ALLOANTIGEN PERSISTENCE IN INDUCTION AND MAINTENANCE OF TRANSPLANTATION TOLERANCE

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The establishment of donor-specific transplantation tolerance universally observed in stable bone marrow (BM)[1] chimeras has stimulated several studies on the tolerogenic role of donor-type cells in the induction and maintenance of an alloantigen-specific unresponsive state. Infusion of allogeneic lymphohematopoietic cells into immunologically immature or immunosuppressed recipients may under certain circumstances lead to the induction of permanent tolerance to allografts, as opposed to the mere prolongation of allograft survival obtained in unreconstituted hosts (1–4). We have studied the mechanism underlying transplantation tolerance induced by total lymphoid irradiation (TLI). In rodents (mice and rats) treated by TLI, tolerance to allogeneic skin grafts can be induced only if donor-type BM cells are also infused (5, 6). Tolerance to perfused organ allografts can be induced in rats (6) and baboons (7) without concomitant infusion of marrow cells, suggesting that the organ itself may provide sufficient tolerogenic signals.

Bone marrow transplantation (BMT) of semiallogeneic combinations in which parental BM cells were infused into TLI-conditioned F_1 hybrids was shown to result in chimeric mice without clinical signs of graft vs. host disease (GVHD) (8). We could not find evidence of donor- or host-type cellular suppression to explain the specific unresponsiveness of the graft against the foreign parental antigens of the F_1 hosts (9).

Splenocytes derived from chimeric mice could transfer tolerance to skin allograft upon injection into irradiated adoptive recipients syngeneic to the original hosts, but could not convey skin tolerance in donor-type adoptive recipients lacking the histocompatibility antigens of the tolerizing hosts (10). These results indicated the possible significance of persistent alloantigen-presenting cells in tolerance.

Intrigued by the possible role of tolerogenic alloantigens in induction and

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[1] *Abbreviations used in this paper:* BM, bone marrow; BMT, bone marrow transplantation; LDA, limiting-dilution analysis; LDC, limiting-dilution culture; pCTL, cytotoxic T lymphocyte precursors; TLI, total lymphoid irradiation; WBI, whole-body irradiation.

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maintenance of transplantation tolerance, we designed an experimental model to help clarify this issue. Our data, presented here, indicate that presentation of tolerogen is not sufficient for tolerance induction. However, once induced, the maintenance of unresponsive state definitely depends on the persistent presence of the tolerogen; alloantigen-specific unresponsiveness identified by limiting-dilution analysis (LDA) could be abrogated simply by transferring the tolerant cells into a tolerogen-free environment.

Materials and Methods

Animals. Inbred BALB/c (H-2^d) (BALB), C57BL/6 (H-2^b) (C57), (BALB/c × C57BL/6)F_1 (H-$2^{d/b}$) (F_1), and C3H/He (H-2^k) (C3H) were purchased from the Hebrew University Animal Farm in Jerusalem, Israel. Recipient mice aged 3–5 mo were subjected to TLI treatment or to whole-body irradiation (WBI), while 6–8-wk-old mice were used as bone marrow donors. All animals were kept in standard, nonprotected animal facilities.

Total Lymphoid Irradiation. Mice were anesthetized with pentrane and a mixture of halotane, N_2O, and O_2 for proper positioning in an apparatus designed to expose the major lymph nodes, thymus, and spleen while shielding most of the skull, ribs, lungs, hind legs, and tail with lead, as previously described (5). TLI consisted of eight exposures of 200 rad/d, six times per week, to a total dose of 1,600 rad delivered from a Phillips x-ray unit (250 kV, 20 mA) at a rate of 50–70 rad/min. The source-to-skin distance was 40 cm, and a 0.2-mm Cu filter was used.

Whole Body Irradiation. WBI was delivered from the same x-ray source as TLI, using different doses, as specified in each experiment.

Cell Irradiation. Single-cell suspensions were irradiated in vitro using a radioactive cobalt source (Gamma Cell 220; Atomic Energy of Canada, Ltd., Toronto, Canada).

Induction of Chimeras. Recipient F_1 mice received TLI (1,600 rad) followed by 200 rad WBI 24 h after the last fraction of TLI. Donor BM cells were isolated by flushing RPMI-1640 (Biological Industries, Kibbutz Beit Haemek, Israel) through the shafts of the femura and tibiae of donor mice. BM cells were washed once, and aliquots of 0.25 ml medium containing 3.0×10^7 live nucleated cells were injected into the lateral tail vein of recipient mice 24 h after WBI.

Assay for Chimerism. The percentage of donor-type cells in treated F_1 recipients was assayed using BALB anti-C57 and C57 anti-BALB antisera in a complement-dependent microcytotoxicity test as previously described (9). All chimeras used in this study displayed >85% donor-type cells in their peripheral blood.

Adoptive Transfer Experiments. Supralethal WBI of 1,800 rad was given to BALB or C57 mice to inactivate all alloreactive cells and their precursors. After 3 h, mice were injected intravenously with $6.0–10.0 \times 10^7$ lymphohematopoietic cells derived from mixtures of BM, spleen, and lymph nodes of either normal mice or long-term chimeras (>100 d after BMT). On the fifth day after irradiation, splenocytes of the adoptively transferred recipients were harvested and analyzed for cytotoxic T lymphocyte precursors (pCTL) in LDA.

