ALLOSUPPRESSOR AND ALLOHELPER T CELLS IN ACUTE AND CHRONIC GRAFT-VS.-HOST DISEASE

V. F_1 Mice with Secondary Chronic GVHD Contain F_1-reactive Allohelper but no Allosuppressor T Cells

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A systemic graft-vs.-host reaction (GVHR)[1] can give rise to a variety of pathological symptoms. One of the possible outcomes is acute GVH disease (GVHD). After a brief initial phase of lymphoid stimulation (1–3),[2] acute GVHD rapidly produces suppressive pathological symptoms, such as pancytopenia accompanied by aplastic anemia and hypogammaglobulinemia (1–7).[2] A different possible consequence of the GVHR consists of long-term stimulatory pathological symptoms and is referred to as chronic GVHD (8). This type of GVHD comprises a persistent lymphoid hyperplasia (1–3, 6, 9),[2] hypergammaglobulinemia (1, 7, 9, 10), and the formation of autoantibodies and pathological lesions reminiscent of systemic lupus erythematosus (SLE) or other types of vascular collagen disease (1–3, 7–10). Either type of GVHD can be experimentally induced by injecting parental strain T lymphocytes into nonirradiated F_1 hybrid mice. Which type of GVHD will develop in these GVH F_1 mice appears to depend on the subset of donor T cells activated in them (2, 3, 7, 11).[2] The stimulatory GVH symptoms can be induced by Lyt-1^+2^--alloreactive donor T helper (Th) cells, whereas induction of the suppressive GVH symptoms requires both alloreactive donor Th and T suppressor (Ts) cells (3, 11).[2] The alloactivated donor Ts cells, which might act by the release of antimitotic factor(s), appear to be the final effector

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[1] *Abbreviations used in this paper:* BMC, bone marrow cell; CML, cell-mediated lympholysis; GVH, graft-vs.-host; GVHD, GVH disease; GVHR, GVH reaction; LGVHD, lethal GVHD; LNC, lymph node cell; LU, lytic unit; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; N F_1, normal BDF_1 hybrid (not undergoing GVHR); NMS, normal mouse serum; GVH F_1 chimera, nonirradiated BDF_1 repopulated by B10 SpC; PFC, plaque-forming cell; PLN, popliteal lymph node; SE, sheep erythrocyte; SLE, systemic lupus erythematosus; SpC, spleen cell; Th, T helper; Ts, T suppressor; Tk, T killer.

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cells causing the pancytopenia of acute GVHD, which, in turn, may terminate as lethal GVHD (7).[2]

In our preceding paper,[2] we analyzed the cellular events leading to acute GVHD after the injection into nonirradiated (C57BL/10 × DBA/2)F_1 (BDF_1) recipients of 10^8 unseparated spleen cells (SpC) obtained from C57BL/10 (B10) donors. After a brief initial activation of Lyt-1^+2^- donor Th cells, beginning in week 2, an activation of F_1-specific Lyt-2^+ donor Ts cells took place. The occurrence of these alloreactive Ts cells was confined to acute GVHD, in time and in the donor-host combinations being compared. In those of the B10-injected BDF_1 mice that did not succumb to acute GVHD but instead recovered from it, a gradual loss of donor Ts cells took place. This gradual disappearance of alloreactive donor Ts cells was paralleled in time by a decrease of the pancytopenia of acute GVHD. By day 60, the GVH F_1 mice showed hematopoietic recovery and exhibited at moderate lymphoid stimulation as well as perivascular and periductal lymphoid infiltrations in liver and salivary glands.[2] We termed this form of chronic GVHD secondary (2°) chronic GVHD to distinguish it from 1° chronic GVHD, which is not preceded by acute GVHD but directly results in a full-blown SLE-like disease (8).[2]

In the present investigation, we used the B10 ⟶ BDF_1 model to further analyze the transition from acute GVHD to 2° chronic GVHD. We found that most of the BDF_1 recipient mice that recovered from acute GVHD were repopulated by lympho-hematopoietic cells of donor (B10) origin. Such mice were termed GVH F_1 chimeras to distinguish them from irradiation chimeras. The takeover in GVH F_1 chimeras of the lympho-hematopoietic tissue by donor cells was found to be mediated by the Lyt-2^+-alloreactive donor Ts cells, which appear to be relatively short-lived. In contrast, donor Th cells alloactivated in the F_1 host were long-lived; they appeared to mediate the symptoms of 2° chronic GVHD.

