HAPTENS CAN SERVE AS SURROGATE TRANSPLANTATION ANTIGENS IN A MANNER THAT DEMONSTRATES H-2 RESTRICTION OF GRAFT REJECTION*

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Contact hypersensitivity (CH) to simple haptens and acute allograft rejection are generally regarded as T cell-mediated responses. However, at the present time, considerable controversy exists concerning the precise phenotype of the effector cells that are the proximate causes of inflammation and tissue destruction. In transplantation immunity, the assumption of a decade ago—that cytotoxic T lymphocytes, predominately of the Lyt-1-23+ type, are the mediators of acute allograft rejection—has been strongly challenged by recent data identifying the T cell subpopulation that mediates delayed-type hypersensitivity reactions (T_{DTH}, Lyt-1^-23-) as an equally (if not more so) important participant in specific graft destruction (1, 2). Similarly, the general belief that T_{DTH} cells mediate contact hypersensitivity (3) has been challenged by Tagart (4) and more recently by Sunday and Dorf (5), who have demonstrated a role for cytotoxic T cells in the development of CH reactions in the skin. In this time of controversy, our laboratory has been involved in the study of both transplantation immunity and contact hypersensitivity and it seemed that useful information might emerge from experiments designed to study both types of cell-mediated immunities simultaneously. The experiments and results to be described examine the possibility that hapten-derived skin grafts can serve as the targets of an immune response elicited by typical contact hypersensitivity-inducing regimens.

Materials and Methods

**Mice.** Adult mice, ages 8-16 wk, of the following strains were used: C57BL/6, BALB/c, C57BL/10 (B10)-H-2^{b}, B20.M-H-2^{f}. These mice, as well as the (B10 × B10.M)F_{1} hybrid, were produced in our animal facility.

**Contact Hypersensitivity.** Immunization to the haptens dinitrofluorobenzene (DNFB) and oxazalone was achieved as described previously (6). Briefly, 25 μl of 0.5% DNFB (or 5% oxazalone) in carrier (4:1 acetone/olive oil) was applied on days 0 and 1 to dry-shaved abdominal skin of recipient mice.

**Skin Grafting.** Skin grafts from clipped body wall skin were prepared as described previously (7). Full thickness grafts were placed on thoracic wall beds and wrapped with plaster of paris bandages. Bandages were removed 8 d later. Graft rejection was assessed by visual inspection. Rejection was determined when all evidence of surface epidermis had disappeared.

**Hapten Derivatization of Skin Grafts.** In some experiments, skin to be used in grafting was shaved in the manner for hapten sensitization and 0.2% DNFB in carrier applied 1-2 h before excision. Grafts were fashioned from this skin in the typical manner. In other experiments.

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grafts in residence on the thoracic wall were painted with 0.2% DNFB (or 1% oxazalone) in 20 µl of carrier, and their subsequent survival was observed.

Neonatal Transplantation Tolerance. Tolerance to alloantigens of the H-2b haplotype was induced in neonatal B10.M mice by intravenous inoculation of (B10 × B10.M)F1 spleen and bone marrow cells as described previously (7). At 8 wk of age these mice received orthotopic B10 skin grafts. Mice accepting their grafts in excess of 60 d were considered to be tolerant.

Results

Toxicity of DNFB for Orthotopically Grafted Skin. DNFB, a hapten used widely for study of contact hypersensitivity, is toxic to the skin in a dose-related fashion when painted epicutaneously. Our first experiments defined a dose range of DNFB that was suitable for the grafting studies we planned to conduct. Dry-shaved abdominal skin of BALB/c mice was painted with 0.2%, 0.5%, 2.0%, and 5% DNFB in carrier (50 µl covering an area 2.5 cm diam). 1 h later the donors were killed, and skin grafts were prepared in conventional fashion. These grafts were then placed on beds prepared on the thoracic cage of syngeneic, normal recipient mice. Plaster of paris bandages were applied for protection. 8 d later the casts were removed and the grafts inspected. Grafts painted with 2 and 5% DNFB proved to be nonviable and were sloughed from their beds. A few of the grafts painted with 0.5% DNFB suffered severe inflammatory reactions, although all eventually healed in place. Grafts painted with 0.2% DNFB were stained light yellow and displayed a mild inflammatory response of similar intensity to that observed in unpainted syngeneic grafts examined at a similar time after grafting. Grafts painted with 0.2% DNFB healed in perfectly at the graft site. By 14 d, all evidence of yellow staining had disappeared by gross inspection. For subsequent experiments, the 0.2% DNFB dose was used routinely to derivatize skin to be used in grafting studies.