Multiple Exposures to Tolerogen-presenting Cells. Normal C57 or BALB splenocytes were washed twice and irradiated (3,000 rad) before injection, either intraperitoneally or both intraperitoneally and intravenously, as specified in each experiment.

Limiting-dilution Cultures (LDC). Splenocytes were plated in LDC as described previously (9). Briefly, varying numbers (usually 250–32,000) of responding spleen cells (either chimeric or from normal mice) were plated in round-bottom microtiter wells (Nunc Roskilde, Denmark) at 16–24 wells/cell dilution with a great excess (10^6) of irradiated (3,000 rad) stimulating (BALB, C57 or C3H) splenocytes and T cell growth factor (TCGF; 5 U/well) (11) in a final volume of 0.2 ml. In mixing experiments, normal and chimeric cells were plated in a 1:1 ratio in LDC. After incubation for 8–9 d, the contents of each well were mixed and 100-μl aliquots from individual wells were transferred to conical-bottom microtiter wells (Nunc) for testing in a 4-h ^{51}Cr-release assay (9), using the

appropriate ^{51}Cr-labeled target cell bearing the H-2 antigens to which the cells were sensitized in LDC.

Cytotoxically negative wells were defined as those in which ^{51}Cr release counts did not exceed three standard deviations of the mean of the ^{51}Cr released in wells originally plated with stimulating cells only (spontaneous release). pCTL frequencies (f) and correlation coefficients (r^2) of regression analysis for Poisson distributions were calculated from plotting the percentage of noncytolytic cultures in each group of wells against the number of cells originally plated in the wells, as described elsewhere (12). In all experiments, r^2 values of all curves were >0.9.

Depletion of T Lymphocytes. 10^7 cells/ml BM cells were incubated with monoclonal anti-Thy-1.2 antibody (CL8600-A; Cedarlane Laboratories, Ontario, Canada; final dilution 1:500). The mixture was incubated for 1 h at 4°C with occasional shaking, after which the cells were pelleted, resuspended in fresh rabbit serum diluted 1:5 (complement), and incubated for 30 min at 37°C in a 5% CO_2 in air incubator. The proportion of T lymphocytes, determined by trypan blue exclusion, indicated 12% dead cells in BM treated by anti-Thy-1.2 antibody, as compared with 4% dead cells in a similar preparation treated with complement alone.

Results

pCTL Frequencies Directed Against Host and Third-party Alloantigens of Normal and Chimeric Splenocytes. BALB → F_1 chimeras containing 90–95% donor-type (BALB) cells in peripheral blood were sacrificed on days 42 and 395 after transplantation. Splenocytes harvested from these chimeras and splenocytes derived from normal BALB mice were plated in LDC, and frequencies of pCTL directed against host-type alloantigens (H-2^b) as well as against unrelated target cells (H-2^k) were estimated.

Table I shows that, at 42 d after transplant, the frequency of anti-H-2^b pCTL in BALB → F_1 chimeric splenocytes is at least 14-fold lower than that observed in normal BALB spleen cells (<1:300,000 and 1:21,000, respectively). The low frequency of anti-H-2^b pCTL was maintained in chimeric splenocytes harvested on day 395 after transplantation (1:81,500 vs. 1:5820 in normal splenocytes).

The frequency of pCTL directed against unrelated H-2^k alloantigens among chimeric splenocytes was eightfold lower on day 42 after transplantation (1:59,000 vs. 1:6800 in normal splenocytes), but was restored to a normal level on day 395 after transplantation (1:11,900 vs. 1:9,770 in normal splenocytes) (Table I).

To ascertain whether suppressor cells in the chimeric cell population are

TABLE I

pCTL Frequencies Directed against Host (H-2^b) and Third-party (H-2^k) Alloantigens of Normal and BALB → F_1 Chimera Splenocytes

Responding cells in LDC	Anti-H-2^b pCTL frequency		Anti-H-2^k pCTL frequency	
	42 d after BMT	395 d after BMT	42 d after BMT	395 d after BMT
Normal BALB	1:21,000	1:5,820	1:6,800	1:9,770
BALB → F_1 chimeras	<1:300,000	1:81,500	1:59,000	1:11,900
1:1 Mixture of normal BALB and BALB → F_1 chimeras	1:30,000	1:6,730	1:4,300	1:6,830

TABLE II

pCTL Frequency of BALB Cells Tolerant to C57 Obtained from BALB → F_1 Chimeras (H-2^d → H-$2^{d/b}$) after Parking in Supralethally Irradiated (1,800 rad) Syngeneic (H-2^d) or Tolerogenic (H-2^b) Recipients