Materials and Methods

Mice. C57BL/10ScSn (B10) (H-$2^{b/b}$, Ig-$1^{b/b}$), B10.D2n (H-$2^{d/d}$), BDF_1 (H-$2^{b/d}$, Ig-$1^{b/c}$), and B10 × B10.BR)F_1 (H-$2^{b/k}$) mice were purchased from Olac 1976 Ltd., Bicester, Oxon, England). BALB/c (H-$2^{d/d}$) and B10.AKM (H-$2^{m/m}$) mice were obtained from the Netherlands Cancer Institute, Amsterdam. Female mice were used throughout; they were 6–10 wk old unless mentioned otherwise.

Preparation of Donor Cells. Single-cell suspensions of donor SpC, lymph node cells (LNC), and bone marrow cells (BMC) were prepared as described (12).

Induction of the GVHR. Unless mentioned otherwise, the GVHR was induced by intravenous injection of 10^8 live B10 SpC into adult, nonirradiated BDF_1 (GVH F_1) mice.

Typing of Serum Ig-1 Allotypes. The procedure of preparing anti-Ig-1 allotype serum has been described elsewhere (13). Briefly, mice were immunized with pertussis vaccine to provide antipertussis IgG. At weekly intervals, B10 (Ig-$1^{b/b}$) pertussis/antipertussis agglutinates were injected subcutaneously into DBA/2 (Ig-$1^{c/c}$) mice, and vice versa. The serum Ig-1 allotypes of individual GVH F_1 mice were determined by immunoprecipitation in agar gel, by applying the anti-Ig-1^b and -Ig-1^c allotype sera at constant dilutions of 1:5 and 1:3, respectively. At these dilutions of the antiallotype sera, equal endpoint titers (1:10) of Ig-1^b and Ig-1^c antigen were found in the sera of normal BDF_1 (N F_1) mice. The anti-Ig-1^c serum could still detect the Ig-1^c antigen provided by one part of N F_1 serum admixed to nine parts of B10 serum. The sera obtained from GVH F_1 mice were assayed undiluted.

Histocompatibility Typing. An anti-H-2^b serum was prepared in B10.D2 mice, and an anti-H-2^d serum was prepared in B10 mice, by injecting lymphoid cells from B10 and B10.D2, respectively. Cells of GVH F_1 mice were typed by a complement (C)-dependent cytotoxicity assay, and the results were expressed as a cytotoxic index (12). Histocompatibility typing of erythrocytes was performed by an indirect Coombs' test by applying the two antisera mentioned above and a rabbit anti-mouse IgG serum (cat. code ØTPN; Behring Werke AG, Marburg/Lahn, Federal Republic of Germany), diluted 1:100.

Preparation of T Cell-depleted SpC (B cells). B cells were prepared by incubating SpC with monoclonal anti-Thy-1.2 (clone F7D5; Olac 1976 Ltd.) and C (12). The percentages of live T lymphocytes after treatmentwith anti-Thy-1.2 and C were determined by indirect immunofluorescence by using a rabbit anti-mouse brain serum in combination with fluorescein isothiocyanate-labeled anti-rabbit IgG (F2190; Dakopatts, Copenhagen, Denmark); the percentage of T cells spared by the anti-Thy-1.2 treatment did not exceed 2%.

