SYNERGISM BETWEEN T AND NON-T CELLS IN THE IN VIVO INDUCTION AND IN VITRO EXPRESSION OF GRAFT-VS.-HOST DISEASE–INDUCED NATURAL SUPPRESSOR CELLS

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We have been using a murine model of graft-vs.-host disease (GVHD)[1] induced across minor histocompatibility antigen (MiHA) barriers to study the immunological changes that occur during chronic GVHD induced by bone marrow (BM) transplantation (1). In our system, we inject B10.D2 (H-2^d, mls b) lymphocytes into irradiated BALB/c (H-2^d, mls b) recipients. These two strains are thus H-2- and mls-identical, and are not reactive in primary mixed leukocyte reaction (MLR) cultures. This model system is very similar to human BM transplantation in which the donor and recipient are matched for HLA identity and nonreactivity in MLR (2, 3).

Previously (4), we have shown that irradiated (600 rad) BALB/c recipients of B10.D2 spleen cells develop a chronic form of GVHD that resembles scleroderma. Furthermore, spleen cells taken from the GVH animals 10 d postinjection are almost totally unresponsive to mitogens (1). These spleen cells also suppress the proliferative response of normal spleen cells to mitogens (1). These changes are profound, and last for many months postinjection (5). We have also shown (5) that, while this GVH suppression appears nonspecific and genetically unrestricted, there are some peculiarities. Early (10-d) GVH suppressor cells are able to suppress the mitogen responses of all mouse strains tested, except for a relatively weak suppression of the lipopolysaccharide (LPS) response of B10.D2 (syngeneic with donor). By day 42, GVH suppressor cells are almost completely unable to suppress the LPS response of B10.D2 spleen cells, while still being able to suppress the mitogen responses of all other mouse strains (including the concanavalin A [Con A] response of B10.D2) (5). The cells that mediate this GVH suppression are not mature T cells, B cells, or macrophages. They can be enriched in the less dense fractions of percoll gradients, but they show no natural killer reactivity. Therefore, they belong to the class of regulatory cells known as natural suppressor (NS) cells (6).

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[1] *Abbreviations used in this paper:* BM, bone marrow; BSS, balanced salt solution; C′, complement; CAS, Con A supernatant; GVHD, graft-vs.-host disease; LN, lymph node; LPS, lipopolysaccharide; MiHA, minor histocompatibility antigen; MLR, mixed lymphocyte response; NS, natural suppressor; TCM, tissue culture medium.

In this report, we investigated the cellular requirements for both the in vivo induction as well as the in vitro expression of this GVH NS cell suppression. While donor T cells are necessary, they are not sufficient in themselves, and a non-T cell population is also required for maximum induction in vivo. The maximum expression of NS cell suppression in vitro also requires T cells. At later times, when GVH suppressor cells are losing potency, the addition of externally generated T cell signals (Con A supernatant [CAS]) allows these suppressor cells to express suppressor function again.

Materials and Methods

Mice. BALB/c (H-2^d, mls b) were purchased from Cumberland View Farms, Clinton, TN. B10.D2/nSn (H-2^d, mls b), C57BL/6 (H-2^d, mls b), and DBA/2 (H-2^d, mls a) were purchased from The Jackson Laboratory, Bar Harbor, ME. C3H/HeN (H-2^d, mls c) were purchased from the Charles River Breeding Laboratories, Wilmington, MA. All mice were female and were used at 6–12 wk of age.

Injection of Transplanted Cells into Host Mice. For the majority of experiments, spleen cells were pooled from donor B10.D2 or BALB/c mice. However, several experiments also used pooled thymus, lymph node (LN) (inguinal and axillary), or BM (from the femur and tibia) cells. Erythrocyte-free cell suspensions were prepared by passage through a stainless steel mesh screen and treatment with Tris-NH_4Cl solution, followed by three washings in balanced salt solution (BSS). After washing, a total of 4 or 5×10^7 trypan blue–excluding B10.D2 or BALB/c cells were injected intravenously in a volume of 1.0 ml BSS into irradiated recipient BALB/c mice. BALB/c recipients of B10.D2 cells are called GVH mice. BALB/c recipients of BALB/c cells are called syngeneic controls.

Irradiation of Recipient Mice. Recipient BALB/c mice were irradiated on day 0 with 600 rad (from a ^{60}Co source [15 rad/min]). They were injected within 3 h after irradiation. After irradiation, all mice were given tetracycline (Polytic, 250 mg/liter; American Cyanamid Co., Wayne, NJ) in their drinking water for the first 14 d. All mice were kept in a laminar flow hood for the duration of the experiment.

Experimental Spleen Cell Suspensions and Medium. At various times after injection, recipient BALB/c mice were killed, their spleens removed and pooled (a minimum of three mice per group). Erythrocyte-free cell suspensions were prepared as above. After washing, the cells were counted and tested for their ability to proliferate in response to mitogens, and for their ability to suppress normal mitogen responses.