Exp.	Adoptively transferred cells	Adoptive recipients	pCTL frequency	
			Anti-H-2^b	Anti-H-2^k
1	BALB	C57	1:150	1:290
	BALB	BALB	1:7,900	1:3,440
2	BALB tolerant to C57	C57	1:245,000	ND
	BALB tolerant to C57	BALB	1:11,500	ND
	Normal fresh BALB splenocytes	—	1:5,900	ND

capable of blocking the cytotoxic response of normal cells, mixtures of normal BALB splenocytes and chimeric cells were plated in LDC. At 42 d after transplantation, the anti-H-2^b pCTL frequency in such mixtures was found to be 1:30,000, similar to that found among normal BALB cells alone (1:21,000). Chimeric cells harvested on day 395 after transplantation also did not suppress the anti-H-2^b responses of normal BALB splenocytes (pCTL frequency of 1:6,730 in the mixed culture as compared with 1:5,820 in normal cells alone). In these mixing experiments, the frequency is stated in terms of normal BALB cells only; as if plated alone. Responses of normal BALB splenocytes against H-2^k alloantigens were equally unaffected by adding chimeric splenocytes harvested at 42 or 395 d after transplantation (Table I). The results strongly suggest that the lower frequency of anti-H-2^b pCTL is probably not mediated by active cell-mediated suppression, and thus supports the concept of functional clonal inactivation.

Adoptive Transfer of Chimeric Cells into Donor- or Recipient-type Secondary Hosts. Anti-host tolerant cells derived from pooled lymphohematopoietic compartments of stable chimeras were "parked" in supralethally irradiated secondary recipient mice (1,800 rad) that were either syngeneic to donor-type or the other parental haplotype of recipient-type cells of the chimera. The total number of cells in the transferred inoculum ranged from 6.0×10^7 to 10.0×10^7 cells/mouse. After 4 d, splenocytes of the secondary recipients were harvested and plated in LDC for the estimation of pCTL frequency. In parallel experiments, lymphohematopoietic cell mixtures obtained from normal mice were transferred into supralethally irradiated allogeneic and syngeneic mice, and after 4 d the splenocytes were analyzed for pCTL frequencies. Results of representative experiments are displayed in Tables II and III. Table II shows that when BALB cells tolerant to C57 (obtained from BALB → F_1 chimeras) were parked in supralethally irradiated C57 recipients, pCTL frequency directed against the tolerated antigen (C57) was clearly low (1:245,000), i.e., the same low level that was observed in the original chimeras (Table I). On the other hand, when the same cells (derived from the same pool of chimeras) were transferred into donor-type mice (BALB), the anti-C57 pCTL frequency increased to 1:11,500, a level comparable to that

TABLE III

pCTL Frequency of C57 Cells Tolerant to BALB Obtained from C57 → F_1 Chimeras (H-2^b → H-$2^{d/b}$) after Parking in Supralethally Irradiated (1,800 rad) Syngeneic (H-2^b) or Tolerogenic (H-2^d) Recipients

Exp.	Adoptively transferred cells	Adoptive recipients	pCTL frequency	
			Anti-H-2^d	Anti-H-2^k
1	C57	BALB	1:38	1:740
	C57	C57	1:520	1:700
2	C57 tolerant to BALB	BALB	1:117,600	ND
	C57 tolerant to BALB	C57	1:24,800	ND
	Normal fresh C57 splenocytes	—	1:4,900	ND

observed in normal controls. In this experiment, normal BALB splenocytes responding in LDC displayed an anti-C57 pCTL frequency of 1:5,900 (Table II).

Similar results were obtained by parking C57 → F_1 chimeric cells in supralethally irradiated BALB and C57 mice (Table III). A low frequency of pCTL (1:117,600) directed against the tolerogen (BALB) was observed in the BALB adoptive recipients, which were syngeneic to the tolerated antigen. On the other hand, a marked increase of anti-BALB pCTL frequency was documented in the C57 adoptive recipients that were syngeneic to donor-type cells of the chimeric mice (pCTL frequency of 1:24,800 as compared with 1:4,900 in normal C57 cells responding against BALB target cells).

Adoptive transfer experiments performed with pooled lymphohematopoietic cells obtained from normal mice were carried out to exclude "trapping" of alloreactive cells in heavily irradiated allogeneic recipients. After parking in the allogeneic host (C57) for 4 d, the adoptively transferred normal BALB cells became sensitized against H-2^b alloantigen, as documented by the high frequency of pCTL directed against both host-type (H-2^b) (1:150) and unrelated (H-2^k) target cells (1:290) (Table II). The high frequency of the anti-H-2^k pCTL can most likely be ascribed to crossreactivity between C57 and C3H mouse strains. After transfer into syngeneic hosts, the same inoculum of BALB cells displayed anti-H-2^b and anti-H-2^k pCTL frequencies (1:7,900 and 1:3,440, respectively), which are comparable to those found among normal BALB splenocytes (Table II). Similar results were obtained when normal C57 cells were adoptively transferred into supralethally irradiated syngeneic or allogeneic hosts (Table III).

The high alloreactive pCTL frequencies observed when normal cells were transferred into the allogeneic adoptive hosts is worth noting for its contrast with those obtained by transfer of chimeric cells. While the allogeneic adoptive host serves to sensitize normal cells, its effect on the genotypically identical allogeneic chimeric cells is that of continued tolerization. The results obtained with normal inocula may serve as indirect evidence against the possibility of in vivo selective depletion of alloreactive clones.

