Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6265–6267, September 1985
Immunology

Evidence that major histocompatibility complex restriction of foreign transplantation antigens occurs when tolerance is induced in neonatal mice and rats

(skin-specific antigens)

LISE DESQUENNE-CLARK*, HIROMITSU KIMURA*, AND WILLYS K. SILVERS†‡

Departments of *Pathology and Laboratory Medicine and †Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Communicated by Elizabeth S. Russell, May 20, 1985

ABSTRACT **Studies on the survival of skin-specific antigen (Skn)-incompatible skin grafts in mice rendered tolerant at birth with major histocompatibility complex (MHC)-incompatible lymph node and spleen or bone marrow cells, as well as studies concerned with the survival of third-party skin grafts in rats rendered tolerant at birth with MHC-incompatible bone marrow cells, indicate that MHC restriction of foreign transplantation antigens occurs when tolerance is induced. Thus, evidence is presented that animals rendered tolerant with MHC-incompatible bone marrow cells depleted of mature T lymphocytes will accept any graft that is homozygous for the bone marrow donor's foreign MHC. Evidence has also been obtained that continuous exposure to foreign transplantation antigens in association with an MHC different from that of the graft may induce unresponsiveness to the same antigens in association with the MHC of the graft.**

Although there is evidence that tolerance of self is major histocompatibility complex (MHC) restricted (1–3), as far as we are aware no one has demonstrated that such restriction also occurs when tolerance is induced at birth to foreign transplantation antigens. We have obtained such evidence. Indeed, the following experiments indicate that if tolerance is induced in neonatal mice and rats with MHC-incompatible bone marrow cells devoid of any already educated T lymphocytes, such animals will accept any graft that is homozygous for the bone marrow donor's foreign MHC.

MATERIALS AND METHODS

A/Ss (hereafter A; H-2^a) and C57BL/6Ss (hereafter B6; H-2^b) mice and (A × B6)F_1 hybrid mice as well as Lewis ($RT1^l$), DA ($RT1^a$), (Lewis × DA)F_1, BN.B4 ($RT1^a$), and (Lewis × BN.B4)F_1 rats were used.

In mice, tolerance was induced by inoculating B6 mice (less than 18 hr old) with 2–5 × 10^7 (A × B6)F_1 male spleen and lymph node cells or bone marrow cells shortly after they had been sublethally irradiated (400 rads of ^{137}Cs irradiation at a dose rate of 88 rads/min; 1 rad = 0.01 gray). The tolerance-inducing inoculum was prepared in Hanks' balanced salt solution as described (4) and was administered intravenously (i.v.) in a standard volume of 0.1 ml of medium through the orbital branch of the anterior facial vein. Tolerance was verified by the permanent acceptance of one-third or one-fourth of a neonatal strain A female heart transplanted in the pinna of the ear (5) 8 weeks after tolerance induction. Animals that accepted these grafts, as determined by monitoring them electrocardiographically (Grass Instruments model 790 polygraph), were challenged with 1-cm^2 adult strain A skin grafts (6) 4 weeks later.

Tolerance was induced by similar procedures in rats. Lewis rats (less than 18 hr old) were inoculated i.v. with 8 × 10^7 (Lewis × DA)F_1 bone marrow cells administered in 0.2 ml of medium. Tolerance was verified by the acceptance of a DA skin graft transplanted 7 weeks after tolerance induction. Tolerant animals were challenged with BN.B4 or BN.B4 and (Lewis × BN.B4)F_1 skin grafts after the DA grafts had been accepted for from 4–7 months. (Lewis × DA)F_1 hybrids were challenged with BN.B4 skin grafts or with (Lewis × BN.B4)F_1 grafts when 6–9 months old. Grafts varied from 2.25 to 4.0 cm^2. DA grafts were placed on the left side of the host's thorax and BN.B4 and (Lewis × BN.B4)F_1 grafts were placed in separate beds on the right side of the thorax (6).

RESULTS AND DISCUSSION

Although, almost from the time that immunological tolerance was discovered, it was known that cell suspensions prepared from different components of the hemolymphopoietic system varied in their capacity to induce tolerance of skin grafts when administered i.v. into MHC-incompatible newborn mice (7) and rats (8), it wasn't until a number of years later that it was realized that tissue-specific antigens were partly responsible for these differences. Thus, the observation that lethally irradiated adult B6 mice restored with MHC-incompatible (B6 × A)F_1 spleen or a mixture of spleen and bone marrow cells persist as chimeras, despite the fact that they reject A strain skin grafts, indicated not only that skin-specific (Skn) antigens occur in this species but also that continued exposure to these antigens is essential if tolerance to them is to be maintained (9, 10). These observations were subsequently shown to apply as well to B6 mice that were inoculated neonatally with (A × B6)F_1 lymphoid cells (11). Such mice accepted A strain hearts but rejected adult A strain skin.