All cultures were carried out in tissue culture medium (TCM), which is RPMI 1640 (Flow Laboratories Inc., McLean, VA) supplemented with 200 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes, and 5% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Clara, CA). Cultures were carried out at 37°C in a humidified atmosphere of 5% CO_2.

Mitogen Proliferation. Mitogens used were Con A (Jackbean; Calbiochem-Behring Corp., San Diego, CA), at a final concentration of 4 μg/ml, and LPS (*S. typhosa*; Difco Laboratories, Detroit, MI), at a final concentration of 10 μg/ml. Spleen cells from either GVH, syngeneic control, or normal mice were placed in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at 2.5×10^5 cells/well in 200 μl of TCM (plus mitogen). They were cultured for a total of 72 h, the final 18 h with 1 μCi of [^{3}H]thymidine (in 10 μl TCM). Cells were harvested, and [^{3}H]thymidine uptake was estimated by liquid scintillation counting.

Suppression of Mitogen Responses by GVH Spleen Cells. In mixing experiments that tested the suppressive ability of GVH spleen cells, 2.5×10^5 normal spleen cells and mitogen were cocultured with various numbers of GVH spleen cells (usually titrated from 4×10^4 to 2.5×10^5 cells/well). The total volume was maintained at 200 μl/well (plus mitogen). These cells were cultured and pulsed as above.

In all proliferation and suppression experiments, each test group was done at least in

triplicate. SD within each replicate was always <10% of the mean, except when the mean was near zero.

Anti-Thy-1.2 Plus Complement (C') Treatment. To eliminate Thy-bearing cells, 3×10^7 spleen cells were incubated for 60 min at 4°C with a 1:250 dilution of anti-Thy-1.2 (New England Nuclear, Boston, MA) in BSS containing 0.3% bovine serum albumin. The cells were then washed once, resuspended in 1 ml of BSS plus 0.3% bovine serum albumin with guinea pig C' (Gibco Laboratories, Grand Island, NY) at a final dilution of 1:15, and incubated a further 60 min at 37°C. After treatment, the cells were washed three times, and the cell concentration readjusted in TCM. This treatment removes, from normal spleen cell populations, the proliferative response to Con A, but not to LPS.

Nylon Wool (NW) Enrichment of LN T Cells. LN cells were passed through an NW column to enrich for T cells, as described by Julius, et al. (7). Briefly, 2×10^8 LN cells at 5×10^7 cells/ml in TCM were applied to 1.8 g of prewashed NW in a 30-ml syringe. The column was incubated for 60 min at 37°C, then cells were eluted from the column with 37°C TCM. ~25% of the cells were recovered by elution.

Con A Supernatant. Lectin-free CAS was produced using BALB/c spleen cells. Spleen cells were resuspended at 10^7 cells/ml in TCM with Con A (4 μg/ml). 50 ml of this suspension were then incubated at 37°C in a 75-cm^2 tissue culture flask (Falcon Labware, Oxnard, CA). After 2 h, the medium was gently poured off and the flask gently washed three times with TCM (without Con A). 50 ml of fresh TCM (without Con A) was then added to the flask, and incubation was continued for an additional 18 h at 37°C. CAS was then harvested and centrifuged to remove cells. The CAS was concentrated using an Amicon filter (cutoff of 10,000 mol wt) to 10 times its original concentration. Concentrated CAS was stored at −70°C until use. It was then reconstituted to its original volume with TCM, and used at final dilutions in culture of 1:10 (i.e. 20 μl in a total of 200 μl), 1:30, and 1:90.

Lectin-free CAS was assayed for its ability to maintain the interleukin 2–dependent T cell line CTLL-20 in culture, and for its lack of lectin by inability to stimulate normal BALB/c spleen cells in culture.

Results

Requirement of T Cells for In Vivo Induction of GVH Suppression. We have previously shown (4) that irradiated (600 rad) BALB/c mice develop chronic GVHD when injected with MiHA-disparate B10.D2 spleen cells. 10 d after injection, there is pronounced splenomegaly, the recipient spleen cells are hyporesponsive to mitogens, and also suppress normal spleen cell proliferative responses to mitogens (1, 4–6).

The first series of experiments were designed to see if T cells in the inoculum are required for the in vivo generation of immune hyporesponsiveness and suppression. Irradiated BALB/c hosts were injected with allogeneic B10.D2 spleen cells, either untreated, or treated with anti-Thy-1.2 plus C' to remove T cells. As shown in Table I, spleen cells from mice that had received T cell–depleted B10.D2 spleen cells (group E) were unable to induce suppression, while spleen cells from mice that were injected with either untreated or C' only–treated B10.D2 spleen cells (groups C and D) were able to suppress mitogen responses. Also, while far from normal, the mitogen responses of the spleen cells from the mice injected with the T-depleted B10.D2 spleen cells (group E) were greatly elevated when compared to those given by the untreated or C' control B10.D2 spleen cell–injected mice (groups C and D).