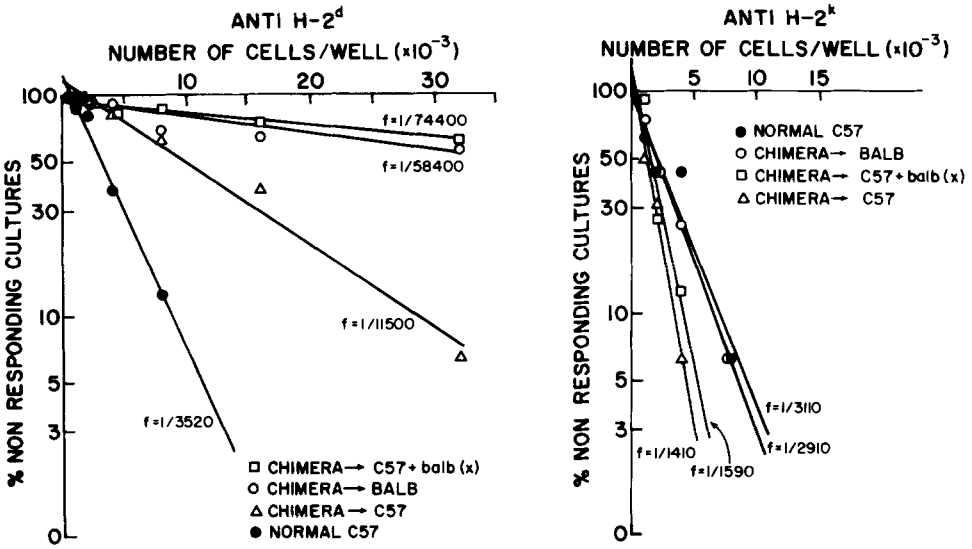
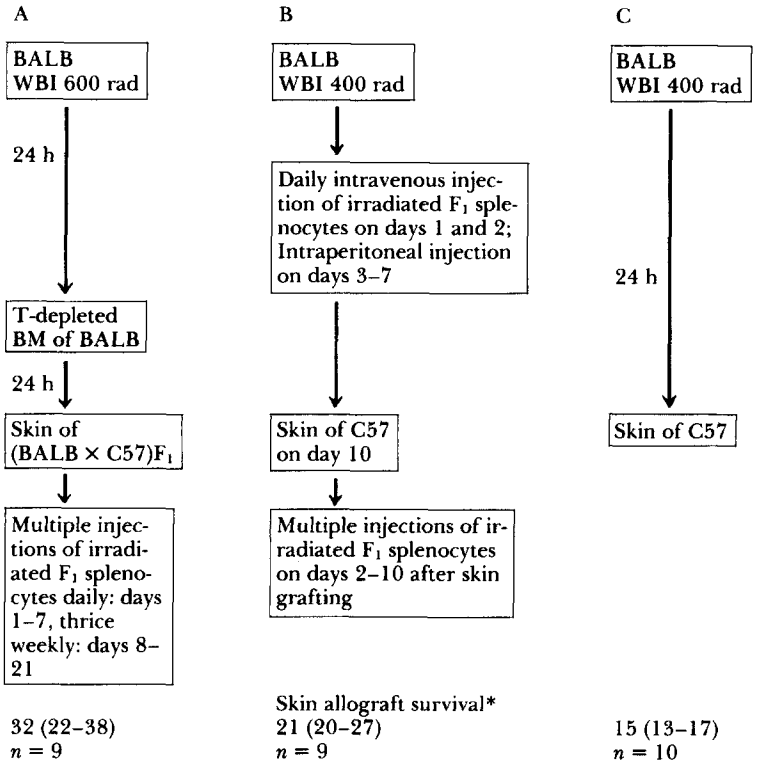


FIGURE 1. LDA of C57 → F_1 chimeric cells (C57 [H-2^b] tolerant to BALB [H-2^d]) after 'parking' for 4 d in supralethally irradiated (1,800 rad) BALB or C57 recipients. Adoptive recipients syngeneic with chimeric donor type cells (C57) were exposed to daily injections of irradiated (3,000 rad) BALB splenocytes. Frequencies of pCTL directed against the tolerizing (H-2^d) and unrelated (H-2^k) alloantigens were determined by LDA.

Control mice subjected to supralethal irradiation (1,800 rad) without lymphohematopoietic reconstitution survived for 5–6 d, and when sacrificed on the fifth day their spleens were totally depleted of cells ($<10^5$ cells). On the other hand, 3–15 × 10^6 cells could be recovered from reconstituted recipients sacrificed on the fifth day. We therefore assume that the cells harvested on the fifth day after supralethal irradiation were derived entirely from the lymphohematopoietic inoculum transferred to the adoptive recipients.

Multiple Exposures of Adoptively Transferred Tolerant Cells to Tolerizing Alloantigens in Adoptive Recipients. Adoptive recipients syngeneic to the chimeric donors were exposed to multiple injections of irradiated tolerogen-bearing splenocytes. Irradiated splenocytes were first introduced 18 h after lymphohematopoietic reconstitution, and followed by daily injections of 6.0–8.0 × 10^7 cells/mouse/d by both intravenous and intraperitonental routes for 3 d. Splenocytes of the adoptive recipients were harvested on the fifth day and analyzed for pCTL frequency directed against tolerogen- and unrelated-type cells.

