INFLUENCE OF SIZE AND GENE DOSAGE ON THE SURVIVAL OF SKIN ALLOGRAFTS ON RATS RENDERED TOLERANT AT BIRTH*

By WILLYS K. SILVERS, NANCY H. COLLINS,‡ AND MINA NAJI

From the Departments of Human Genetics and Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

In most studies on the phenomenon of immunological tolerance as it applies to the cellular antigens responsible for transplantation immunity, neonatal mice or rats are inoculated with suspensions of living, allogeneic cells and, when immunologically mature, challenged with skin allografts of the same genetic origin as the putative tolerance-conferring stimulus. If such grafts are accepted permanently, the animals are judged to be highly or completely tolerant, whereas if the grafts outlive those on untreated recipients but are eventually rejected, the hosts are considered partially or incompletely tolerant. Although there is evidence that exposure to the test graft may sometimes augment the degree of unresponsiveness induced at birth (1), it has generally been assumed that such grafts are indicators of, rather than contributors to, the immunological states of their hosts. We here provide evidence that this assumption is incorrect. Our results indicate that the attributes of the test grafts that putatively tolerant rats are challenged with influence their immune response. Our findings also indicate that the survivals of these grafts are determined by the same factors that operate when only weak histoincompatibilities prevail.

Materials and Methods

Rats. Major histocompatibility complex (MHC)-incompatible BN/Ss (BN; $RT1^n$) and Lewis/Ss (Lew; $RT1^l$) rats, as well as their F_1 hybrids (Lew/BN), were used. The median survival time (MST) of BN (or Lew/BN) skin on adult Lew hosts is ~10 d (1).

Because there was no difference in the ability of bone marrow cells from reciprocally produced F_1 hybrids to induce tolerance of skin, or of skin from these hybrids to survive on tolerant hosts, the results using them have been pooled. There also was no evidence of a sex difference in tolerance susceptibility. Hence, the results with males and females have likewise been pooled.

Tolerance Induction. Tolerance was induced by inoculating rats <20 h old intravenously with suspensions of bone marrow cells prepared from adult F_1 hybrid animals according to procedures described elsewhere (2).

Skin Grafting. Animals were grafted when 2 mo old. Grafting entailed the transfer of full-thickness ventral-trunk skin. The preparation of the grafts, as well as the operative technique, have been described elsewhere (3). First grafts were always transplanted to the right thorax, and second grafts to the left. When animals were grafted simultaneously with two grafts of

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different sizes and/or origins, their positions on the right and left sides of the thorax were alternated.

Grafts of two size ranges were used. "Small" grafts were carefully prepared to measure 1 cm^2 and "large" grafts were initially 6.25 cm^2 (2.5 × 2.5). However, when these grafts were remeasured at primary inspection (9 d post-grafting) to assess more accurately their size, the small grafts varied from 0.75 to 1.5 cm^2 and the large grafts from 4.0 to 6.25 cm^2. H-Y-incompatible grafts were avoided. A survival time of 100 d was adopted as a criterion of permanent survival. In experiments in which putatively tolerant rats were each challenged with a single graft, litters were "split," with half receiving BN and the remainder receiving Lew/BN skin.

MST. The MST of grafts were determined using a computer program using probit transformation (4).

Results

Influence of Graft Origin and Size on Survival on Putatively Tolerant Hosts. These experiments were initiated when, in the course of producing tolerant rats for another study, we obtained a considerably higher percentage of "highly tolerant" animals than anticipated on the basis of prior results (1, 5–7). In trying to account for this success, it occurred to us that our previous protocol for assessing tolerance had been modified. Instead of challenging Lew rats with BN skin, they were test grafted with Lew/BN skin. Moreover, the size of the grafts was considerably larger than those previously used. To determine whether either or both of these factors were responsible for the discrepancy, Lew rats were injected at birth with 10, 20, or 100 × 10^6 Lew/BN bone marrow cells and, when mature, challenged with either large or small BN or Lew/BN skin grafts. The results (Table I) clearly indicate that the origin and size of the grafts play a major role in determining their survival. Large Lew/BN grafts are the most readily, and small BN grafts the least likely, to be accepted.