Preparation of Nylon-Wool-purified T Cells. T cell-enriched populations (T cells) were prepared by passing SpC, or SpC plus LNC, through nylon wool (14).

Anti-Lyt Treatment. Cell suspensions depleted of Lyt subsets were prepared as described elsewhere (11). Briefly, SpC were treated twice with either a 1:1,000 diluted mouse monoclonal anti-Lyt-1.2 (IgG2b) (NEI-017; New England Nuclear, Boston, MA) and C or a 1:1,000 diluted mouse monoclonal anti-Lyt-2.2 (IgM) (NEI-006; New England Nuclear) and C. After the cells were treated with either normal mouse serum (NMS) plus C, anti-Lyt-2.2 plus C, or anti-Lyt-1.2 plus C, live T cells comprised 35, 20, and 4%, respectively, of the total number of live SpC. The ability and/or inability of the respective cell populations to induce allohelp and/or allosuppression in vitro and in vivo has been described elsewhere (11). The term Lyt-1^-2^+ cells is an operational one, as defined by treatment with anti-Lyt-1.2 plus C; it does not preclude the possible existence of a low concentration of Lyt-1.2 on the so-called Lyt-1^-2^+ cells.

Primary Anti-Sheep Erythrocyte (SE) Response In Vitro. Allohelper and allosuppressor activities of GVH F_1 SpC were assayed as described by Pickel and Hoffman (15), with some modifications.[2] Briefly, a constant number of 5×10^6 B cells or SpC obtained from N F_1 mice or control mice were co-cultured with increasing numbers of SpC or T cells obtained from GVH F_1 mice; 5×10^5 SE were added as the antigen. On day 4, the direct plaque-forming cell (PFC) responses of triplicate cultures were determined by Jerne plaque assay.

Mixed Lymphocyte Reactions (MLR). MLR were performed as described elsewhere (16). Briefly, MLR were set up in four replicate cultures with 5×10^5 responder and 7.5×10^5 1,500 rad-irradiated stimulator cells per well. The MLR were incubated for 4 d, and [^{3}H]thymidine was present during the last 4 h.

Cell-mediated Lymphocytotoxicity Assay (CML). The CML activity of SpC was determined as described (12). Briefly, responder cells (12×10^6) were incubated for 5 d together with 3,300 rad-irradiated stimulator cells (12×10^6) in 5 ml of culture medium. The CML activity of the cultures was measured in a 4-h chromium-release assay using concanavalin A blasts as target cells. Results are expressed as lytic units (LU) per culture (17), one LU being equal to the number of responder cells that gives 25% specific lysis of the target cell population.

Popliteal Lymph Node (PLN) Assay. The PLN assay was performed as described (18). On day 10 after the injection of donor SpC into one hind footpad of a nonirradiated recipient mouse, both PLN were removed and weighed. The strength of the reactive PLN enlargement was calculated as the PLN index, being the ratio of the lymph node weight from the injected side over that from the noninjected side. For the measurement of B cell stimulation in a PLN, cell suspensions were prepared from it and the total number of IgM- and IgG-producing cells per 10^5 PLN cells was determined by protein A PFC assay.

Protein A PFC Assay. This assay was performed as described elsewhere (19).

Assay for the Mortality Induced by Transferred GVH F_1 SpC. At the age of ~3 mo, the prospective recipient mice were given a lethal 950 rad whole-body irradiation, delivered by 662 kV gamma rays emitted from a ^{137}Cs source at a dose of 375 rad/min. Within 3 h after irradiation, the mice were injected intravenously with the donor cells indicated. On days 1–7 after irradiation, the mice received drinking water that contained polymyxin B (12,500 U/liter) and oxytetracycline (100 mg/liter). Mice were inspected twice a week for symptoms of acute GVHD or death.