Finally, it should be noted that the viable cell recovery per spleen of BALB/c host mice injected with T-depleted B10.D2 spleen cells (group E) was substantially lower than those injected with whole B10.D2 spleen cells (groups C and D). In

TABLE I

T Cells Are Required in the B10.D2 Spleen Cell Inoculum to Induce Suppression in BALB/c Hosts

Group	Cell type injected*	Donor cell treatment‡	Viable cell recovery per spleen§	Con A–induced proliferation‖	LPS-induced proliferation‖	Suppression of Con A–induced proliferation¶	Suppression of LPS-induced proliferation¶
				cpm			
A	Normal BALB/c	—	1.7×10^8	325,000	110,000	356,000	117,000
B	None	—	5×10^6	98,000	4,000	333,000 (6)	90,000 (23)
C	B10.D2	—	1.01×10^8	500	200	5,000 (99)	4,400 (96)
D	B10.D2	C′	8.0×10^7	400	2,000	45,000 (87)	18,000 (84)
E	B10.D2	anti-Thy + C′	2.2×10^7	31,000	36,000	359,000 (−1)	98,000 (17)
F	BALB/c	—	2.9×10^7	143,000	29,000	348,000 (2)	96,000 (18)
G	BALB/c	C′	3.3×10^7	123,000	29,000	363,000 (−2)	93,000 (20)
H	BALB/c	anti-Thy + C′	2.3×10^7	81,000	28,000	363,000 (−2)	22,000 (30)

BALB/c (600 rad) mice were injected on day 0, and their spleens were assayed 10 d later.

* 5×10^7 of the indicated spleen cells were injected into BALB/c hosts on day 0. Normal BALB/c are mice neither irradiated nor injected. Experimental mice all received 600 rad.

‡ Before injection, spleen cells were treated with anti-Thy-1.2 + C′ to remove T cells.

§ A minimum of three mice per group were killed, their spleen cells were pooled, and the total number of viable cells per spleen was determined by trypan-blue exclusion.

‖ 2.5×10^5 cells/well + 4 μg/ml Con A or 10 μg/ml LPS.

¶ 10^5 GVH spleen cells were added to 2.5×10^5 normal BALB/c cells/well + Con A or LPS. Percent suppression (in parentheses) was calculated vs. 3.5×10^5 normal BALB/c spleen cells/well with the appropriate mitogen.

fact, the cell recovery is about the same as seen in the syngeneic control BALB/c → BALB/c mice (groups F, G, and H). There was also substantial splenomegaly in the mice that had received either the untreated or C′ only–treated allogeneic B10.D2 spleen cells. This is not readily apparent in the viable cell recovery because a large percentage (50–70%) of the total cell number in these suppressive spleens at this time are, in fact, dead cells (our unpublished observations).

Relative Efficiency of Cells from Various Lymphatic Organs in Inducing GVH Suppression. Since the previous experiments had shown that T cells are required for the in vivo induction of GVH suppression (as well as maximum hyporesponsiveness to mitogens and splenomegaly), the next series of experiments was designed to test the ability of B10.D2 cells from either the spleen, BM, LN, or thymus to induce suppression in irradiated BALB/c hosts. As shown in Table II, those mice that had been injected with either BM (group D) or spleen cells (group E) had many more viable spleen cells than animals injected with either thymus or LN cells (groups B and C, respectively).

With regard to mitogen responsiveness, spleen cells from group E mice, which had received B10.D2 spleen cells, were unresponsive to both Con A and LPS. Group D mice, which had received BM cells, were relatively hyporesponsive, although comparable to those seen in the appropriate syngeneic control (group H). The spleen cells from mice that received thymus (group B) or lymph node (group C) were only moderately hyporesponsive.

With regard to suppressive abilities, spleen cells from recipients of B10.D2

TABLE II

Source of B10.D2 Cells Used to Induce Suppression in BALB/c Hosts

Group	Cell type injected*	Viable cell recovery per spleen‡	Con A–induced proliferation§	LPS-induced proliferation§	Suppression of Con A–induced proliferation‖	Suppression of LPS-induced proliferation‖
					cpm	
A	Normal BALB/c	1.6×10^8	257,000	91,000	329,000	101,000
	B10.D2					
B	Thymus	3×10^6	53,000	2,400	306,000 (7)	97,000 (4)
C	LN	8×10^6	37,000	65,000	291,000 (11)	53,000 (47)
D	BM	9.0×10^7	2,800	16,000	165,000 (50)	58,000 (42)
E	Spleen	7.1×10^7	400	700	9,300 (97)	5,000 (95)
	BALB/c					
F	Thymus	5×10^6	82,000	2,400	334,000 (−1)	108,000 (−7)
G	LN	4×10^6	208,000	27,000	324,000 (2)	91,000 (10)
H	BM	1.1×10^8	9,500	20,000	302,000 (8)	92,000 (9)
I	Spleen	3.8×10^7	33,000	12,400	342,000 (−4)	83,000 (18)

BALB/c (600 rad) mice were injected on day 0, and their spleens were assayed 10 d later.

* 5×10^7 cells from the indicated organ were injected into BALB/c hosts on day 0. Normal BALB/c are mice neither irradiated nor injected.