The influence that gene dosage has on graft survival is best exemplified by the fate of small grafts on rats inoculated at birth with 100 × 10^6 cells and by the survival of large grafts on 20 × 10^6 cell recipients. At the higher dosage, 14 of 17 (82%) small Lew/BN grafts survived for >100 d, whereas all 16 small BN grafts were rejected; at the lower dosage, 12 of 18 (67%) large Lew/BN grafts but only 3/19 (16%) similarly sized BN grafts survived for >100 d ($P < 0.01$). Indeed, at every dosage at which the survivals of large and small Lew/BN and BN grafts were compared, the hybrid grafts did better.

As inferred from the above results, the size of the graft also plays a significant role in determining its survival on putatively tolerant hosts. In fact, graft size seems to be as important a factor as gene dosage. This is illustrated by the survivals of large and small BN grafts on Lew recipients of 100 × 10^6 cells. Whereas 14 of 20 (70%) large BN grafts were retained in perfect condition for >100 d on such recipients (and 5 of the grafts that were scored as rejected nevertheless persisted, although they were recognized only by the persistence of a few pigmented hairs), as noted above, all 16 small BN grafts were rejected. It is also exemplified by the survivals of large and small Lew/BN grafts on Lew recipients of 20 × 10^6 cells. Although, as also noted above, 12 of 18 (67%) large Lew/BN grafts survived for >100 d on these hosts, only 3 of 25 (12%) small Lew/BN grafts were not rejected ($P < 0.01$).

Survival of First and Second Large BN or Lew/BN Grafts on Putatively Tolerant Hosts. To determine how rats that accepted or rejected large Lew/BN or BN skin grafts responded to second large Lew/BN or BN grafts, they were either regrafted when the

TABLE I

Survival of Large and Small‡ BN or Lew/BN Skin Grafts on Lew Rats Inoculated at Birth with Lew/BN Bone Marrow Cells*

Number of cells inoculated ($\times 10^6$)	Graft size	Graft donor	Number of hosts	Survival times	MST§
				d	
100	Large	BN	20	12, 13,‖ 2 × 24,‖ 42,‖ 70,‖ 14 × >100	>100
		Lew/BN	19	40, 18 × >100	>100
	Small	BN	16	13, 15, 21, 22, 25, 2 × 26, 29, 34, 2 × 35, 40, 42, 47, 75, 97	>29.3
		Lew/BN	17	22, 29, 61, 14 × >100	>100
20	Large	BN	19	9, 2 × 10, 2 × 11, 2 × 13, 18, 21, 2 × 22, 26, 2 × 53, 84, 92, 3 × >100	>23.4
		Lew/BN	18	11, 21, 23, 35, 71, 72, 12 × >100	>100
	Small	BN	41	4 × 9, 6 × 10, 3 × 12, 3 × 13, 2 × 14, 15, 17, 2 × 18, 19, 20, 21, 3 × 22, 2 × 25, 26, 2 × 28, 30, 32, 33, 2 × 34, 37, 42, >100	>16.6
		Lew/BN	25	6 × 9, 2 × 12, 14, 15, 2 × 22, 2 × 26, 27, 30, 31, 33, 35, 2 × 36, 97, 3 × >100	>19.5
10	Large	BN	16	4 × 9, 3 × 10, 11, 7 × 12, 18	10.3
		Lew/BN	15	4 × 9, 10, 11, 3 × 12, 13, 14, 15, 2 × 22, 26	11.4

* From 4.0 to 6.25 cm^2.

‡ From 0.75 to 1.5 cm^2.

§ In calculating the MST, grafts that were not rejected were scored as surviving for 100 d.

‖ These grafts were scored as rejected on the day noted. However, subsequently some pigmented hairs were observed emerging from the scar tissue which formed.

initial graft had been in residence for 100 d, or 4–6 wk after the first graft had been rejected. The results (Table II) to a large degree depended upon the number of cells used to induce tolerance. When unresponsiveness was induced with 100×10^6 cells, all animals that had accepted initial grafts, regardless of their origin, accepted second grafts. This was even the case for the five animals regrafted with Lew/BN skin whose initial BN grafts were recognized only by the persistence of a few pigmented hairs. Indeed, when three of these animals were subsequently grafted a third time with a second large BN graft, although the grafts were partially rejected, they all recovered and eventually attained almost their original size.

