of 200 × 10^6 MHA-anti-CB lymphoid cells injected intracutaneously without exception develop this severe cutaneous disorder within 10 days (1). Thus the capacity to express epidermolysis upon similar challenge was selected as the means by which an animal with chronic GVH disease could be characterized as refractory or not. An initial panel of 17 F1 hosts was available for testing, 14 of whom had received their GVH-inciting lymphoid cells intravenously 40–150 days previously (Table II). None of these latter animals evinced the slightest evidence of epidermolysis when challenged intracutaneously with 200 million MHA-anti-CB cells, whereas the three remaining hosts in the panel, having received their original donor cells 195 days previously, did exhibit mild epidermolysis of insufficient severity to procure their demise. It would appear that hamsters with chronic GVH disease become refractory to a recrudescence of GVH reactivity upon rechallenge with lymphoid cells of original donor genotype.

In searching for an explanation for this phenomenon, it was considered that F1 hybrid hosts might be capable of mounting an immune response to unshared recessive antigens on the original donor cells and that this hypersensi-
tivity could preclude the subsequent engraftment of donor cells. To test this idea, a panel of 12 putative refractory F₁ hybrids were grafted orthotopically with skin from both MHA and CB parental strain donors. No animal showed the slightest inclination to reject either type of skin (all grafts survived indefinitely); therefore, it seemed extremely unlikely that an obscure host immunity could have prevented epidermolysis by prejudicing the "take" of a subsequent inoculum of MHA lymphoid cells. Moreover, the indefinite survival of CB skin grafts on these animals merely reemphasized the earlier observation that as the GVH reaction unfolds, even the attacking donor lymphoid cells seem to lose their immunologic commitments, in this case, against CB transplantation antigens.

Another hypothesis to account for refractoriness takes cognizance of the immunologic incompetence of GVH-afflicted hamsters, maintaining that a deficiency in the central and/or efferent limb of the immunologic reflex arc precludes the mediation and expression of further GVH reactivity. Evidence bearing on this idea was sought by challenging each of 20 MHA-refractory F₁ hybrids intracutaneously with 200 × 10⁶ lymphoid cells from CB strain donors sensitized to MHA antigens (Table III). 18 of these animals developed severe epidermolysis and died, indicating that they had sufficient immunologic competence and efferent "mediators" to exhibit epidermolysis if the challenge were genetically appropriate, i.e., immunologically competent cells from the other parental strain.

On suspicion that the appearance of refractoriness might depend upon the time elapsed after initiation of GVH disease, a series of experiments was conducted in which small groups of F₁ recipients of intravenously injected MHA lymphoid cells were rechallenged intracutaneously with MHA-anti-CB cells at regular intervals thereafter: 4, 7, 14, 21, 28, 35, 49, and 100 days. Intracutaneous inoculation sites containing 10 × 10⁶ cells were scored 24, 48, and 72 hr later as immune lymphocyte transfer reactions (ILTR), and the animals were scrutinized for the appearance of epidermolysis. Their

<table>
<thead>
<tr>
<th>Interval between first and second injection (i.v.)/ (i.c.)</th>
<th>No. of animals tested</th>
<th>No. with epidermolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td></td>
<td></td>
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<tr>
<td>40</td>
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<td>150</td>
<td>2</td>
<td>0/2</td>
</tr>
<tr>
<td>195</td>
<td>3</td>
<td>3/3</td>
</tr>
</tbody>
</table>
ability to display ILTR's became attenuated within 7 days after initiation of GVH disease (Table IV), and by 35 days intracutaneous injections of donor cells incited no response whatever. Similarly, as early as 7 days, some animals exhibited relative refractoriness, which persisted through the first 28 days after which the typical cutaneous syndrome, even in mild form, could no longer be elicited. This absolute refractoriness persisted throughout the 100 day limit on the experiment. It was particularly interesting to note that in every case, a positive ILTR served as a harbinger of epidermolysis, and in only two instances did a negative ILTR precede the appearance of epidermolysis. This highly impressive association between intense local GVH reactions and epidermolysis strongly suggested that the two events might be causally related.