Susceptibility of Hapten-derivatized Skin Grafts to Acute Rejection in Hapten-immune Recipients. In the first experiments, skin grafts were prepared from normal abdominal wall skin of C57BL/6 and BALB/c mice, and placed on thoracic walls of syngeneic mice. On the following day, the abdomens of the graft-bearing mice were dry-shaved and painted with 0.5% DNFB. Control panels of grafted mice were painted with 10% oxazalone. These epicutaneous paintings were repeated on the subsequent day. On the 8th d after grafting, the plaster bandages were removed. On the next day (9 d after grafting) the surface of the grafts was painted with 0.2% DNFB in carrier. Care was taken to avoid spread of the hapten solution across the margins of the graft on to body wall skin. The surface of these grafts was observed by gross inspection over the next several days. DNFB-painted grafts on DNFB-immune recipients developed intense inflammatory reactions within 24 h (see Table I). This inflammatory crisis became particularly acute at 48 h, at which time mild stroking of the graft surface with a fine forceps easily dislodged the epidermal covering, exposing raw dermis. On C57BL/6 mice, the majority of DNFB-derivatized grafts were destroyed. Similarly, among DNFB-immune BALB/c mice, six of nine grafts were destroyed (rejected). In grafts that were not destroyed within 72 h of hapten painting, the inflammatory reaction gradually subsided and these grafts healed in place. Several grafts were shrunken in size and none grew fresh crops of fur during the subsequent 30-d observation period. By contrast, DNFB-painted grafts on oxazalone-immune recipients developed only mild inflammatory reactions within 24 h. Moreover, these
Survival of DNFB-derivatized Skin Grafts on Hapten-immune Recipient Mice

| Strain | Immune to | Number grafted | Number with crises | Number rejected |
|--------|-----------|----------------|-------------------|-----------------|
| C57BL/6 | DNFB      | 10             | 10                | 8               |
|        | Oxazalone | 9              | 0                 | 0               |
| BALB/c | DNFB      | 9              | 9                 | 6               |
|        | Oxazalone | 10             | 0                 | 0               |

0.2% DNFB was applied to skin graft 9 d after graft was placed on thoracic wall.

reactions promptly subsided and even vigorous stroking of the graft surface with forceps failed to separate the epidermis from dermis. All of these grafts healed in place and the majority demonstrated new fur growth during the subsequent observation period. Thus, it would appear that hapten-immune animals can respond to syngeneic skin grafts that have been derivatized with the same hapten by mounting an immunologically specific inflammatory response that is deleterious to the graft.

We next investigated whether the time interval between grafting and application of hapten was crucial to the rejection process. Panels of C57BL/6 mice were grafted orthotopically with normal, syngeneic abdominal skin. Their plaster bandages were removed 8 d later. On day 21 after grafting, the abdominal skin of these animals was shaved and painted with an immunogenic dose of DNFB. This was repeated 24 h later. On the 27th d after grafting, 0.2% DNFB in carrier was applied carefully to the surface of the grafts. As observed by gross inspection, these healed-in grafts developed mild inflammatory changes during the subsequent 24 h, but the response was neither intense nor sustained. In no instance were these grafts rejected. Thus, the ability of hapten-immune mice to reject syngeneic skin grafts painted with hapten is dependent upon the time interval that elapses between grafting and challenge of the graft with hapten. We presume, but have no direct evidence, that the vulnerability of fresh skin grafts to hapten-specific rejection relates to the healing-in process itself.