Fig. 1 presents results obtained with C57 → F_1 chimeric cells that were adoptively transferred into supralethally irradiated C57 mice with or without multiple injections of irradiated BALB splenocytes. Here again, when BALB-tolerant C57 cells were transferred into heavily irradiated C57 mice, the low frequency of pCTL directed against BALB target cells clearly increased, and an almost normal alloreactive response was documented (1:11,500). However, multiple exposures of supralethally irradiated secondary hosts (C57) to irradiated tolerogen-bearing cells (BALB) resulted in a marked decrease in pCTL frequency directed against BALB (1:74,400), thus leading to the reestablishment of the



* Median (range in parentheses) in days.

FIGURE 2. Design of experimental protocols used to investigate potential role of multiple exposures of irradiated alloantigen-presenting cells in induction of unresponsiveness to allogeneic skin grafts.

tolerant state, as indicated by the low frequency of anti-BALB pCTL found in the original chimeras.

A low frequency of anti-BALB pCTL (1:58,400) could be maintained when the chimeric inoculum was transferred into supralethally irradiated BALB mice. Response to unrelated alloantigen (H-2^k) was similar in all three experimental groups (1:1,410, 1:1,590, and 1:2,910, respectively; Fig. 1).

Presentation of Tolerogen-bearing Cell in Induction of Tolerance. In previous sections we have brought evidence to support the notion that persistence of tolerogen-presenting cells is required for the maintenance of transplantation tolerance. It was of interest as well to study the role of tolerogen-presenting cells in induction of tolerance. We therefore designed the following experiments, detailed in Fig. 2.

Lethally irradiated BALB mice (600 rad) were reconstituted with T cell–depleted syngeneic BM and F_1 skin was grafted after 24 h. Multiple injections of irradiated F_1 spleen cells were given intraperitoneally every day for the first week, followed by three injections per week over the next 2 wk. Skin rejection by all nine mice tested was observed between days 22–38 with a median of 32 d (Fig. 2*A*).

Sublethally irradiated BALB mice (400 rad) were subjected to multiple injections of irradiated F_1 splenocytes on days 1 and 2 (intravenously) and on days 3–7 (intraperitoneally) after WBI (Fig. 2*B*). Skin of C57 mice was grafted on day 10 after WBI, followed by continued multiple daily intraperitoneal injections of irradiated F_1 splenocytes on days 2–10 after skin grafting. Skin rejection by all nine mice was observed on days 20–27, with a median of 21 d (Fig. 2*B*).

Sublethally irradiated (400 rad) BALB mice were transplanted with skin of C57 mice 24 h after WBI (Fig. 2*C*). This group of mice served as control for skin graft survival after sublethal irradiation. Skin rejection by all 10 mice tested was observed on days 13–17, with a median of 15 d. The mild delay of skin rejection observed in Fig. 2*B* as compared with Fig. 2*C* was found to be statistically significant ($p < 0.01$). It was impossible to induce permanent survival of skin allograft by multiple injections of irradiated splenocytes bearing the same H-2 antigens as the skin graft, although permanent tolerance to skin allografts was observed in one experiment using nonirradiated spleen cells in similar experimental conditions (data not shown).

Discussion

Tolerant mice that were conditioned with TLI and transplanted with allogeneic BM cells display markedly low frequencies of pCTL directed against host-type alloantigens. The low frequency of antihost-specific cytotoxic activity demonstrated on day 42 after transplant was maintained on day 395, while at the same time normal levels of cytotoxic cells could be raised against unrelated alloantigens, indicating the existence of specific transplantation tolerance at the cellular level. No evidence was found for the presence of regulatory cells to account for chimeric hyporeactivity in previous studies (9), as well as by 1:1 mixing experiments in the present study. We have previously shown (10) that when chimeric cells (C57 → BALB) were adoptively transferred to sublethally irradiated recipients (C57) that were syngeneic to donor-type cells of the original chimeras, skin allografts of the host-type (BALB) of the chimera were always rejected. On the other hand, irradiated host-type secondary recipients (BALB) permanently accepted skin allograft derived from mice syngeneic to donor-type cells (C57) of the original chimeras. Similar studies were carried out in lethally irradiated adoptive recipients of C57 → BALB chimeric cells to minimize the possibility of skin graft (BALB) rejection by residual host-type (C57) immunocompetent cells. The results of these studies indicated that BALB skin allografts were always rejected by the secondary (C57) recipients (our unpublished data). In other words, tolerance was broken upon transfer into a tolerogen-free environment. These results could be interpreted in two ways. The first is that residual alloreactive cells of the adoptive secondary recipients were radioresistant and could thus cause rejection of allogeneic skin. This interpretation implies that chimeric spleen cells are not capable of suppressing rejection induced by a relatively small number of residual alloreactive cells of the recipient. The presence of radioresistant alloreactive cells is well established in both animals and man (13, 14). Lethally irradiated (1,200–1,440 rad) patients with malignant hematological disorders who also receive high-dose cyclophosphamide (120 mg/kg) are still capable of rejecting BM grafts obtained from HLA-identical

siblings (15). The second possible explanation is that tolerant cells obtained from the chimeric spleen (C57 → BALB) may have lost their alloantigen-specific unresponsiveness in the new milieu of the H-2-incompatible adoptive host (C57). If this were the situation, it could be suggested that the new environment to which the chimeric cells were transferred lacks an essential constituent to maintain tolerance. Determining the mechanism of loss of specific unresponsiveness in allogeneic hosts might help in understanding the mechanism of tolerance maintenance. We therefore designed an experimental system to exclude any chance of residual reactivity of host lymphoid cells that might play a role during skin allograft rejection.