‡ A minimum of three mice per group were killed, their spleens pooled, and the total number of viable cells per spleen was determined by trypan-blue exclusion.

§ 2.5×10^5 cells/well + 4 μg/ml Con A or 10 μg/ml LPS.

‖ 10^5 GVH spleen cells were added to 2.5×10^5 normal BALB/c cells/well + Con A or LPS. Percent suppression (in parentheses) was calculated vs. 3.5×10^5 normal BALB/c spleen cells/well with the appropriate mitogen.

spleen cells (group E) suppressed normal lymphocyte proliferative responses to Con A and LPS. Spleen cells from BM-injected mice (group D) were moderately suppressive, but spleen cells from mice injected with LN or thymus cells (groups C and B) showed little suppressive ability. Fig. 1 shows a typical titration with GVH or syngeneic control spleen cells in the suppression of the Con A response of normal BALB/c spleen cells. 2.5×10^5 normal BALB/c cells are easily suppressed by as few as 4×10^4 GVH spleen cells from mice that had received a B10.D2 spleen inoculum, while recipients of BM cells are suppressive only at 1.0 or 2.5×10^5 cells. The least suppressive cells are those from mice receiving thymus or LN cells. In contrast, Fig. 1*B* shows that spleen cells of the syngeneic controls are not significantly suppressive at any cocultured dose.

Thus, although results in Table I show that T cells are required to induce hyporesponsiveness and suppression, the results in Table II and Fig. 1 show that neither thymus nor LN cells (both of which are mostly T cells) are able to induce hyporesponsiveness and suppression alone.

T and Non-T Cells Are Required for In Vivo Induction of Maximum GVH Suppression. The previous results indicated that T cells are required, but not sufficient for in vivo induction of GVH suppression. Also, the injection of a mixture of two cell populations (one [LN] rich in mature T cells, the other [BM] rich in stem cells, but neither capable of inducing maximum suppression alone) produced a synergistic effect in the induction of suppression (data not shown). We next asked if a purified T cell population could show synergy with a T cell–depleted population for the in vivo induction of GVH suppression. This was done by injecting irradiated BALB/c recipients with mixtures of B10.D2 spleen

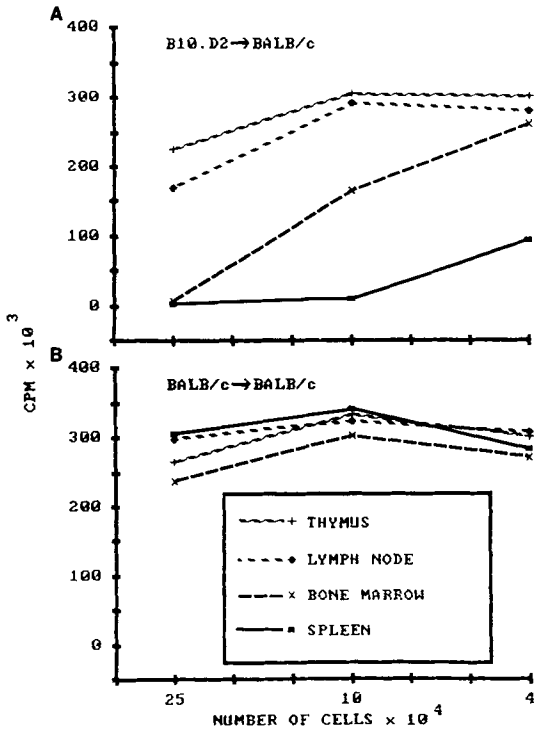

FIGURE 1. Titration of GVH spleen cell suppressive activity: Source of B10.D2 cells used to induce suppression in irradiated BALB/c hosts. BALB/c hosts were irradiated on day 0, then injected intravenously with 5×10^7 nucleated cells from either the thymus, LN, BM, or spleen of B10.D2 (*A*) or BALB/c (*B*) mice. 10 d later, three or more mice were killed per group, and their spleens were pooled. 25, 10, or 4×10^4 GVH or control spleen cells were cocultured with 25×10^5 indicator spleen cells from BALB/c plus Con A (4 μg/ml) cultures for 72 h, the final 18 h with [^{3}H]thymidine (indicator culture minus suppressors gave 295,000 cpm). The cultures were then harvested and counted for ^{3}H incorporation. cpm are expressed on the vertical axis, with the number of added GVH spleen cells on the horizontal axis.

cells depleted of T cells by anti-Thy-1.2 plus C′ treatment, and B10.D2 LN cells enriched for T cells by passage through an NW column.

Table III shows the results. When untreated B10.D2 spleen cells were given to irradiated BALB/c recipients (group B), the recipient spleens were unresponsive to Con A and LPS, and also suppressed the Con A and LPS responses of normal cells. If the donor graft was anti-Thy-treated spleen cells (group C), the unresponsiveness and suppression were much less evident. Donor NW nonadherent LN cells (group D) induced only modest unresponsiveness and suppression. However, mixtures of T-depleted spleen and nonadherent LN cells (groups E–G) produced significant unresponsiveness, and potent suppression. As few as 5% LN T cells added to 95% T-depleted spleen cells produced more unresponsiveness and more suppression than could be expected from the sum of their separate abilities.