Irradiation Chimeras. Untreated BDF_1 mice, ~3 mo old, were submitted to 950 rad whole-body irradiation and given antibiotics as described above. The mice were then injected with 10^7 live B10 BMC that had been treated with monoclonal anti-Thy-1.2 and C. No deaths due to GVHD were observed in the recipients. About 3 mo after the repopulation, the F_1 recipients were tested by histocompatibility typing. More than 95% of their SpC were found to be of B10 origin; these irradiation chimeras were used for further studies.

Results

Complete Lympho-hematopoietic Repopulation of Nonirradiated BDF_1 Recipients by B10 SpC. A systemic GVHR was induced in groups of BDF_1 recipient mice by injecting 10^8 B10 SpC. As reported earlier (2, 12),[2] at 2–6 wk after the induction of GVHR, the GVH F_1 mice invariably showed symptoms of acute GVHD, including weight loss, cellular depletion of the lympho-hematopoietic tissue, and anemia. The percentage of GVH F_1 that succumbed to acute GVHD, however, varied from experiment to experiment, the mortality ranging from 20 to 80%. The survivors of acute GVHD recovered and no longer showed clinical symptoms of GVHD.

In three groups of GVH F_1 mice that had survived for >100 d, the presence of donor cell chimerism was assayed. This was done by determining the Ig allotype(s) present in the sera of the GVH F_1 mice and typing their erythrocytes for the presence of $H\text{-}2^b$ and $H\text{-}2^d$ antigens. In 70–80% of the GVH F_1 mice tested (Table I), neither the host-derived $Ig\text{-}1^c$ allotypic markers in the serum nor the $H\text{-}2^d$ alloantigens on the erythrocytes were detectable. In contrast, the $Ig\text{-}1^b$ allotype and $H\text{-}2^b$ antigens, which are common to both host and donor, were readily detectable in all these GVH F_1 mice (Table I). These findings indicate that most of the GVH F_1 mice that had recovered from acute GVHD were repopulated by lympho-hematopoietic cells of donor origin. In a group of

TABLE I

Demonstration of Lympho-hematopoietic Takeover by B10 Donor Cells ($Ig\text{-}1^{b/b}$, $H\text{-}2^{b/b}$) in the Majority of GVH F_1 Mice Surviving Acute GVHD

Experiment*	Number of mice with indicated Ig-1 allotype in the serum‡/number tested				Number of mice with indicated H-2 antigens on their erythrocytes§/number tested			
	Day 100		Day 200		Day 100		Day 200	
	$Ig\text{-}1^b$	$Ig\text{-}1^c$	$Ig\text{-}1^b$	$Ig\text{-}1^c$	$H\text{-}2^b$	$H\text{-}2^d$	$H\text{-}2^b$	$H\text{-}2^d$
1	32/32‖	8/32	31/31	7/31	32/32	8/32	31/31	7/31
2	29/29‖	9/29	—¶	—	29/29	8/29	—	—
3	38/38‖	11/38	—	—	38/38	11/38	—	—

* Each group of BDF_1 recipients consisted initially of 40 mice.

‡ An Ig-1 allotype was considered to be lacking when it was not detectable in the undiluted serum by the Ouchterlony test.

§ The sensitivity of the applied indirect Coombs' test was such that the anti-$H\text{-}2^d$ serum still detected one part of BDF_1 erythrocytes mixed to four parts of B10 erythrocytes.

‖ The $Ig\text{-}1^b$ allotype in these mice was still detectable at a serum dilution of 1:10.

¶ Not tested.

GVH F_1 mice that were autopsied at day 100 after the induction of GVHR, repopulation was confirmed by histocompatibility typing of their SpC (Table II). From the same GVH F_1 mice, we also typed cell suspensions consisting of either BMC, thymus cells, or LNC: whereas the cytotoxic indices obtained after treatment with the host-specific anti-H-2^d serum and C were 3, 1, and 2, respectively, those obtained with anti-H-2^b serum and C were 98, 94, and 97.