A supralethal irradiation dose of 1,800 rad was given to adoptive hosts before transfer of tolerant chimeric cells. Adoptive recipients were of two different genotypes, one syngeneic to host-type cells of the original chimeras and the other providing the chimeric cells with an environment lacking the tolerance-inducing H-2 haplotype. In another experiment, the chimeric cells in the tolerogen-free adoptive recipients were exposed to repeated injections of irradiated tolerogen-presenting cells.

The results of these experiments indicated that persistence of cells bearing the tolerogen is mandatory to maintain immunological unresponsiveness towards a specific alloantigen. Low frequencies of pCTL directed against the tolerogenic cells were maintained only in tolerizing-type adoptive recipients or in those recipients that received multiple injections of tolerogen-presenting cells. Whenever unresponsive cells were parked in hosts lacking the specific tolerogen, pCTL frequencies increased markedly in the adoptive recipients within 4 d. Frequencies of pCTL directed against unrelated alloantigens were close to normal regardless of the presence of the tolerogen-type cells in the adoptive recipients.

It could be argued that the low anti–host-type pCTL frequency observed in the adoptive transfer experiments (chimeric C57 → F_1 or BALB → F_1 cells transferred to BALB or C57 recipients, respectively) could result from a selective in vivo adsorbance of chimeric alloreactive cells directed against the adoptive hosts (C57 anti-BALB and BALB anti-C57, respectively). This explanation seems unlikely, because the original chimeric cells displayed a low frequency of pCTL against the tolerogen-type cells. Moreover, normal cells subjected to the same experimental conditions developed a very high anti–allogeneic pCTL frequency as a result of in vivo sensitization occurring in the adoptive hosts, rather than allospecific immunodepletion. This is in agreement with previously published data (16, 17) showing that hypoalloreactivity occurred during the first 24–48 h after priming with allogeneic lymphocytes, while marked hyperresponsiveness occurred on days 4 and 5 after priming. Because, in our experimental model, the splenocytes were harvested on the fifth day after infusion, negative selection caused by depletion of alloreactive clones is not a likely explanation for the hyporeactivity observed in chimeric cells parked in H-2-incompatible milieu.

Our results clearly indicate the requirement for the persistence of tolerizing alloantigens in maintaining already-induced tolerance. Repeated administration of irradiated alloantigen-presenting cells into sublethally irradiated recipients was insufficient for the induction of permanent tolerance as documented by

rejection of skin allografts in sublethally and lethally irradiated recipients (Fig. 2).

Several reports have already suggested the crucial role of donor cells in allograft tolerance (1–4, 18, 19). Luberof and Silver (20, 21) demonstrated the requirement for persistence of donor cells for maintenance of neonatally induced tolerance. By injecting tolerant hosts with cytotoxic antidonor isoantisera or transfer of antitolerogen-sensitized lymphoid cells, they could frequently abolish tolerance. Maki et al. (22) have shown that donor T cells with suppressive activity might also play a role in enhancement of skin allografts in mice.

In another experimental system, the importance of donor lymphoid cells was affirmed in the transfer of tolerance into secondary hosts. Dorsch and Roser (23), using neonatally induced tolerance in rats, could not transfer tolerance to secondary hosts if the small proportion (1–2%) of donor F_1 cells was removed from the transferred inoculum in a host vs. graft tolerance model. Pierce et al. (24), using fractionated WBI (a conditioning treatment somewhat similar to TLI) to establish allogeneic chimeras, have shown that cells responsible for tolerance transfer were of donor origin, and that these donor-derived cells were not only necessary but also sufficient for tolerance transfer. In those adoptive recipients, when donor lymphoid cells were omitted from the transferred inoculum, the skin allografts were uniformly rejected.

In what way the tolerizing cells effect tolerance maintenance is still unknown. Tutschka et al. (25) have provided evidence that tolerogen-bearing cells are crucial for the continuous presence of suppressor cells responsible for maintenance of transplantation tolerance. In their experimental model, chimeric splenocytes that were transferred to a tolerogen-free environment displayed a marked clonal reduction of suppressor cells, while reexposure to the tolerogen-type cells caused clonal expansion of the memory suppressor cells.

Stephenson et al. (26, 27) and Dorsch et al. (23) suggested that the tolerogen-bearing cells may serve as a source for alloreactive stimulation of the suppressor cells, which probably suppress via an antiidiotypic mechanism directed against a determinant present on the antitolerogen receptor of the alloreactive cells. This interpretation is entirely compatible with results presented by Stockinger (28) and Heeg et al. (29), who demonstrated removal of suppressor cells by adsorbance of tolerant cells on monolayers of syngeneic blasts bearing receptors for the given tolerogen. In these experimental systems, the tolerogen-presenting cells may be essential in antiidiotypic interactions that could play an important role in transplantation tolerance. By this mechanism, small numbers of suppressor cells may be sufficient for continuous presentation of tolerogenic signals, although suppressive effects might be undetected in standard suppression assays of limited sensitivity.