Genetics of Suppressive Ability of GVH Spleen Cells. The next series of experiments was designed to explore the role of T cells in the expression of this GVH-induced suppression in vitro.

TABLE III

T and Non-T Cells Are Required for In Vivo Induction of Maximum Suppression

Group	Injected cells			Viable cell recovery per spleen	Con A–induced proliferation§	LPS-induced proliferation§	Suppression of Con A–induced proliferation‖	Suppression of LPS-induced proliferation‖
	Untreated spleen cells	Anti-Thy-1.2 + C′–treated spleen cells	NW-NA LN cells					
						cpm		
A	Normal BALB/c			1.35×10^8	311,000	92,000	356,000	93,000
B	4×10^7 B10.D2			8.3×10^7	400	300	35,000 (90)	5,000 (95)
C	—	4×10^7	—	3.1×10^7	39,000	41,000	320,000 (10)	69,000 (25)
D	—	—	2×10^7	4×10^6	18,000	4,300	264,000 (26)	52,000 (43)
E	—	2×10^7	2×10^7	5.1×10^7	600	300	85,000 (76)	5,000 (95)
F	—	3.3×10^7	6.7×10^6	6.9×10^7	600	400	67,000 (81)	9,000 (90)
G	—	3.8×10^7	2.2×10^6	7.0×10^7	400	1,300	83,000 (77)	26,000 (72)
H	—	—	—	6×10^6	149,000	12,000	346,000 (3)	76,000 (18)

BALB/c (600 rad) mice were injected on day 0, and their spleens were assayed 10 d later.

* B10.D2 cells of the indicated type were injected into BALB/c hosts on day 0. Normal BALB/c are mice neither irradiated nor injected. The cell types injected were either untreated spleen cells or anti-Thy-1.2 + C′–treated spleen cells (i.e. T cell–depleted), or NW-nonadherent (NW-NA) LN cells (i.e. T cell–enriched), or a mixture of the latter two.

‡ A minimum of three mice per group were killed, their spleens cells were pooled, and the total number of viable cells per spleen was determined by trypan-blue exclusion.

§ 2.5×10^5 cells/well + 4 μg/ml Con A or 10 μg/ml LPS.

‖ 10^5 GVH spleen cells were added to 2.5×10^5 normal BALB/c cells/well + Con A or LPS. Percent suppression (in parentheses) was calculated vs. 3.5×10^5 normal BALB/c spleen cells/well with the appropriate mitogen.

Previously (5), we have shown that both 10- and 42-d GVH spleen cells are relatively nonspecific in their ability to suppress the mitogen response of normal lymphocytes. It was apparent that, while both 10- and 42-d GVH spleen cells could suppress the Con A responses of all mouse strains tested by almost 100%, they were not able to do the same with LPS responses. Day 10 GVH spleen cells were weak in suppressing a B10.D2 (syngeneic to donor) LPS response. By day 42, GVH spleen cells were unable to suppress the B10.D2 LPS response, while still able to suppress other LPS responses. This is shown in Fig. 2. This figure represents the selective loss with time of the ability to suppress a B10.D2 or self LPS response (GVH spleen cells are almost entirely of donor origin, data not shown). This result suggested that maximum in vitro expression of NS cell activity may require T cell signals. In the suppression of LPS responses, T cell activation would have to occur, probably through MLR-type reactions, while in the case of suppression of Con A responses, the added mitogen itself would allow for T cell activation, and therefore all strains could be suppressed. This would be most apparent at later times during GVHD, when the GVH NS cells have become somewhat quiescent.

Removal of T Cells from Normal Responder Population in Mitogen Assay Decreases Ability of 40-d GVH Spleen Cells to Suppress. This hypothesis implies that if T cells are removed from the normal responding indicator population (one where B10.D2 can act as a stimulus for an MLR), then the suppression generated by relatively quiescent 40-d GVH suppressor cells should be less than the same culture containing normal T cells. Results from one such LPS suppression experiment are shown in Table IV. This shows that, while removal of T cells