H-2 typing of the spleens of GVH F_1 mice killed at regular intervals showed that host cells had almost completely disappeared from their spleens as early as day 14 after induction of GVHR (Table II). The rapid disappearance of the host-derived Ig-1^c allotype from the sera of another group of GVH F_1 mice (Table II) agrees with this observation. Once established, the repopulation of GVH F_1 mice was a stable condition in that it lasted for at least 200 d (Table I).

Lympho-hematopoietic Repopulation of Nonirradiated BDF$_1$ Recipients Is Mediated by Non–Lyt-separated Alloreactive T Cells. Next, we analyzed the cellular requirements of lympho-hematopoietic takeover. As expected, we found that the injection of 5 × 10^7 B10 LNC, a population deficient in hematopoietic stem cells, failed to repopulate, but caused lethal GVHD (LGVHD) in 70% of the BDF_1 recipients. Whereas GVH mortality in the F_1 recipients was significantly reduced by the addition of B10 BMC as a source of stem cells to the inoculum of B10 LNC, this was not the case when BMC from BDF_1 mice were added to B10 LNC (data not shown). In the next experiment, we demonstrated that lympho-hematopoietic repopulation was mediated by unseparated alloreactive B10 T cells (Table III). First, three groups of BDF_1 mice were injected with 10^8 live cells of one of the following sources: (*a*) B10 SpC, pretreated with NMS and C, (*b*) B10 SpC, pretreated with anti-Thy-1.2 and C, or (*c*) SpC obtained from B10 → BDF_1 irradiation chimeras. The SpC obtained from irradiation chimeras were shown to be specifically tolerant of BDF_1 antigens in both MLR (Table IV) and allogeneic T-B cell cooperation (Fig. 1). At about day 100 after the induction of GVHR, we determined whether the host-specific Ig-1^c allotype was still present in sera from the recipients. In contrast to B10 SpC pretreated with NMS, neither anti-Thy-1.2–pretreated B10 SpC nor B10 SpC that were tolerant of BDF_1 recipients were able to cause the lympho-hematopoietic repopulation (Table III).

TABLE II

Kinetics of Lympho-hematopoietic Takeover by B10 Donor Cells (Ig-$1^{b/b}$, H-$2^{b/b}$) in GVH F_1 Mice

Method	Antiserum used	Time after the induction of the GVHR (days)						
		0	7	14	28	60	100	150
		Number of positive animals/number tested						
Typing of Ig-1 allotypes in serum	Anti-Ig-1^c	10/10	—*	10/10	2/10	2/10	2/10	2/10
	Anti-Ig-1^b	10/10	—	10/10	10/10	10/10	10/10	10/10
		Mean cytotoxic index‡						
C-dependent cytotoxic test	Anti-H-2^d	95	56	6	2	−2	2	1
	Anti-H-2^b	98	91	99	97	97	92	94

* Not tested.

‡ Each number represents the mean cytotoxic index of 6 to 12 individually tested GVH F_1 spleens.

TABLE III

Lympho-hematopoietic Repopulation Requires Unseparated Alloreactive Donor T Cells

Donor SpC*		Number of BDF_1 recipients[‡] with indicated Ig-1 allotype in the serum/number tested	
Mice	Pretreatment	$Ig\text{-}1^b$	$Ig\text{-}1^c$
B10	NMS + C	15/15	2/15
B10	Anti-Thy-1.2 + C	15/15	15/15
Irradiation chimeras	None	15/15	15/15
B10	Anti-Lyt-1.2 + C	14/14	14/14
B10	Anti-Lyt-2.2 + C	15/15	15/15

* 10^8 live SpC were injected.

‡ Each group initially consisted of 15 mice; they were tested at about day 100 after the injection of donor cells.