On the other hand, the response of Lew rats inoculated with 20×10^6 cells to second grafts was much more variable. Although second grafts on rats that had rejected first grafts usually were rejected acutely, there were two exceptions. One rat rejected its initial BN graft in 18 d yet accepted a second Lew/BN graft for 72 d; another similarly treated animal accepted a second BN graft for 53 d after having accepted its initial BN graft for only 26 d. Another observation unique to this panel of hosts was the deleterious influence second grafts sometimes had on the survival of first transplants. 6 of 14 animals bearing flourishing first grafts rejected both grafts within 15 d of being regrafted. In fact, in only three animals were we unable to detect an immune response against either graft.

Survival of Small BN and Lew/BN Grafts Transplanted to the Same Host. Because there

TABLE II

Survival of First and Second Large BN or Lew/BN Skin Grafts on Lew Rats Inoculated at Birth with Lew/BN Bone Marrow Cells*

Number of cells inoculated ($\times 10^6$)	First graft	Second graft‡	Number of hosts	Survival first graft/survival second graft
				d
100	BN	Lew/BN	16	12/11, 13§/>100,‖, 2 × 24§/>100‖, 42§/>100, 70§/>100, 10 × >200/>100
	Lew/BN	BN	13	13 × >200/>100
	BN	BN	4	4 × >200/>100
	Lew/BN	Lew/BN	5	5 × >200/>100
20	BN	Lew/BN	11	9/8, 10/8, 11/8, 13/8, 18/72, 2 × 22/8, 84/10, 92/10, 115/15, >200/>100
	Lew/BN	BN	12	35/9, 71/9, 72/9, 109/9, 110/9, 112/9, 112/10, >200/9,¶, 3 × >200/>100,** >200/>100
	BN	BN	6	11/9, 13/9, 21/14, 26/53, 2 × 53/9
	Lew/BN	Lew/BN	6	11/8, 21/8, 23/8, 110/9, >200/>100,** >200/>100

* From 4.0 to 6.25 cm^2.

‡ Recipients challenged with second grafts 4–6 wk after first graft scored as rejected or after first graft had survived for 100 d.

§ These grafts were scored as rejected on the day noted. However, subsequently some pigmented hairs were observed emerging from the scar tissue that formed.

‖ These animals were also subsequently challenged with a second BN graft and although these grafts were partially rejected they recovered, attained almost their original size (with numerous hairs) and survived for >100 d.

¶ This graft was scored as rejected on the day noted. However, subsequently a few pigmented hairs were observed emerging from the scar tissue that formed.

** These grafts were partially rejected but recovered and eventually attained ~50% of their original size.

TABLE III

Survival of Small BN and Lew/BN Skin Grafts Transplanted Simultaneously to Lew Rats Inoculated at Birth with Lew/BN Bone Marrow Cells*

Number of cells inoculated ($\times 10^6$)	Number of hosts	Survival BN graft/survival Lew/BN graft
		d
100	26	11/11, 14/14, 28/28, 28/36, 65/65, 72/72, 82/69, 89/82, 2 × 97/>100, 98/98, >100/89, >100/97, 13 × >100/>100
20	20	11/13, 11/14, 21/22, 22/22, 23/23, 27/28, 27/30, 30/34, 2 × 31/31, >32/>32,‡ 36/36, >36/>36,‡ 42/42, 63/63, 70/71, 97/97, 98/98, 2 × >100/>100

* From 0.75 to 1.5 cm^2.

‡ Animal died.

was a discrepancy in the survivals of BN and Lew/BN grafts when transplanted separately, their fates were determined when transplanted to the same host. Accordingly, Lew rats that had been inoculated at birth with 20 or 100 × 10^6 cells were grafted with a small BN graft on one side of their thorax and with a similarly sized Lew/BN graft on the other. The results (Table III) indicate that although concomitant exposure to both grafts had no significant effect on the survival of the F_1 hybrid transplants, i.e., their survivals were similar to those grafted alone, it promoted the

TABLE IV

Survival of Small and Large‡ Lew/BN Skin Grafts Transplanted Simultaneously to Lew Rats Inoculated at Birth with 20 × 10^6 Lew/BN Bone Marrow Cells*

Number of hosts	Survival small graft/survival large graft
	d
16	10/10, 14/14, 18/18, 27/27, 31/33, 11 × >100/>100

* From 0.8 to 1.5 cm^2.
‡ From 4.0 to 6.25 cm^2.