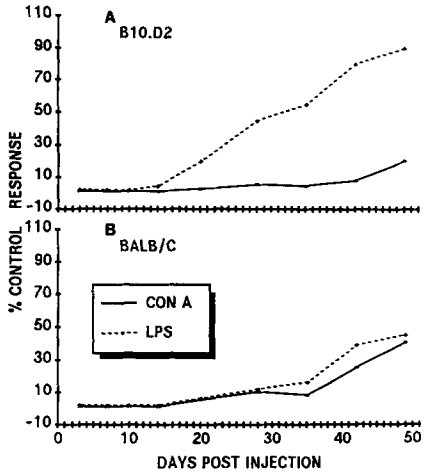

FIGURE 2. Suppressive ability over time of spleen cells from B10.D2 → BALB/c GVH animals towards normal B10.D2 (*A*) or BALB/c (*B*) lymphocyte proliferative responses to the mitogens Con A or LPS. BALB/c hosts were irradiated on day 0 with 600 rad, then injected intravenously with 5×10^7 B10.D2 spleen cells. At various times subsequently, three or more mice per day were killed, and their spleens were pooled. 2.5×10^5 GVH spleen cells were cocultured with 2.5×10^5 BALB/c (syngeneic to host) (*B*) or B10.D2 (syngeneic to donor) (*A*) spleen cells plus either Con A (4 μg/ml) or LPS (10 μg/ml) for 72 h, the final 18 h with [^{3}H]thymidine. The cultures were then harvested and counted for ^{3}H incorporation. Percent control response was calculated vs. the 2.5×10^5 normal BALB/c or B10.D2 Con A or LPS proliferative response, whichever was appropriate. The percent of control response is expressed on the vertical axis, with the time in days postirradiation/injection on the horizontal axis.

TABLE IV

Removal of T Cells from Normal Spleen Cell Population Reduces Ability of GVH Spleen Cells to Suppress

Group	Responder population	Anti-Thy-1.2 + C′ treatment	40-d GVH spleen cells added	Suppression of LPS-induced proliferation
				cpm
A	B10.D2	−	−	93,000
B		−	+	76,000 (18)
C		+	−	89,000
D		+	+	77,000 (13)
E	C57BL/6	−	−	121,000
F		−	+	64,000 (47)
G		+	−	102,000
H		+	+	84,000 (18)

2.5×10^5 normal spleen cells (with or without anti-Thy-1.2 + C′ treatment to remove T cells) were cocultured with 2.0×10^5 40-d B10.D2 → BALB/c (600 rad) GVH spleen cells with 10 μg/ml LPS.

* Percent suppression (in parentheses) was calculated vs. 4.5×10^5 normal spleen cell population (with or without anti-Thy-1.2 + C′ treatment).

from the normal B10.D2 population had little effect on the ability of B10.D2 → BALB/c GVH spleen cells to suppress (18 → 13%), the same treatment caused the suppressive ability of GVH spleen cells to fall from 47% to 18% with C57BL/6 populations. This result shows that T cells are important for in vitro expression of GVH suppression; in this case, the C57BL/6 T cells responding to the B10.D2 → BALB/c GVH spleen cells generate enough T cell signals to allow suppression to express itself (group F vs. E). When T cells are removed, suppression is greatly diminished (group H vs. G). When B10.D2 → BALB/c GVH suppressor cells are cultured with B10.D2 responder cells plus LPS (groups A–D), there are no MLR signals to activate the suppressors, and removal of responder T cells makes no difference.

CAS Enhances Suppressive Ability of 40-d GVH Spleen Cells. If GVH suppressor cells are taken at 40 d postinjection, when the suppressor cells are relatively quiescent (see Fig. 2 and ref. 5) and are added to the LPS response of normal spleen cells plus lectin-free CAS, the suppression should be more than the same culture minus lectin-free CAS. The results in Fig. 3 show that this is indeed the case.

The suppressive ability of the 40-d GVH spleen cells roughly parallels the proliferative responses in either a 1- or 2-way MLR between normal B10.D2 and these three strains (i.e. little suppression of B10.D2, moderate suppression of C57BL/6, and good suppression of DBA/2 LPS responses). The addition of the CAS (in itself not suppressive of normal LPS responses) greatly enhances the suppressive ability of these GVH suppressor cells. In fact, with a 1:10 or 1:30 dilution of CAS, the suppression of all three strains becomes essentially equal, thus indicating that the GVH suppressor cells are capable of suppressing lym-

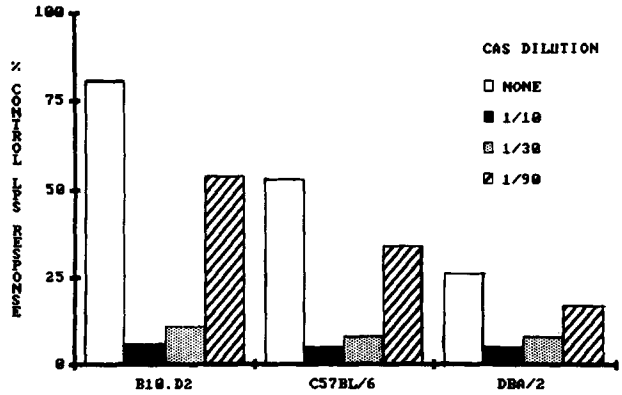

FIGURE 3. CAS enhances the suppressive ability of 40-d GVH spleen cells. BALB/c hosts were irradiated on day 0, then injected intravenously with 5×10^7 B10.D2 spleen cells. 40 d later, spleens were taken and pooled. 2.0×10^5 GVH spleen cells were cocultured with 2.5×10^5 normal spleen cells from the various mouse strains plus LPS (10 μg/ml) for 72 h, the final 18 h with [^{3}H]thymidine. Some of these cultures were done with a 1:10, 1:30, or 1:90 final dilution of CAS (not suppressive, at these dilutions, of normal LPS responses). The cultures were then harvested and counted for ^{3}H incorporation. Percent control response was calculated vs. the 4.5×10^5 normal spleen cell LPS proliferative responses. The percent control response is expressed on the vertical axis, with the various strains used on the horizontal axis.

phocytes from any strain if the proper T cell signals are present (from the CAS in this case).