TABLE IV

MLR Responses of GVH F_1 Chimera Cells Exposed to Various Stimulator Cells

Responder cells		Mean cpm obtained with stimulator cells indicated*					
Mice	Cell type[‡]	B10	GVH F_1 chimera	BDF_1	(B10 × B10.BR)	B10.D2	B10.AKM
		Day 100 after induction of GVHR					
B10	SpC	370	787	13,730	8,966	14,416	17,303
B10	T cells	214	546	114,660	64,375	62,711	91,834
GVH F_1 chimera[§]	SpC	678	987	29,141	12,918	22,417	22,336
GVH F_1 chimera	T cells	458	739	104,865	32,285	57,202	76,052
Irradiation chimera	SpC	513	—[‖]	563	5,640	—	6,329
Irradiation chimera	T cells	1,114	—	1,135	55,342	—	93,400
		Day 210 after induction of GVHR					
B10	T cells	438	399	137,467	45,771	64,555	121,380
GVH F_1 chimera	T cells	744	671	5,433	28,173	5,588	51,538

* For the sake of readability, standard deviations are not shown; they never exceeded 10% of the mean values.

‡ In each group, T cells were prepared by pooling SpC and LNC obtained from four mice and passing them over nylon wool.

§ GVH F_1 SpC were pooled from four mice.

‖ Not tested.

To determine which T cell subpopulations were involved, we then injected groups of BDF_1 recipients with cells from B10 SpC that had been pretreated with either anti-Lyt-1.2 and C or anti-Lyt-2.2 and C. Neither anti-Lyt-1.2– nor anti-Lyt-2.2–pretreated B10 SpC were able to repopulate the F_1 recipients (Table III). Thus, lympho-hematopoietic repopulation of nonirradiated BDF_1 mice required inocula containing alloreactive B10 T cells that had not been separated into Lyt subsets and, in addition, included syngeneic (B10) stem cells.

Persistence in Long-term GVH F_1 Chimeras of F_1-specific Donor Th Cells. Next, we analyzed the functional properties of cells derived from the long-term GVH F_1 chimeras. Unless indicated otherwise, the experiments described in this section and all subsequent sections were performed with SpC that were obtained from GVH F_1 chimeras killed at day 100–150 after the induction of GVHR. These SpC consisted of >95% B10 donor cells. First, we studied whether B10 Th cells, obtained from GVH F_1 chimeras, could still react against host-type alloantigens.

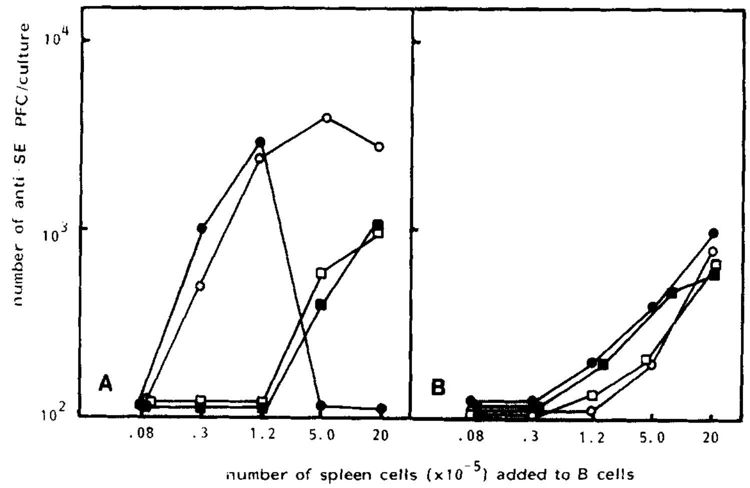

FIGURE 1. Helper activity in the spleens of GVH F_1 chimeras killed ~100 d after the induction of GVHR. SpC obtained from a GVH F_1 chimera provide allohelp to BDF_1 B cells (*A*), but only syngeneic help to B10 B cells (*B*). The SpC titrated to the SE-stimulated B cell cultures were obtained from (●) a B10 mouse, (■) an irradiation chimera, (○) a GVH F_1 chimera, (□) an N F_1 mouse.