TABLE V

Survival of Small BN and Large‡ Lew/BN Skin Grafts Transplanted Simultaneously to Lew Rats Inoculated at Birth with 20 × 10^6 Lew/BN Bone Marrow Cells*

Number of hosts	Survival small BN graft/survival large Lew/BN graft
	d
21	2 × 9/9, 9/11, 12/12, 18/18, 25/26, 26/26, 27/27, 35/36, 39/>100, >43/>43,§ >50/>50,§ 9 × >100/>100

* From 0.8 to 1.5 cm^2.
‡ From 4.0 to 6.25 cm^2.
§ Animal died.

TABLE VI

Survival of First Small BN and Second Large‡ Lew/BN Skin Grafts on Lew Rats Inoculated at Birth with 20 × 10^6 Lew/BN Bone Marrow Cells*

Number of hosts	Survival small BN graft/survival large Lew/BN graft§	MST
	d	
19	2 × 9/8, 9/9, 10/8, 10/11, 11/18, 13/8, 14/8, 14/9, 17/19, 18/10, 19/12, 21/14, 28/10, 32/12, 33/15, 34/13, 34/15, 37/18	15.7/10.4

* From 0.75 to 1.5 cm^2.
‡ From 4.0 to 6.25 cm^2.
§ Recipients challenged with large Lew/BN graft 2–22 d after small BN graft scored as rejected.

survival of the BN grafts. This was especially seen in recipients that had received 100 × 10^6 cells. Whereas 16 such hosts all rejected small single BN grafts (see Table I), 15 of 26 (58%) of these grafts survived for >100 d when accompanied by an F_1 hybrid transplant. Recipients of 20 × 10^6 cells also accepted BN grafts more readily when they were accompanied by Lew/BN transplants. Thus, if one defines animals accepting their test graft for at least 14 d, i.e., >3 SD in excess of the MST on untreated hosts, as tolerant (1), 18 of 20 (90%) recipients of 20 × 10^6 cells that were grafted bilaterally displayed some level of tolerance of the small BN graft, compared with 25 of 41 (61%) rats challenged with the BN graft alone ($P < 0.05$).

It also should be noted that when both BN and Lew/BN grafts were rejected, there was usually no more than a few days difference in their survivals, and in the few

animals (all 100×10^6 cell recipients) in which this was not the case, neither graft appeared to be favored.

Survival of Large and Small Grafts Transplanted to the Same Host. If the relationship between graft size and graft acceptance is solely a function of antigen dosage, then small grafts should fare as well as large ones when both are transplanted to the same putatively tolerant hosts. To determine whether this is the case, panels of Lew rats that had received 20×10^6 Lew/BN cells were challenged with a large F_1 hybrid graft on one side of their thorax and with either a small BN or F_1 hybrid graft on the other. The results (Tables IV and V) are in accord with the contention that the amount of antigen the host is challenged with rather than the size of the grafts per se determine their fate. Whereas only 3 of 25 (12%) small Lew/BN and 1 of 41 (2%) small BN grafts survived for >100 d when grafted alone (see Table I), 11 of 16 (69%) and 9 of 19 (47%) of these grafts, respectively, survived for >100 d when transplanted along with large F_1 hybrid grafts ($P < 0.01$). Indeed, with one exception (see Table V), the small grafts survived as well as the large grafts. On the other hand, concomitant exposure to both grafts had no significant effect on the survival of the large grafts.

Survival of Large Grafts on Rats That Have Rejected Small Grafts. Although the above results demonstrate that the survival of skin grafts on putatively tolerant hosts depends upon their size (amount of antigen) and genotype (homozygous or heterozygous), they do not provide any definitive information as to why these factors are important. Accordingly, the last experiment addressed this question. In this experiment, 19 Lew recipients of 20×10^6 cells that had rejected small BN grafts were regrafted with large Lew/BN transplants. It was reasoned that inasmuch as >60% of these large grafts were accepted when transplanted to previously ungrafted but similarly treated Lew recipients (see Table I), they should also be accepted by a similar proportion of the previously grafted animals unless exposure to the first grafts had altered their immune response. As indicated in Table VI, all the second grafts were rejected within 19 d.