In an effort to procure rejection of all rather than only some hapten-derivatized grafts in this experimental system, we explored two additional protocols. In the first, 0.2% DNFB was painted on the shaved abdominal skin of donor C57BL/6 mice 1 h before killing. Grafts were prepared from these donors and placed orthotopically on syngeneic recipients. These animals were immunized to DNFB by abdominal skin painting over the next 2 d. When the protective dressings were removed on day 8 after grafting, the survival of the grafts was observed. As the data in Table II indicate, ~50% of these grafts were rejected. By contrast, no DNFB-derivatized grafts placed on oxazalone-immunized mice were rejected.

In the second approach, DNFB-derivatized grafts were placed on syngeneic BALB/c recipients who were immunized to DNFB 1 and 2 d later. The protective dressings were removed on day 8 after grafting; the grafts were once again challenged with 0.2% DNFB 24 h later. All of these grafts developed intense, inflammatory reactions and all succumbed to this process within 48 h of the second painting with hapten (data presented in Table II). Control mice received DNFB-derivatized skin grafts but were not immunized on their abdominal skin with the hapten. However, when their grafts were rechallenged with 0.2% DNFB after removal of the plaster casts, 50% of these grafts developed severe inflammation and two were rejected. We have evidence (8, and manuscript in preparation) that grafts painted with 0.2%
Table II

Influence of Time of DNFB Application on Survival of Syngeneic Skin Grafts on DNFB-immune Mice

| Immune status of recipients | Time of DNFB application to syngeneic skin grafts* | Number grafted | Number with crisis | Number rejected |
|-----------------------------|--------------------------------------------------|----------------|--------------------|-----------------|
| C57BL/6 immune to DNFB      | Before graft preparation                         | 20             | 12                 | 11              |
| C57BL/6 normal              | Same as above (control)                          | 9              | 0                  | 0               |
| BALB/c immune to DNFB       | Before graft preparation and 9 d later           | 9              | 9                  | 0               |
| BALB/c normal               | Same as above (control)                          | 10             | 5                  | 2               |

* DNFB painted at concentration of 0.2% in carrier.

Table III

Evidence of H-2 Restriction of Effector Cells Responsible for Hapten-specific Graft Rejection

| DNFB-immune recipients | Donor of test graft* | Number grafted | Number with crisis | Number rejected |
|------------------------|----------------------|----------------|--------------------|-----------------|
| B10.M                  | B10.M                | 8              | 8                  | 7               |
| B10.M tolerant of B10  | B10                  | 6              | 0                  | 0               |

* DNFB was applied to test grafts 9 d after grafting.

DNFB are capable in their own right of inducing typical contact hypersensitivity in recipient mice, and we presumed that this accounts for the "inappropriate" rejection of some control grafts in this experiment.

H-2 Restriction of Effector Cells Mediating Hapten-specific Graft Rejection. The availability of this assay system made it possible to design an experiment to test whether the cells that effect contact hypersensitivity are restricted in vivo by the products of H-2. B10.M mice were rendered tolerant at birth by intravenous inoculation of 15 × 10⁶ (B10 × B10.M)F₁ lymphohematopoietic cells. At 8 wk of age, these animals were grafted with B10 skin. Mice retaining their grafts beyond 60 d were regarded as fully tolerant. B10.M mice thus tolerant of B10 alloantigens received orthotopically fresh B10 skin grafts. Control B10.M mice received syngeneic B10.M grafts. Both panels of mice were immunized to DNFB by abdominal skin painting 1 and 2 d after grafting. Their casts were removed on day 8 and the grafts were challenged with 0.2% DNFB the next day. Every hapten-challenged syngeneic B10.M graft developed crisis and seven of eight were rejected within 72 h (see Table III). By contrast, little or no inflammation was observed in the DNFB-painted (but tolerated) B10 grafts and none were rejected. These results confirm that haptns when derivatized to skin can serve as surrogate transplantation antigens and dramatically address the in vivo requirement for H-2 identity of effector cells and hapten-derivatized graft for the rejection response to be consummated. Since the degree of hematopoietic chimerism in our tolerant mice is between 0.5 and 2%, the hapten-specific effector cells produced by skin painting are almost exclusively of B10.M type (9). Moreover, the vast majority of antigen-presenting cells are similarly of B10.M origin (10). Thus, DNFB-specific, H-2 restricted effector cells, when confronted by DNFB derivatized H-2b skin, are unable to initiate an immunologically specific inflammatory reaction.