This was done by measuring the effects of SpC from GVH F_1 chimeras on the primary anti-SE response, in vitro, of B cells syngeneic either to the host (BDF_1) or to the GVH donor (B10). Graded numbers of SpC obtained from GVH F_1 chimeras, normal B10 or BDF_1 mice, or B10 → F_1 irradiation chimeras were added to SE-stimulated B cell cultures. The results of such an experiment are shown in Fig. 1; they are representative of six identical experiments, each of which was done using SpC from an individual GVH F_1 chimera. Essentially similar results were obtained when nylon-wool-purified T cells instead of SpC were used (data not shown). The addition of low numbers of normal B10 SpC to semiallogeneic BDF_1 B cells led to a positive allogeneic effect (Fig. 1 *A*), which is known to be caused by Lyt-1^+2^--alloreactive Th cells (20, 21). When the number of normal B10 SpC was further increased, a negative allogeneic effect occurred, caused by Lyt-2^+-alloreactive Ts cells (20, 21). The addition of low numbers of SpC obtained from GVH F_1 chimeras to the BDF_1 B cells led, as did the addition of normal B10 SpC, to a positive allogeneic effect. However, no negative allogeneic effect ensued after the addition of high numbers of SpC from the GVH F_1 chimeras. These findings indicate that host-specific donor Th cells were still present in the spleens of long-term GVH F_1 chimeras, whereas alloreactive Ts cells were no longer detectable. The loss of alloreactive Ts cell activity from the spleens of GVH F_1 chimeras (day 100) was also apparent in the observation that they failed to suppress, but instead slightly stimulated, the anti-SE PFC response of N F_1 SpC (Fig. 2). For comparison, the suppressive effects resulting from co-culture of N F_1 SpC and GVH F_1 SpC obtained at days 10 and 38 after the induction of GVHR are shown (Fig. 2).

T Cells of Long-term GVH F_1 Chimeras Show Split Tolerance Towards Host Alloantigens. Next, we examined whether the SpC of GVH F_1 chimeras were able to

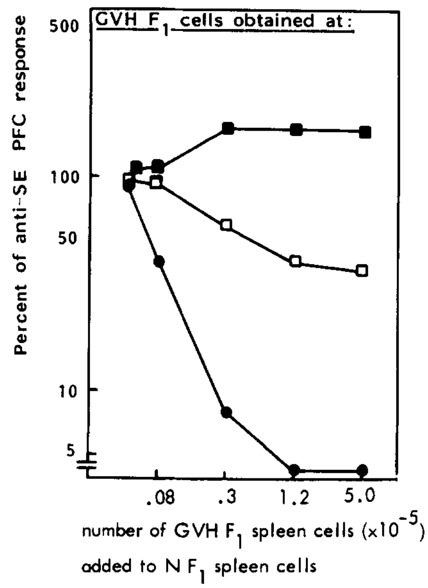

FIGURE 2. Suppressor activity in the spleens of BDF_1 recipients at various times after the induction of GVHR. (●) 10 d, (□) 38 d, or (■) 100 d (GVH F_1 chimera) after the induction of GVHR with B10 SpC. Results are expressed are the percentage of PFC in control cultures (1,860 PFC/culture) to which no GVH F_1 SpC were added. Results representative of five identical experiments are shown.

respond in MLR and CML to host or third-party alloantigens. GVH F_1 chimera cells, obtained at day 100 after the induction of GVHR, responded to host-type and third-party alloantigens with about equal strength (Table IV). At 210 d after the induction of GVHR, GVH F_1 chimera cells still showed a significant, albeit reduced, response against host-type alloantigens (Table IV). Thus, donor T cells, able to proliferate in response to host-type alloantigens, did persist in GVH F_1 chimeras for at least 6 mo. In marked contrast, the T cells obtained from these long-term GVH F_1 chimeras showed hardly any CML activity after restimulation with irradiated host-type (BDF_1) cells (Table V). Out of the total of seven individually tested GVH F_1 chimeras, five showed no detectable CML activity at all, and two others showed only a very weak CML activity. As is evident from Table V, the lack of CML activity against host-type alloantigens was specific. SpC obtained from GVH F_1 chimeras did not suppress the anti-BDF_1 CML response of N B10 SpC (Table V). This indicates that a lack of cytotoxic T cell precursors rather than the presence of suppressor cells was responsible for the specific inability of GVH F_1 chimeras to generate T killer (Tk) cells against host-type alloantigens.