An essential prerequisite to the development of ILTR’s in hamster skin is adequate numbers of genotypically appropriate circulating lymphocytes. In

### TABLE III

<table>
<thead>
<tr>
<th>Interval between first injection (i.v.) and second injection (i.c.)</th>
<th>No. of animals tested</th>
<th>No. with epidermolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td></td>
<td></td>
</tr>
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<td>45</td>
<td>6</td>
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<td>8/8</td>
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<tr>
<td>70</td>
<td>6</td>
<td>4/6</td>
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### TABLE IV

<table>
<thead>
<tr>
<th>Days after inoculation*</th>
<th>No. of animals tested</th>
<th>No. with positive ILTR's</th>
<th>No. with epidermolysis</th>
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</thead>
<tbody>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
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<td>7</td>
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</tr>
<tr>
<td>100</td>
<td>5</td>
<td>0</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* (CB × MHA)F1 hybrids received 200 million MHA-anti-CB lymphoid cells intravenously on day 0, and were then challenged intracutaneously with 200 million MHA-anti-CB lymphoid cells at the above intervals.
† Developed very mild epidermolysis.
Refractory State and Immunologic Competence of GVH

In a previous report it was shown that F₁ recipients of 200 million MHA cells injected intravenously developed severe lymphopenia within 28-35 days, and it seemed reasonable to suspect that the appearance of refractoriness in hamsters with chronic GVH disease may result from a deficiency of target tissue, i.e., host lymphoid cells. In an attempt to validate this hypothesis, several experiments were carried out in an effort to abolish the refractory state by providing additional target F₁ lymphoid cells.

Reconstitution Experiments in Refractory Hamsters.—In the first experiment in this series, a panel of six intravenous recipients of 200 million MHA-anti-CB lymphoid cells, 35 or more days postinoculation (hereafter referred to as refractory), were injected intravenously with one donor equivalent of (CB × MHA)F₁ bone marrow cells on the presumption that the diminished supply of circulating lymphoid cells in these animals could thus be repleted. When this panel was challenged intracutaneously 2-7 days later (Table V) with 200 X 10⁶ MHA-anti-CB cells, no sign of epidermal disease was observed. Although direct evidence was not obtained, it was suspected that the injected hematopoietic cells may not have established effective residence, by virtue of the persistent GVH potential of the original MHA lymphoid cells or their progeny residing within these hosts.

In an attempt to insure the engraftment of F₁ bone marrow cells, 10 refractory F₁ hosts were exposed to 900 R whole body X-irradiation, and each was reconstituted 24 hr later with one donor equivalent of (CB × MHA)F₁ hematopoietic cells. Every animal survived the irradiation and was challenged intracutaneously 21 days later with 200 million MHA-anti-CB lymphoid cells. Severe epidermolysis and death ensued within 3 wk in all hosts, indicating that systemic reconstitution with F₁ hematopoietic stem cells had taken place, and that refractoriness had been abolished (Table V).

Lastly, an attempt was made to effect a similar reversion of refractoriness by supplying the putative target lymphoid cells locally in the inciting cutane-
ous inoculum. \( F_1 \) hybrids made refractory by the previous intravenous injection of MHA–anti-CB cells were injected intracutaneously with a mixture of monodisperse lymphoid cells derived from MHA–anti-CB and (CB × MHA)\( F_1 \) donors. As expected (Table V), intense inflammatory reactions appeared at the cutaneous inoculation sites of five animals, and shortly thereafter full-blown epidermolysis developed, demonstrating that the refractory state had in this instance been circumvented by providing target lymphoid cells locally as well as systemically.

**DISCUSSION**

There appear to be three ways in which a GVH process can result in the development of refractoriness (see Table VI): by inducing alterations in (a) host reactivity, (b) original donor cell reactivity, and/or host antigenicity. It is reasonably well established that, since the genes operating at histocompatibility loci function as codominants, \( F_1 \) hybrids are genetically incapable of mounting a specific immunologic response to grafts of tissues and cells from either parental strain.

However, there are unequivocal reports of somewhat unique genetic relationships wherein this general rule seems not to apply, e.g. hematopoietic cells from C57BL murine donors survive only very poorly in lethally irradiated (A × C57BL)\( F_1 \) hybrids (12), and circumstantial evidence has been advanced which implies that an immunologic mechanism is responsible (13). In the case of Syrian hamsters, the ease with which (CB × MHA)\( F_1 \) hybrid hosts can be spared irradiation sickness by the provision of MHA hematopoietic cells indicates that this particular genetic relationship behaves according to traditional precepts. Moreover, the failure of MHA-refractory animals to reject MHA skin homografts attests to the validity of this interpretation.

As the data reported here indicate, the humoral and cellular modalities of
immunologic responsiveness are deficient in refractory hamsters. However, these deficiencies are relative rather than absolute, and the observation that MHA-refractory animals can develop epidermolysis upon intracutaneous challenge with CB-anti-MHA lymphoid cells indicates that neither putative depletion of nonspecific mediators, nor relative immunologic incompetency is responsible in any major way for the appearance of the refractory state.