A similar situation occurs in rats, where, in the limited number of MHC-incompatible strain combinations that have been tested, adult F_1 hybrid lymph node cells, either alone or mixed with spleen cells, have been shown to be effective in inducing high degrees of tolerance of hearts but not skin (12, 13). However, because in this species MHC-incompatible bone marrow cells are highly effective in rendering newborn rats highly tolerant of skin grafts (14, 15), it was assumed that any antigen(s) specific to skin must also be expressed by marrow cells (16).

It occurred to us that this discrepancy between mice and rats might be attributed to the fact that whereas *both*

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MHC, major histocompatibility complex; Skn antigen, skin-specific antigen; APC, antigen-presenting cells.

‡To whom reprint requests should be addressed.

lymphoid cells and bone marrow cells had been tested for their capacity to induce tolerance of skin in neonatal rats, only lymphoid cells had been tested in the A to B6 mouse strain combination. Accordingly, we have inoculated newborn B6 mice either with a mixture of lymph node and spleen cells or with bone marrow cells prepared from (A × B6)F_1 hybrid donors. The results (Table 1) clearly demonstrate that there is a highly significant difference ($P < 0.001$) in the ability of these inocula to induce tolerance of A strain skin. Whereas bone marrow cells usually render their recipients permanently tolerant of A skin, as previously reported, lymphoid cells do not.

Although the most obvious explanation for these results is that in both mice and rats putative Skn antigens are not confined to the integument but are also expressed by bone marrow cells, we offer what we believe is a more likely explanation: that MHC restriction is involved. We contend that when neonatal mice or rats are inoculated with MHC-incompatible bone marrow cells, two independent events occur. Immunological tolerance is induced to the foreign transplantation antigens present in the inoculum and, because donor cells migrate to the thymus, the entire T-cell repertoire of the tolerant animal, including chimeric donor T cells, is restricted to the host's MHC (17). When tolerance is induced with bone marrow cells this restriction frequently results in the acceptance of Skn-incompatible donor strain skin grafts. However, this is not the case in animals rendered tolerant with lymphoid cells because such suspensions usually include a sufficient number of already educated T cells to reject the graft. Thus, we argue that, after the inoculation of (A × B6)F_1 bone marrow cells into newborn B6 mice, strain A (H-2^a) tissue-specific antigens, regardless of whether they are present in the inoculum or not, are responded to only when they are associated on the same cell with the MHC of B6 (H-2^b). Hence, when these mice are challenged with strain A skin grafts, the grafts are accepted because their Skn antigens are not recognized in association with the MHC of the graft (H-2^a) but are responded to only in association with the MHC of the host (H-2^b).

Although our hypothesis does not appear to be in accord with some of the original (9, 18) and subsequent (10, 19) studies on Skn antigens, in which lethally irradiated B6 or (B6 × A)F_1 × B6 backcross mice, restored with a mixture of (B6 × A)F_1 bone marrow and spleen cells, or with bone marrow cells alone, rejected A strain skin, we believe these studies are not analogous with ours. Unlike neonatally treated mice and rats, the hemolymphopoietic systems of these adult radiation chimeras are almost entirely (>95%) of donor (F_1 hybrid) origin (9), and hence they undoubtedly include a sufficient number of already educated effector T cells to mediate graft rejection. Indeed, the fact that these radiation chimeras reject A strain skin grafts despite the fact that they are repopulated with (B6 × A)F_1 bone marrow cells provides convincing evidence that bone marrow cells do not express Skn antigens. The recent observation (20) that genetically anemic W/W^v mice and lethally irradiated wild-type mice, cured and populated by grafted marrow cells from MHC-compatible but Skn-incompatible donors, reject donor strain skin grafts also supports this notion.

Table 1. Survival of strain A skin grafts on B6 mice rendered tolerant at birth with (A × B6)F_1 lymphoid or bone marrow cells

Origin of tolerance-inducing inoculum	No. hosts	Graft survival times, days
Spleen and lymph node	15	11, 14, 15, 21, 23, 25, 25, 32 32, 32, 32, 35, 56, 99, >100
Bone marrow	10	45, 48, 56, 84, six >100

It follows that if, as a result of inducing tolerance in newborn mice with MHC-incompatible bone marrow cells, Skn antigens are MHC restricted, such restriction should apply to all transplantation antigens. To determine if this was the case we challenged eight Lewis rats rendered tolerant at birth with MHC-incompatible (Lewis × DA)F_1 bone marrow cells, and bearing DA skin grafts of long standing, with BN.B4 female skin grafts—i.e., skin grafts that, except for their $RT1^a$ haplotype, originated from animals that were genetically unrelated to DA. We also determined the survival of BN.B4 skin grafts on 6 (Lewis × DA)F_1 males.