Discussion

These experiments involve a murine GVHD model system where irradiated BALB/c recipients are injected with H-2- and mls-identical, but MiHA-disparate B10.D2 lymphocytes. This results in a chronic form of GVHD. Splenomegaly occurs early in these animals (4), although with time the spleens gradually empty of lymphocytes (5). The spleen cells taken from these mice are hyporesponsive to both Con A and LPS, and suppress normal spleen cell responses to these mitogens (1, 5). The suppressor cells are not mature T cells, B cells, or macrophages, but belong to a class called natural suppressor (NS) cells (6).

B10.D2 spleen cells injected into irradiated BALB/c recipients induce splenomegaly, mitogen unresponsiveness, and suppression. However, B10.D2 spleen cells depleted of T cells by anti-Thy-1.2 plus C′ treatment induce no splenomegaly, only moderate mitogen hyporesponsiveness, and little, if any, suppressive activity in the recipient spleen cells (Table I). This shows the absolute requirement of T cells in the in vivo development of this GVH suppression. Nevertheless, the results of Table II show that B10.D2 thymus or LN cells alone cause little if any suppression. This is not unexpected with thymocytes, as they are primarily immature T cells that may be unable to respond well to BALB/c MiHA. However, LN cells are primarily mature T cells that are capable of causing a fatal GVHD in this system (our unpublished observation) yet do not induce suppression. Thus, T cells are required for in vivo induction of GVH suppression, but they are not sufficient.

Further experiments showed that T cells interacted with a non-T cell population in the inoculum to induce the GVH suppression (Table III). A B10.D2 spleen cell population depleted of T cells showed tremendous synergism with a T cell–enriched LN cell population in the in vivo induction of GVH suppression. As few as 2.2×10^6 (5%) NW nonadherent B10.D2 LN cells injected with 3.8×10^7 (95%) anti-Thy plus C′–treated B10.D2 spleen cells produced near-maximal suppressive activity. Thus, it seems that although T cells are required (but not sufficient) for in vivo induction of GVH suppression, only a relatively small number of them are sufficient for maximum induction. Preliminary experiments indicate that the cell population with which the T cells synergize is a non-T, non-B, and nonmacrophage population.

Previous results (5) show the nonspecificity of these GVH suppressor cells at days 10 and 42, but there was a selective loss with time of the ability of GVH spleen cells to suppress a B10.D2 (self) LPS response while still being able to suppress the LPS and Con A responses of all other strains, including the Con A response of B10.D2 (Fig. 2). However, this inability to suppress a B10.D2 LPS response can be overcome by the addition of CAS to the cultures (Fig. 3). In fact, CAS enhances the suppressive ability of GVH suppressor cells in general.

These results have led us to the following hypothesis: NS cells are derived from the donor inoculum. Their proliferation and maturation into functional NS cells require an immunocompromised host (1, 5), as well as T cell signals. T cell signals are also necessary for maximum suppression by NS cells in vitro.

These NS cells then inhibit the proliferation of stimulated cells. In the GVH mice, the immunocompetent donor B10.D2 T cells react with a large number of allogeneic BALB/c host determinants, thus producing large amounts of activated T cell signals for an extended period of time. Activated T cell signals expand the NS cell population also derived from the donor. This sequence explains the need for both T cells and non-T cells in the donor B10.D2 inoculum. This mechanism also explains the progressive loss of cells in the spleens of these GVH animals (5). As host cells respond to various antigens, the NS cells inhibit their proliferation, and consequently, fewer and fewer cells survive. This may also explain in part the increased susceptibility to infections during GVHD (8–10). T cells responding to foreign antigens may actually be functionally eliminated by NS cells.

This mechanism also explains the apparent selective loss with time of the ability of B10.D2 → BALB/c GVH suppressor cells to suppress a B10.D2 LPS response. At a later time in this model system (e.g. the 40 d used in this paper), the NS cell population would be relatively quiescent because of lowered levels of activated T cell signals *in vivo*, as most reactive T cells will have been inhibited/eliminated by this time. However, it may be that a population of T cells becomes activated to a certain stage of development before the NS cell population becomes capable of their downregulation, this being one explanation for the chronic nature of this type of GVHD. Therefore, to show maximum suppressive activity in vitro, T cell activation is also needed. When these GVH NS cells are added to syngeneic B10.D2 indicator spleen cells with LPS, there is little if any T cell activation, and therefore only minimal suppression. However, if allogeneic indicator lymphocytes are used with LPS, then T cells in the responding population can recognize the foreign determinants present on the GVH (B10.D2) population, resulting in T cell signal production, followed by activation of NS cells, and inhibition of allogeneic LPS responses. The need for T cells in the normal population is shown in Table IV, where the LPS response of a T cell–depleted C57BL/6 population was much less suppressed than one in which T cells are present. In the case of the NS cell suppression of a Con A response, recognition of antigenic differences is not necessary, because Con A activates T cells in large numbers. Therefore, B10.D2 NS cells are able to suppress a syngeneic B10.D2 Con A response, but not an LPS response. Also, the addition of externally-generated T cell signals, in the form of CAS, can turn a situation where little or no suppression is found (i.e. suppression by 40-d GVH NS cells of a B10.D2 LPS response) to a situation where maximum suppression is possible (Fig. 3). Preliminary work indicates that CAS functions by activating the NS cells, not just by causing the NS cells to proliferate (although this activity may also be attributed to CAS [11]).