Discussion

To our knowledge, these are the first experiments to demonstrate that haptns, derivatized to intact tissue cells, can function in vivo as transplantation antigens.
Contact hypersensitivity, induced by conventional methods, can express itself when hapten-immune mice are confronted by the hapten, derivatized to syngeneic orthotopic skin grafts. The expression ranges from intense, self-limited inflammatory reactions within the grafted skin to outright destruction (rejection) of the derivatized tissue. This result confirms that the effector cell population elicited by exposure to sensitizing haptens includes cells capable of provoking skin graft rejection. We presume, but have no direct evidence, that these cells are typical DTH effectors. This conclusion would be concordant with an ever-increasing body of recent evidence that ascribes to T_{DTH} cells a predominant role in acute allograft rejection (1), and would link the pathogenesis of contact hypersensitivity and acute transplantation rejection by a common effector modality.

The ability of hapten-immune recipients to reject syngeneic skin derivatized at the time of grafting is modest compared to their ability to reject grafts derived immediately after removal of protective dressings. We suspect that this difference relates to the gradual decay in the former group of cell surface hapten concentration during the time elapsed after painting. The fact that syngeneic skin grafts, allowed to heal in place for 27 d before derivation with DNFB, were not rejected by DNFB-immune mice implies that the vulnerability of haptenated grafts is related in part to whether they have had adequate time to "heal" in place. "Healing-in" grafts are more susceptible to rejection than are grafts that have been in residence for prolonged intervals before induction of transplantation immunity (11-13).

Perhaps the most interesting finding to emerge from these studies is that DNFB-immune mice, rendered H-2 tolerant by neonatal inoculation of semiallogeneic hematopoietic cells, are incapable of rejecting hapten-derivatized skin grafts prepared from donors bearing the tolerated H-2 antigens. It appears that the T_{DTH} effector cells, induced by abdominal skin painting with hapten, recognized the hapten in association with H-2 determinants of the skin (the host) and are subsequently restricted in their ability to express DTH to tissues bearing the same H-2 antigens. Since in our experiments hapten-derivatized grafts bearing only "tolerated" H-2 determinants are not rejected, we conclude that the phenomenon of H-2 restriction of T_{DTH} effector cells operates in vivo. This is more than a trivial conclusion. It is very difficult to devise experimental models to test whether the phenomenon of H-2 restriction operates in transplantation immunity. A recent publication from Silvers et al. (14) claims that rejection of minor H incompatible skin and parathyroid grafts is H-2 restricted; these authors have taken advantage of the restricted tissue distribution of polymorphic minor histocompatibility antigens to test this possibility. In vivo H-2 restriction has also been demonstrated in protection of mice against challenge with minor H-incompatible tumor cells (15) and in the induction of graft-vs.-host disease directed at minor H antigens (16). Yet others have failed to find evidence of in vivo H-2 restriction in tissue transplantation (17). Resolution of this question is important since minor immunogenetic disparity will ultimately become important in clinical organ transplantation.

Summary

Hapten-immune mice are capable of rejecting syngeneic skin grafts that are derivatized with the relevant hapten, but only if the hapten is applied while the graft is "healing in." This model system was used to demonstrate that the hapten-specific
immune effectors responsible for rejection are restricted by H-2 determinants of the recipient. Thus, haptens can be used in vivo as surrogate transplantation antigens for the study of immunopathogenic mechanisms in transplantation immunity.

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