The results are summarized in Table 2. Although only one BN.B4 graft was permanently accepted by a Lewis animal tolerant of DA (and this graft displayed no signs of rejection and is currently, at >200 days standing, bearing a luxuriant crop of hair), we believe the fact that all of them outlived similar grafts on the (Lewis × DA)F_1 rats provides further evidence that MHC restriction of foreign transplantation antigens occurs when tolerance is induced at birth. Moreover, if this assumption is correct, we believe that the most likely explanations for the ultimate demise of all but one of the BN.B4 grafts was either cross-reactivity (21)—i.e., some BN.B4 antigens recognized in association with an $RT1^1$ MHC may share specificities with these same antigens associated with an $RT1^a$ MHC—and/or, more likely, the occurrence of a significant population of already educated effector T cells in the tolerance-inducing inoculum.

We also believe that the fact that the one tolerant Lewis rat that permanently accepted its BN.B4 graft subsequently accepted a (Lewis × BN.B4)F_1 hybrid graft—i.e., a graft whose transplantation antigens should be recognized in association with the MHC of the host and hence rejected—is significant (this graft, which remains in impeccable condition after surviving >100 days, was transplanted after the BN.B4 graft had been accepted for 100 days). Indeed, we think it likely that this situation is analogous to reports that endocrine grafts, deficient in antigen-presenting cells (APC), not only are accepted by MHC-incompatible recipients but also may induce unresponsiveness to subsequent *fresh* grafts of the same tissue (22–24). Thus, we suggest that in both these situations continuous exposure to the graft's foreign transplantation antigens in association with the MHC of the host may induce unresponsiveness to the same antigens in association with the MHC of the graft.

Finally, to obtain additional information on these phenomena, we challenged a panel of six tolerant male Lewis rats, derived from the same pool as those noted above (and also bearing well-established DA skin grafts), with two grafts, a BN.B4 graft and a (Lewis × BN.B4)F_1 hybrid graft. We also challenged six (Lewis × DA)F_1 hybrid males with (Lewis × BN.B4)F_1 hybrid skin. We believe that the results of this experiment (Table 3) not only are in accord with the thesis that MHC restriction accompanies the induction of tolerance but also suggest that even the simultaneous presence of a BN.B4 graft on a Lewis rat tolerant of DA may enhance the survival of a (Lewis × BN.B4)F_1 graft. Evidence for restriction is provided by the fact that in no case did a (Lewis × BN.B4)F_1 graft outlive a BN.B4 graft on a tolerant recipient,

Table 2. Survival of BN.B4 skin grafts on (Lewis × DA)F_1 hybrids and on Lewis rats rendered tolerant at birth with (Lewis × DA)F_1 bone marrow cells

Recipients	No.	Graft survival times, days
(Lewis × DA)F_1	6	10, 10, 10, 11, 11, 11
Tolerant Lewis	8	14, 14, 16, 16, 17, 19, 38, >200*

*This rat was challenged with a (Lewis × BN.B4)F_1 hybrid graft after the BN.B4 graft had survived for 100 days. The hybrid graft has survived >100 days.

Table 3. Survival of (Lewis × BN.B4)F_1 skin grafts on (Lewis × DA)F_1 rats and of (Lewis × BN.B4)F_1 and BN.B4 grafts on Lewis rats rendered tolerant at birth with (Lewis × DA)F_1 bone marrow cells

Recipients	No.	Survival of F_1 hybrid graft/ survival of BN.B4 graft, days
(Lewis × DA)F_1	6	12, 12, 13, 13, 13, 13
Tolerant Lewis	6	16/20, 16/24, 17/23, 18/52, 20/20, 53/53

and this is exactly what one would expect if only the transplantation antigens of the hybrid graft were recognized in association with RT1[1]. In such a situation, while both the immunologically competent cells of the host and the already educated T cells in the tolerance-inducing inoculum would be expected to react against the F_1 graft, only the latter would be expected to react against the BN.B4 transplant. Evidence that the BN.B4 grafts may have enhanced the survivals of the (Lewis × BN.B4)F_1 hybrid grafts is indicated by the fact that the latter survived significantly longer on the tolerant animals than on the F_1 rats. Unfortunately, because of a paucity of tolerant Lewis recipients we have not yet been able to include an important "control panel" in this experiment, namely tolerant rats exposed to only a single (Lewis × BN.B4)F_1 graft.