If immune tolerance is maintained by antiidiotypic interactions, it might fit well into the idiotypic network concept developed from Jerne's postulates to explain the general balance between unresponsiveness and responsiveness of the immune system (30).

Our experiments may also suggest an alternative explanation that we favor, that induction/maintenance of unresponsiveness occurs already at the level of pCTLs. The rapid loss (within 4 d) of the unresponsiveness of pCTL to alloan-

tigens in the absence of tolerogen, and the demonstration of its partial or complete restoration by tolerogen-bearing cells may indicate that maintenance of the unresponsive state may be due simply to blocking of alloantigen receptors on pCTL by soluble determinants of MHC antigens known to be present in serum and other body fluids. Shedding of such MHC determinants by metabolically active and dividing cells may certainly be explained by persisting cells, whereas irradiated, short-lived cells may serve as a short-term and limited source of tolerizing MHC determinants. The absence or, rather, undetectability of alloantigen-specific suppressor cells in parental → F_1 chimeras (9) suggests that the state of tolerance in TLI-induced chimeras is maintained by functional, alloantigen-dependent, clonal reduction.

In summary, we have brought evidence showing the importance of persistent presentation of tolerizing allogeneic cells in maintenance of tolerance to alloantigens at the cellular level by limiting-dilution analysis.

Further studies on the mechanism of induction and maintenance of transplantation tolerance, currently underway, are essential for understanding one of nature's most fundamental enigmas: the discrimination between self and nonself. Clearly, better understanding of some of these fundamental mechanisms might provide a way to induce true transplantation tolerance to allografts.

Summary

Infusion of parental bone marrow cells into F_1 hybrids conditioned by total lymphoid irradiation (TLI) results in chimeras with a high percentage of donor-type cells, and without clinical signs of graft-vs.-host reaction. In these chimeras, a state of tolerance has been shown to be associated with paucity of cytotoxic T lymphocyte percursors (pCTL) reactive with host-type alloantigens. To determine whether the presence of tolerizing alloantigens is essential for maintenance of unresponsiveness, lymphohematopoietic cells obtained from such tolerant chimeras were transferred into supralethally irradiated recipients of two different genotypes: in one case the adoptive recipients were syngeneic with host-type cells, and in the other they were syngeneic with donor-type cells of the original chimeras, thus providing the chimeric cells with a tolerogen-free environment. After "parking" for 4 d in syngeneic donor-type mice, the transferred cells displayed a marked increase in the frequency of pCTL directed against tolerizing alloantigens, whereas a low pCTL frequency directed against the same H-2 target cells was maintained in allogeneic tolerizing-type adoptive recipients. Multiple injections of adoptive donor-type mice with tolerizing-type cells of the original chimera reestablished a low level of cytotoxic precursors. Cytotoxic activity against unrelated alloantigens was independent of the presence of tolerogen-presenting cells in the adoptively transferred mice. Our experimental model suggests that persistence of cells bearing tolerizing alloantigens is an essential requirement for maintenance of previously established tolerance.

