DEFICIT OF SPECIFIC THYMUS-DEPENDENT LYMPHOCYTES IN TRANSPLANTATION TOLERANCE IN THE RAT*

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The cellular basis for transplantation tolerance (TT) is poorly understood and has been the subject of much recent investigation (1, 2). A basic question, which has not heretofore been explored experimentally, is whether tolerant animals harbor tolerant cells that can under suitable circumstances be redeemed from a suppressed state to one of renewed immunologic competence. According to the clonal selectionist view of tolerance (3), recovery from tolerance at the level of the whole animal is due to regeneration of previously deleted clones from stem cells, and not redemption of tolerant cells from a suppressed state. The present study suggests that this hypothesis is substantially correct insofar as the thymus-dependent cells (T cells) responsive to major histocompatibility (H) antigens in the rat are concerned.

The local renal graft-vs.-host reaction (GVHR) technique (4) was utilized because it permits ready identification and estimation of the proliferative response of donor lymphocytes to host strain H antigens. Cells that react in this capacity are called H-ARC (histocompatibility antigen-reactive cells), and in accord with the present results and those of others (5) are predominantly T cells. Under the conditions of the experiments the effective antigens are determined by genes in the AgB chromosome region, the major histocompatibility locus in the rat (6).

Previous work has established clearly that specific unresponsiveness is induced among H-ARC by injection of newborn parental strain rats with genetically tolerant F_1 hybrid hematopoietic cells, and that this unresponsiveness is a property of the H-ARC themselves rather than one imposed by serum of the chimera (5–7, footnote 1). In the present study we relied upon sex chromosome markers to identify the origins of proliferating cells in GVHR, induced by cells from intact and thymectomized donors in which TT had been abolished adoptively. The purpose was to determine whether H-ARC that had recovered from a paralyzed state contributed to recovery from TT.

Materials and Methods

Inbred Lewis (L), Brown Norway (BN), (LBN)F_1 hybrid and Lewis × Buffalo [(LBf)F_1] hybrid rats were obtained from Microbiological Associates, Inc., Bethesda, Md., and Dr. W. K. Silvers. Each strain differs from the others at the AgB locus.

Chimerism and TT were induced in L rats less than 24 h old by intravenous injection of

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50–100 million (LBN)F_1 bone marrow and spleen cells (three donor equivalent femoral and tibial marrow: one donor equivalent spleen) from 2–3-mo old males. Each putative chimera was orthotopically grafted with (LBN)F_1 ear or belly skin within 6 wk. Over 90% of the injected rats accepted their test grafts in cosmetically perfect condition for over 30 days. No delayed rejections beyond that time were observed. Such chimeras are designated L-LBN hereinafter.

TT was abrogated by intraperitoneal injection of 9–27-wk old L-LBN rats with 100–200 million spleen and cervical lymph node cells from normal 2–4-mo old L rats. The formerly tolerated skin allografts were rejected within 15–25 days by over 90% of these chimeras. Rats in which TT had thus been abolished are hereinafter designated L-LBN-TA.

Renal GVHR were induced in 3–4-mo old (LBN)F_1 and (LBf)F_1 hosts, as described elsewhere (4), by a mixture of splenic and cervical lymph node cells (5–20%) in doses of 25–50 $\times$ 10^6 cells/0.1–0.15 ml, or blood lymphocytes 10^7 cells/0.1 ml. The suspensions were prepared in chilled Hanks' balanced salt solution (HBSS). The centrifuged lymphoid cells (0.4–1.2 ml packed vol) from the L-LBN were washed three times in 15-ml vol of HBSS.

The hosts were sacrificed on the 7th day at which time over 95% of the locally proliferating cells are of donor origin by chromosome marker techniques (4, 6, footnote 1). Karyotype analysis of proliferating cells derived from the infiltrate of the local GVHR was performed as described elsewhere (4). Up to 30–50 metaphases were usually scored from each reaction. The slides were coded and analyzed by observers unaware of the respective sexes of the primary and adoptive sources. The yield of analyzable mitoses from the (LBN)F_1 hosts was often lower than from the (LBf)F_1 controls, because the reactions in the former were smaller than in the latter. This difference is probably related to a cell-associated suppressor mechanism in L-LBN-TA rats that limits the proliferative response of H-ARC to the alloantigens of the formerly tolerated strain (8).

The Principle of the Experiment.—Upon adoptive termination of TT there are three possible sources of H-ARC that could proliferate in GVHR in hosts of the formerly tolerated strain. These are: (*a*) H-ARC that were present, but blocked while TT was in effect; (*b*) H-ARC that develop from thymic precursors after TT is abrogated; (*c*) H-ARC that were adoptively conferred.