Discussion

These results demonstrate that the attributes of the test grafts putatively tolerant rats are challenged with influence their immune response. Large grafts are more readily accepted than small grafts and F_1 hybrid grafts survive better than homozygous parental strain allografts. Indeed, the behavior of MHC-incompatible skin grafts on putatively tolerant rats bears a striking similarity to the behavior of grafts that are only incompatible with respect to weak transplantation antigens (8, 9). When only weak histoincompatibilities prevail, there is also a direct relationship between graft size and graft survival (10, 11) as well as evidence for a gene dosage effect (12). There are other analogies, too. Tolerant rats that accept first MHC-incompatible grafts usually accept second grafts and the same applies to female rats that have accepted male skin isografts, i.e., grafts incompatible only with respect to the weak H-Y antigen (11, 13). Moreover, in both instances animals that have manifested an immune response usually reject subsequent grafts in an accelerated fashion, regardless of their size (11, 13). Still another similarity is the manner in which putatively tolerant rats and those challenged with H-Y-incompatible skin respond to small and large grafts transplanted simultaneously. In both situations the total amount of incompatible skin, rather than the size of each graft, appears to be the most important factor in determining their fate (11).

In retrospect, failure to recognize the key role that test grafts play in the immune response of neonatally treated rats is not surprising because, when tolerance was first discovered, it was believed to result solely from the specific elimination of clones of cells that normally mediate rejection (14, 15). Although there are situations in accord with this explanation (16, 17) and, in fact, in such cases the attributes of the graft would not be expected to influence its survival, we now know that most instances of tolerance represent a heterogeneous state involving not only the specific elimination of cells potentially harmful to the graft but also their proliferation, the activity of suppressor cells, and perhaps the participation of blocking serum factors as well (18–25). Indeed, when one looks at tolerance as the outcome of all of these mechanisms, it is not at all surprising that the attributes of the graft can either foster its induction or influence the response of the host in favor of immunity.

If one assumes that the primary difference between MHC and non-MHC transplantation barriers is the number of cells available to react with the graft, elimination of some of these cells in MHC-incompatible situations, as would be expected to occur after neonatal exposure of Lew rats to Lew/BN bone marrow cells, could make them comparable. In fact, if the degree of clonal deletion is directly related to the number of cells inoculated at birth, one might expect test grafts to have a more profound influence on the immune state of Lew rats inoculated with 20×10^6 than with 100×10^6 F_1 hybrid cells. This seems to be the case. Whereas after the inoculation of 100×10^6 cells all rats that accepted large Lew/BN or BN grafts for 100 d subsequently accepted second large Lew/BN or BN grafts, ~40% of the 20×10^6 cell recipients that accepted their initial grafts for 100 d rejected these grafts, as well as second grafts, within 15 d of regrafting.

It also should be noted that because rats challenged with both BN and Lew/BN grafts are exposed to considerably more antigen than recipients of single small grafts, it is difficult to assess what influence the genotypes of the two grafts have on their survival. Moreover, because Lew/BN grafts fare better than similarly sized BN grafts, the relationship between graft size and graft dosage cannot be attributed simply to the availability of more antigen. Indeed, the better survival of F_1 hybrid skin grafts on putatively tolerant Lew rats could be related to the ease with which the survival of Lew/BN but not BN kidneys are immunologically enhanced in Lew adults (26).

Finally, the possible involvement of skin-specific antigens should not be overlooked. Such antigens are known to occur in mice (27, 28) and could explain many of the results reported here.

Summary

The attributes of the test grafts with which putatively tolerant rats are challenged influence their immune response. Lewis (Lew) rats inoculated at birth with Lew/BN F_1 hybrid bone marrow cells accept large skin allografts more readily than small allografts, and F_1 hybrid skin grafts survive better than BN transplants. The results indicate that the survivals of these major histocompatibility complex-incompatible grafts are determined by the same factors that operate when only weak histoincompatibilities prevail.

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