With regard to postulated alterations in the reactivity of original donor cells, it was found that suspensions of F1 bone marrow cells failed to erase refractoriness except when preceded by whole body X-irradiation. Clearly not all of the donor cells had committed immunologic suicide, but instead at least some remained viable within their hosts in a manner that had to be suppressed in order for a graft of F1 marrow cells to take. Yet, evidence of moderate alterations in the reactivity of donor cells was obtained; F1 hybrids harboring these cells failed to reject grafts of PD4 hamster skin with normal vigor, even if the original donors had been specifically sensitized to PD4 isoantigens. While this evidence implied that the lymphoid cells in the initial donor inoculum had indeed undergone a functional alteration, the role this transformation played in the development of refractoriness remained obscure.

It is the major thesis of this report that alterations in antigenicity of the host are primarily responsible for the emergence of refractoriness in F1 hybrid hamsters with GVH disease. Syrian hamsters may be unique among animals studied to date in the almost absolute requirement for lymphoid cells as antigenic targets in a variety of cellular immune reactions of homologous type. In immune lymphocyte transfer reactions in normal hamsters, circulating lymphoid cells and not parenchymatous cutaneous cells provide the antigenic stimulus (14), while in skin tests performed in lethally irradiated hamsters, target lymphoid cells must be admixed with immunocompetent attacking cells in order to evoke a positive local inflammatory reaction (9).

Similarly the expression of systemic GVH disease in hamsters, as evidenced by the epidermolytic syndrome, is almost totally dependent upon the presence of circulating leukocytes of appropriate antigenic constitution within the host, whose own genotype, incidentally, may even be irrelevant (2). It is not too surprising, therefore, that the virtual ablation of host lymphatic tissues procured by the inoculum of attacking cells should leave insufficient numbers of lymphoid cells to serve as immunologic targets for a subsequent inoculation of attacking cells isologous to the original donor strain. However, there did appear to be sufficient MHA cells persisting within these hosts to allow a subsequent inoculation of lymphoid cells from the other parental strain, CB, to perceive them as targets, overcome refractoriness, and evoke epidermolysis. In this report, reconstitution experiments in which viable F1 leukocytes were provided either systemically or locally as adjuncts to the rechallenge inoculum of MHA cells successfully nullified the refractory state, thereby reenforcing
the notion that host lymphoid cells play a pivotal role in the pathogenesis of the acute GVH syndrome and one of its sequela, the refractory state.

It would be inappropriate to extrapolate directly from these findings to account for comparable experimental results in other species. Unlike in the hamster, refractoriness in rats and mice extends to both parental strains (3, 15), irrespective of which had served as original donor. In addition, it has been suggested that in these latter species, donor cell-secreted isoantibodies (in a manner reminiscent of enhancement) gradually blunt the continued attack of donor effector cells, thus allowing their hosts to recover and exhibit refractoriness to rechallenge. Hamsters, at least the strains employed in these experiments, do not appear to make isoantibodies to transplantation antigens (16), yet many of them do in fact recover from acute GVH disease and become refractory. The homology between the experience with hamsters and with isoantibody-producing species may be that if lymphoid cells are the major target tissue in GVH disease, irrespective of species, attenuation of the disease is obtained either by obliteration of that tissue or by masking its presence with specific immunoglobulins. Donor cells themselves are able to effect both results, and by the same means render their hosts refractory to rechallenge.

SUMMARY

The so-called refractory state, one sequela of acute graft-versus-host disease, has been studied in adult (CB X MHA)F1 hybrid Syrian hamsters inoculated with sublethal numbers of MHA-anti-CB lymphoid cells. Intracutaneous challenge of these animals with 200 million MHA-anti-CB lymphoid cells after the acute syndrome subsided failed to evoke epidermal necrolysis, whereas a similar challenge administered to normal F1 recipients invariably resulted in lethal epidermolysis. Moreover, the gradual attrition of lymphatic tissues in these hosts and their fading capacity to display adequately immune lymphocyte transfer reactions in the skin coincided with increasing evidence of host refractoriness, suggesting a causal interrelationship. It was possible to circumvent refractoriness by challenging these animals intracutaneously with MHA-anti-CB cells if: (a) the hosts had been lethally irradiated and reconstituted with F1 hematopoietic cells, or (b) the intracutaneous inocula contained admixed F1 lymphoid cells. This evidence provides additional support for the hypothesis that in GVH disease donor lymphocytes attack primarily host lymphoid cells bearing offending homologous antigens. The GVH process can continue so long as these lymphocyte-bound antigens persist within the host, and will abate only as the aggregate host lymphatic mass is effectively destroyed (hamsters) or its antigenic determinants are masked by isoantibodies (rats, mice, man?). At this point, insufficient target tissues remain for rechallenge to incite significant recrudescence of the disease.

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REFERENCES