The experimental protocol, in which the contribution of each to the regeneration of the donor's competence is distinguished, was as follows. TT was abolished in L-LBN by lymphoid cells from a normal L rat differing in sex from the chimera. Hereinafter H-ARC indigenous to the rat in which TT had been induced and abolished are referred to as from the primary (1°) source, and cells conferred adoptively as from the adoptive source. The L-LBN-TA was then used as a donor to induce renal GVHR in (LBN)F_1 male and (LBf)F_1 female hosts. The origins of the proliferating cells in the local reactions could thus be determined by analysis of sex chromosome markers in metaphase preparations. Due to sex chromosome polymorphism both host types have a single subacrocentric X chromosome marker, because neither the Y chromosome from the BN paternal strain nor the X from the Bf maternal strain are distinguishable from other acrocentrics. By contrast the metaphases of L origin (primary or adoptive source) have two subacrocentric X's or one of these plus a distinguishable small acrocentric Y as chromosome markers (4).

In some cases, the L-LBN chimera was subjected to adult thymectomy just before receipt of the adoptively transferred cells. This procedure was intended to block the subsequent maturation of a new set of H-ARC (9). Thus primary source metaphases in GVHR induced in the (LBN)F_1 hosts by cells from thymectomized L-LBN-TA should represent H-ARC that had been present, but reversibly suppressed, in the chimera before abolition of TT.

RESULTS

The results of the chromosome marker analyses from GVHR induced by cells from L-LBN-TA donors are shown in Table I. The earliest detectable

donor-type metaphases in (LBN)F_1 hosts were from the adoptive source. In contrast, almost all of the metaphases from (LBf)F_1 hosts were from the 1° source. After 1 mo the percentage of 1° source metaphases increased dramatically in the (LBN)F_1 hosts. Thus it is clear that whatever conditions block the development or the activity of 1° source H-ARC responsive to BN strain, AgB factors in tolerant L-LBN no longer pertain in the L-LBN-TA after about 1 mo.

TABLE I

Origins of Proliferating Cells in Renal GVHR Induced by Lymphocytes from Formerly Tolerant Donors (L-LBN-TA)

Sex of L-LBN-TA donor	Interval adoptive transfer to GVH test	GVH test cell source‡	(LBN)F_1 hosts			(LBf)F_1 hosts		
			1°	Adoptive	Host	1°	Adoptive	Host
	days							
F	4	SL	0	0	0	28	2	0
M	16	SL	0	7	0	49	2	0
F	21	B‖	0	8	3	–	–	–§
M	27	B	0	0	0	30	0	0
		SL	5	29	1	29	1	0
F	33	SL	31	9	3	–	–	–§
F	33	B	22	12	7	59	1	0
F	42	B¶	22	4	1	29	0	1
M	50	B	0	0	0	19	1	0
		SL	1	0	0	17	0	0
F	56	SL**	18	20	0	19	0	0
M	60	SL	14	21	2	25	3	0
F	120	SL	13	17	0	–	–	–
Sum as	≤27		9	83	8	96	4	0
percent	≥33		59	36	5	97	3	1

Each horizontal entry above represents test of cells from one or two donors in one to four hosts of each strain.

‡ B = blood leukocytes; SL = spleen and cervical lymph node cells.

§ – = sample lost or not tested.

‖ Pooled leukocytes from two donors.

¶ Repeat test same two donors.

** Final test with spleen and node cells from one of these.

In contrast to the above, 1° source metaphases were never detected in numbers above background (i.e. the percent proliferating host cells) in local GVHR in (LBN)F_1 hosts in cases where the donor had been thymectomized (Table II). This deficit occurs specifically among 1° source H-ARC sensitive to BN strain antigen. Adoptive source H-ARC sensitive to the same antigen were readily detected, and the vast majority of metaphases in the lesions in (LBf)F_1 hosts were 1° source type, as with the intact donors. The above findings apply irrespective of the source of GVH initiating cells, i.e., peripheral blood or spleen plus lymph node.

DISCUSSION

The present results are predicted by Burnet's clonal selection theory (3). Thus lymphocytes from tolerant donors are specifically unresponsive in GVH assays (and also in mixed lymphocyte reactions in vitro) because there is a specific deficit in the set of H-ARC that would normally be responsive to the foreign AgB antigens expressed by allogeneic cells resident in the chimera. This

TABLE II

Origins of Proliferating Cells in Renal GVHR Induced by Cells from Adult Thymectomized L-LBN-TA Donors

Sex of L-LBN-TA donor	Interval adoptive transfer to GVH test	GVH test cell source‡	(LBN)F_1 hosts			(LBf)F_1 hosts		
			1°	Adoptive	Host	1°	Adoptive	Host
	days							
F	21	B§	0	34	1	22	5	2
	42	B‖	1	47	2	–	–	–
	56	SL¶	0	17	2	18	0	2
	56	SL¶	0	14	0	30	2	8
F	42	B**	0	29	0	32	7	0
	56	SL‡‡	2	67	1	28	2	0
	56	SL‡‡	0	36*	1	28	2	0
F	60	SL	5	84	1	–	–	–
M	60	SL	0	48	2	54	6	0
F	120	SL	0	1	0	49	6	0
	120	SL	2	6	4	58	2	0
Sum as percent			2.5	94.0	3.5	87.9	8.8	3.3

Each entry represents test of cells from one donor unless otherwise noted in cell source column.