Additional experiments provide still further evidence that MHC restriction of foreign transplantation antigens occurs when tolerance is induced at birth. Thus, three A strain mice rendered tolerant at birth (after sublethal irradiation) with 2 × 10^7 (A × B6)F_1 bone marrow cells have accepted C3H.SW (H-2^b) skin grafts for >50 days [two similarly tolerant mice rejected these grafts in 21 and 35 days, and eleven (A × B6)F_1 males rejected them within 20 days]. It is undoubtedly significant that one of the recipients currently maintaining a perfect transplant was inoculated with F_1 cells putatively devoid of T cells (by anti-θ treatment).

Clearly, if the interpretations of our results are correct, and in this regard we would like to call attention to similar findings by Miyamoto and his colleagues (25), they conform with our previous observation that when strong non-MHC histoincompatibilities prevail, removing APC from the graft will ensure the indefinite survival of only MHC-*incompatible* transplants (26).

We thank Mary P. Happ for helpful discussions and Susan Raab for technical assistance. We also appreciate Dr. Donald Bailey's helpful suggestions. This work was supported by U.S. Public Health Service Grant CA-18640. L.D.-C. is a trainee, Veterinary Medical Scientist Program (National Institutes of Health Grant 5T32 GM 07170).

1. Groves, E. S. & Singer, A. (1983) *J. Exp. Med.* **158,** 1483–1497.
2. Matzinger, P., Zamoyska, R. & Waldmann, H. (1984) *Nature (London)* **308,** 738–741.
3. Rammensee, H.-G. & Bevan, M. J. (1984) *Nature (London)* **308,** 741–744.
4. Billingham, R. E. (1961) in *Transplantation of Tissues and Cells*, eds. Billingham, R. E. & Silvers, W. K. (Wistar, Philadelphia), pp. 87–106.
5. Jirsch, D. W., Kraft, N. & Diener, E. (1974) *Cardiovasc. Res.* **8,** 145–148.
6. Billingham, R. E. (1961) in *Transplantation of Tissues and Cells*, eds. Billingham, R. E. & Silvers, W. K. (Wistar, Philadelphia), pp. 1–26.
7. Billingham, R. E. & Silvers, W. K. (1961) *J. Exp. Zool.* **146,** 113–130.
8. Billingham, R. E., Defendi, V., Silvers, W. K. & Steinmuller, D. (1962) *J. Natl. Cancer Inst.* **28,** 365–435.
9. Boyse, E. A., Lance, E. M., Carswell, E. A., Cooper, S. & Old, L. J. (1970) *Nature (London)* **227,** 901–903.
10. Boyse, E. A., Carswell, E. A., Scheid, M. P. & Old, L. J. (1973) *Nature (London)* **244,** 441–442.
11. Silvers, W. K., Wachtel, S. S. & Poole, T. W. (1976) *J. Exp. Med.* **143,** 1317–1326.
12. Barker, C. F., Lubaroff, D. M. & Silvers, W. K. (1971) *Science* **172,** 1050–1052.
13. Perloff, L. J., Naji, A., Silvers, W. K. & Barker, C. F. (1983) *Transplant. Proc.* **15,** 841–844.
14. Billingham, R. E. & Silvers, W. K. (1962) *J. Cell. Comp. Physiol.* **60,** 183–200.
15. Silvers, W. K. & Billingham, R. E. (1970) *Transplant. Proc.* **2,** 152–161.
16. Silvers, W. K., Lubaroff, D. M., Wilson, D. B. & Fox, D. (1970) *Science* **167,** 1264–1266.
17. Bevan, M. J. (1977) *Nature (London)* **26,** 417–418.
18. Lance, E. M., Boyse, E. A., Cooper, S. & Carswell, E. A. (1971) *Transplant. Proc.* **3,** 864–868.
19. Wachtel, S. S., Thaler, H. T. & Boyse, E. A. (1977) *Immunogenetics* **5,** 17–23.
20. Harrison, D. E. & Mobraaten, L. E. (1984) *Immunogenetics* **19,** 503–509.
21. Bevan, M. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2094–2098.
22. Bowen, K. M., Prowse, S. J. & Lafferty, K. J. (1981) *Science* **213,** 1261–1262.
23. Zitron, I. M., Ono, J., Lacy, P. E. & Davie, J. M. (1981) *Transplantation* **32,** 156–158.
24. Donohoe, J. A., Andrus, L., Bowen, K. M., Simeonovic, C., Prowse, S. J. & Lafferty, K. J. (1983) *Transplantation* **35,** 62–67.
25. Miyamoto, M., Sano, T., Suzuki, K. & Fukumoto, T. (1984) *Transplantation* **38,** 284–287.
26. Silvers, W. K., Bartlett, S. T., Chen, H.-D., Fleming, H. L., Naji, A. & Barker, C. F. (1984) *Transplantation* **37,** 28–32.