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References

1. Wood, M. L., A. P. Monaco, J. J. Gozzo, and A. Liegeois. 1971. Use of homozygous allogeneic bone marrow for induction of tolerance with anti-lymphocyte serum: dose and timing. *Transplant. Proc.* 3:676.
2. Caridis, D. T., A. Liegeois, I. Barrett, and A. P. Monaco. 1973. Enhanced survival of canine renal allografts of ALS-treated dogs given bone marrow. *Transplant. Proc.* 5:671.
3. Thomas, J. M., F. M. Carver, M. B. Foil, W. R. Hall, C. Adams, G. B. Fahrenbruch, and F. T. Thomas. 1983. Renal allograft tolerance induced with ATG and donor bone marrow in outbred rhesus monkeys. *Transplantation (Baltimore).* 36:104.
4. DeFazio, S., W. C. Hartner, A. P. Monaco, and J. J. Gozzo. 1986. Mouse skin graft prolongation with donor strain bone marrow and anti-lymphocyte serum. *Transplantation.* 41:26.
5. Slavin, S., S. Strober, Z. Fuks, and H. S. Kaplan. 1977. Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts. *J. Exp. Med.* 146:34.
6. Slavin, S., B. Reitz, C. P. Bieber, H. S. Kaplan, and S. Strober. 1978. Transplantation tolerance in adult rats using total lymphoid irradiation: permanent survival of skin, heart and marrow allografts. *J. Exp. Med.* 147:700.
7. Myburgh, J. A., J. A. Smit, J. H. Stark, and S. Browde. 1984. Total lymphoid irradiation in kidney and liver transplantation in the baboon: prolonged graft survival and alteration in T cell subsets with low cumulative dose regimens. *J. Immunol.* 132:1019.
8. Slavin, S., Z. Fuks, L. Weiss, and S. Morecki. Mechanisms of tolerance in chimeric mice prepared with total lymphoid irradiation. 1980. *ICN-UCLA Symp. Mol. Cell. Biol.* 17:383.
9. Morecki, S., B. Leshem, M. Weigensberg, S. Bar, and S. Slavin. 1985. Functional clonal deletion versus active suppression in transplantation tolerance induced by total lymphoid irradiation. *Transplantation (Baltimore).* 40:201.
10. Slavin, S., S. Morecki, M. Weigensberg, S. Bar, and L. Weiss. 1986. Functional clonal deletion vs suppressor cell-induced transplantation tolerance in chimeras prepared with a short course of total lymphoid irradiation. *Transplantation.* 41:680.
11. Leshem, B., B. Gotsman, and E. Kedar. 1984. In vitro elicitation of cytotoxic response against non-immunogenic murine tumor by allosensitization. *Cancer Immunol. Immunother.* 17:117.
12. McDonald, H. R., J. C. Cerottini, J. E. Ryser, J. L. Maryanski, C. Taswell, M. B. Widmer, and K. T. Brunner. 1980. Quantitation and cloning of cytolytic T lymphocytes and their precursors. *Immunol. Rev.* 51:93.
13. Gassmann, W., H. U. Wottge, M. von Kolzynski, and W. Mueller-Ruchholtz. 1986. Immune reactivity after high-dose irradiation. *Transplantation.* 41:380.
14. Korngold, R., J. R. Bennink, and P. C. Doherty. 1981. Early dominance of irradiated host cells in the responder profiles of thymocytes from P $\rightarrow$ F_1 radiation chimeras. *J. Immunol.* 127:124.
15. Slavin, S., R. Or, E. Naparstek, et al. 1986. New approaches for prevention of rejection and graft vs host disease in clinical bone marrow transplantation. *Isr. J. Med. Sci.* 22:264.
16. Dorsch, S., and B. Roser. 1974. The clonal nature of allo-antigen-sensitive small lymphocytes in the recirculating pool of normal rats. *Aust. J. Exp. Biol. Med. Sci.* 52:45.
17. Ryan, J. J., A. Ahmed, P. D. Kind, C. B. Thompson, A. K. Berning, and K. W. Sell. 1984. Recognition and response to alloantigens in vivo. I. Negative and positive

selection of MLR reactivity in murine peripheral blood lymphocytes to major histocompatibility complex and Mls antigens. *J. Immunol.* 133:606.

18. Noguchi, M., K. Onoe, M. Ogasawara, K. Iwabuchi, L. Geng, K. Ogasawara, R. A. Good, and K. Morikawa. 1985. H-2 incompatible bone marrow chimeras produce donor H-2 restricted Ly-2 suppressor T-cell factor(s). *Proc. Natl. Acad. Sci. USA.* 82:7063.
19. Desquenne-Clark, L., H. Kimura, and W. K. Silvers. 1985. Evidence that major histocompatibility complex restriction of foreign transplantation antigens occurs when tolerance is induced in neonatal mice and rats. *Proc. Natl. Acad. Sci. USA.* 82:6265.
20. Lubaroff, D. M., and W. K. Silvers. 1970. The abolition of tolerance of skin homografts in rats with isoantiserum. *J. Immunol.* 104:1236.
21. Lubaroff, D. M., and W. K. Silvers. 1973. The importance of chimerism in maintaining tolerance of skin allografts in mice. *J. Immunol.* 111:65.
22. Maki, T. M., R. Gottschalk, M. L. Wood, and A. P. Monaco. 1981. Specific unresponsiveness to skin allografts in anti-lymphocyte serum-treated marrow injected mice: participation of donor marrow derived suppressor cells. *J. Immunol.* 127:1433.
23. Dorsch, S., and B. Roser. 1982. Suppressor cells in transplantation tolerance. II. Identification and probable mode of action of chimeric suppressor T cells. *Transplantation.* 33:525.
24. Pierce, G. E., and L. M. Watts. 1985. The role of donor lymphoid cells in the transfer of allograft tolerance. *Transplantation (Baltimore).* 40:702.
25. Tutschka, P. J., A. D. Hess, W. E. Beschrner, and G. W. Santos. 1982. Suppressor cells in transplantation tolerance. III. The role of antigen in the maintenance of transplantation tolerance. *Transplantation.* 33:510.
26. Stephenson, S. P., and B. Roser. 1985. The molecular basis of transplantation tolerance. *Transplant. Proc.* 17:1145.
27. Stephenson, P., S. Dorsch, B. Roser, U. Godden, and J. Herbert. 1983. The identity of suppressor cells in neonatal tolerance. *Transplant. Proc.* 15:850.
28. Stockinger, B. 1984. Cytotoxic T-cell precursors revealed in neonatally tolerant mice. *Proc. Natl. Acad. Sci.* 81:220.
29. Heeg, K., and H. Wagner. 1985. Analysis of immunological tolerance to major histocomptibility complex antigens. I. High frequencies of tolerogen-specific cytotoxic T lymphocytes precursors in mice neonatally tolerized to class I major histocompatibility complex antigens. *Eur. J. Immunol.* 15:25.
30. Jerne, N. K. 1984. Idiotypic networks and other preconceived ideas. *Immunol. Rev.* 79:5.