We have shown in previous work (5, 6) on this GVH system that the BALB/c → BALB/c syngeneic control mice also show some hyporesponsiveness and suppression. Although this suppression is much smaller in magnitude and more transient than the GVH suppression (5), the effector suppressor cell is also a NS cell (6). Syngeneic suppression and GVH suppression may in fact be instances of the same basic mechanism, as outlined above. In the syngeneic control mice, there should be few activated T cell signals generated by cells reacting to foreign determinants, although it is possible that some signals could

be generated by T cells reacting to increased levels of various syngeneic differentiation antigens which occur during the repopulation that follows irradiation.

This GVH suppression appears to be similar to that found in other systems where BM/lymphoreticular cells are rapidly proliferating: in neonatal mice (12), in adult mice or humans given total lymphoid irradiation (13, 14), with irradiation and autologous BM transplants in dogs (15), and after ^{89}Sr (16) or cyclophosphamide treatment in mice (17). In these cases, nonspecific suppressors of normal cell proliferation have been found, and these suppressors bear the phenotype of natural suppressor (or null) cells (13–15, 17, 18). Thus, it appears that NS cells may be present in all cases where rapid proliferation of stem cells is occurring, including the case of B10.D2 → BALB/c (irradiated).

It is possible that the suppressor mechanism we describe here could also be responsible for the inactivation/elimination of autoreactive cells that normally occurs in the neonate (19), or may occur in adult BM (20, 21), both of which have a relatively large NS cell component (13, 18). Early in T cell development, either in the neonate or in adult BM, pre-T cells that react with self antigens would become activated to release signals into the local environment. The NS cells, a natural component of these environments, could then respond to these activated T cell signals and downregulate the autoreactive T cell, with functional clonal elimination being the result.

Among the many interesting and potentially useful implications of this hypothesis is that it may further our understanding of the immunology of BM transplants and thus possibly widen the applications of this procedure (2, 3, 8, 22, 23). Also, if NS cells are involved, one may be able to prolong as well as enhance the neonatal period during which tolerance and graft acceptance can be induced. This may be possible by the addition of the proper T cell signals. Finally, in adults, procedures such as whole body irradiation followed by autologous (15), syngeneic (5, 6), or MiHA-disparate (1, 5, 6) reconstitution seem to open a window of time during which NS cells can be activated, and allow a period for the induction of acquired immunological tolerance. According to our data, this window of opportunity may be opened wider by the addition of the proper T cell signals.

Summary

We have been studying the mitogen hyporesponsiveness and immunosuppression induced in chronic murine graft-vs.-host disease (GVHD) induced across minor histocompatibility (MiHA) barriers. In this system, donor and recipient mice are major histocompatibility complex– and mls-identical, and are nonreactive in primary mixed leukocyte reactions. Spleen cells from B10.D2 (H-2^d, mls b) mice were injected into irradiated (600 rad) BALB/c (H-2^d, mls b) recipients. Recipient spleen cells are hyporesponsive to mitogens, and contain natural suppressor (NS) cells. We investigated the cellular requirements for both the in vivo induction and the in vitro expression of this GVH suppression. T cells are required in the graft, but they are not sufficient to induce suppression, and a non-T cell population is also required for maximum induction in vivo. T cells are also required for the maximum expression of NS cell suppressive ability in vitro. Early in the course of GVH, the suppressor cells are able to suppress the

Con A and LPS response of all mouse strains tested (except for the relative difficulty in suppressing the B10.D2 LPS response). Later, they become almost completely unable to suppress the B10.D2 LPS response, while still being able to suppress the Con A and LPS response of all other strains tested (including the B10.D2 Con A response). This inability to suppress a B10.D2 LPS response can be brought back to almost complete suppression by the addition of concanavalin A supernatant (CAS).

We present a hypothesis to explain what may be a common mechanism for GVH-induced suppression, total lymphoid irradiation–induced suppression, and neonatal tolerance. These situations all include rapidly proliferating lymphohematopoietic stem cell populations, and also have large numbers of NS cells. NS cells can suppress proliferating lymphoid populations, and their development and activity are greatly enhanced by T cell signals such as are supplied by donor T cells in chronic GVHD. Thus, NS cells may feed back on and downregulate self-reactive T cells or T cells responding to introduced foreign antigens.

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