‡ B = blood leukocytes; SL = spleen and cervical lymph node cells.

§ Pooled leukocytes from three donors.

‖ Pooled leukocytes from same three donors as above (repeat test).

¶ Two of same three donors tested individually.

** Pooled leukocytes from two donors.

‡‡ Same donors tested individually.

deficit is indicated by the paucity of 1° source metaphases from GVHR induced by lymphocytes from intact L-LBN-TA donors soon after adoptive abolition of tolerance, and by their virtual absence from reactions induced by lymphocytes from adult thymectomized L-LBN-TA in (LBN)F_1 hosts. The specific cellular deficit in H-ARC in the tolerant animal is thus made manifest by its continuance after tolerance is adoptively terminated. The deficit revealed by these data would have been obscured if there had been a considerable number of reversibly inactivated H-ARC in the tolerant animal, because such cells would have been encountered as 1° source metaphases in GVHR in (LBN)F_1 hosts. The deficit would also have been obscured were large numbers of the cells that proliferate in the local GVHR responding to stimuli other than foreign AgB

antigens of the host, and/or if H-ARC were not comprised of subsets (or clones) recognizing different AgB antigens (5, 6). While the present results are adequate to show that irreversible inactivation is the eventual fate of almost all specific H-ARC when TT is induced under the conditions of the experiment, the possibility remains that a small population of reversibly tolerant cells was obscured by the background noise of about 5% mitoses not derived from donor H-ARC.

Recovery of the previously depleted set of H-ARC in L-LBN-TA proceeds slowly over a matter of months (8, footnote 1). Weak reactivity, attributable to adoptively conferred H-ARC during the 1st mo after adoptive transfer, becomes stronger as 1° source H-ARC begin to appear in intact L-LBN-TA.[1] However, as noted above, these 1° source cells are not detected if the chimera has previously been thymectomized. This suggests that the specific deficit among autochthonous H-ARC in the tolerant L-LBN chimera is repaired after termination of TT by a thymus-dependent mechanism. Presumably this repair proceeds by the maturation and peripheralization of thymic precursors of H-ARC (5, 9). The time required for the appearance of 1° source H-ARC responsive to formerly tolerated antigen was approximately that required for differentiation of marrow-derived stem cells into peripheral T cells via the thymus, as determined by chromosome marker studies in mice (9). An alternative explanation of our data would be that thymic hormone is required for the recovery of tolerant T cells, but this seems unlikely in view of the time required for the appearance of specific 1° source H-ARC in the intact donors.

One might question whether recuperating tolerant cells missed detection because we waited too long after adoptive transfer before utilizing the L-LBN-TA as donors for the induction of GVHR. Perhaps so, but individual H-ARC are normally either long-lived or sporadically dividing cells that leave similarly competent progeny (10). Recuperated tolerant cells would thus have been missed on account of experimental delay only if they were short-lived end cells, but in that case they could be regarded as biologically insignificant.

One final point, which merits comment, is that the H-2 antigens that stimulate proliferation of mouse H-ARC in vitro may be serologically silent. Such H-2 factors have been called "lymphocyte-defined" antigens (11). If the same situation holds true in the rat, it seems probable that the tolerance detected in the present study was tolerance to T lymphocyte-defined AgB antigen. The exact role of such lymphocyte-defined antigens in elicitation of various manifestations of alloaggression has yet to be defined. There is preliminary evidence that the T cells that mediate cytotoxicity in vitro sometimes recognize different alloantigens from those that stimulate proliferation of H-ARC (11). Thus there is not necessarily a conflict between studies showing tolerance among H-ARC, and those of the Hellströms and Wegmann that indicate that "forbidden clones" of cytotoxic lymphocytes are often present in various allogeneic chimeras (1, 12). Other possible interpretations of the latter phenomenon have been discussed elsewhere (2).

SUMMARY

Recovery from adoptively terminated transplantation tolerance was studied by utilizing formerly tolerant rats as donors of lymphocytes in local renal graft-vs.-host reactions (GVHR). The origin of the proliferating lymphocytes in the GVHR was studied by means of sex chromosome markers. A deficit of specifically reactive lymphocytes, while tolerance was in effect, was revealed by the continuing absence of autochthonous specifically reactive cells after tolerance was abolished in adult thymectomized chimeras. The findings are consistent with Burnet's hypothesis of the cellular basis of tolerance, but apply only to the T lymphocytes of donor origin which normally proliferate in these GVHR.

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