T Cells of B10 → F_1 Irradiation Chimeras Are Completely Tolerant of the Host. We then asked whether the type of split tolerance found in long-term GVH F_1 chimeras also existed in irradiation chimeras, as stated by Sprent et al. (22). Evidently, it did not, however, because SpC obtained from irradiation chimeras showed neither allohelper (Fig. 1) nor proliferative activity (Table IV) in response to host-type antigens.

TABLE V
CTL Responsiveness of SpC Obtained from GVH F_1 Chimeras

Responder SpC	Target cells	Mean number of LU/culture ± SEM
B10*	BDF_1	688 ± 110
B10*	$(B10 \times B10.BR)F_1$	483 ± 72
GVH F_1 chimera‡	BDF_1	8 ± 5
GVH F_1 chimera‡	$(B10 \times B10.BR)F_1$	503 ± 114
B10 + GVH F_1 chimera§	BDF_1	555ǁ
		555
B10 + GVH F_1 chimera§	$(B10 \times B10.BR)F_1$	555ǁ
		1,100

* Cells obtained from seven normal B10 mice that were tested individually.
‡ Cells obtained from seven GVH F_1 chimeras that were tested individually; these mice were taken at days 100–120 after the induction of the GVHR.
§ Mixtures containing 8×10^6 normal B10 SpC and 4×10^6/SpC obtained from a GVH F_1 chimera were co-cultured for 5 d in MLR. Two GVH F_1 chimeras were studied; they had been taken from the group of seven chimeras described in footnote ‡.
ǁ Results of two individual experiments.

GVH Reactiviy of Adoptively Transferred SpC Obtained from Long-term GVH F_1 Chimeras. Having demonstrated the split tolerance of GVH chimera cells in vitro, we then examined their ability to react against host alloantigens in two GVH assays in vivo, the PLN assay and the GVH mortality assay. First, we asked whether the B10 T cells present in GVH chimeras were still able to cause PLN enlargement as well as B cell activation when confronted with BDF_1 alloantigens. Both types of reactivity were indeed found. Moreover, both the PLN enlargement and the B cell activation were caused by cells reacting specifically against the DBA/2 part of the BDF_1 recipients (Table VI). More than 95% of the total Ig secretion induced in the PLN was of the IgG isotype.

Then we tested the ability of GVH F_1 chimera SpC to induce mortality in lethally irradiated BDF_1 recipients. To avoid mortality due to a hypothetical lack of hematopoietic stem cells in the donor cell inoculum, the SpC obtained from the GVH F_1 chimeras were mixed with BMC from the same mice. The cell suspensions thus obtained did not contain detectable numbers of BDF_1 cells, whereas the percentage of T cells in the cell mixture was similar to that in a mixture prepared from normal B10 Spc plus BMC. The injection of 30×10^6 GVH F_1 chimera cells into 950 rad-irradiated BDF_1 recipients did not lead to a significant incidence of LGVHD; 9 out of 10 recipients survived for >80 d (Table VII, group 4) without showing symptoms of secondary disease. At day 80 after the cell transfer, the sera of these secondary BDF_1 recipients contained only the Ig-1^b allotype, indicating that they were repopulated by donor (B10) cells. As expected, all BDF_1 recipients of normal B10 SpC died within 40 d after the induction of GVHD (Table VII, group 1). The inability of GVH F_1 chimera cells to induce GVH mortality in irradiated BDF_1 recipients was specific, since $(B10 \times B10.BR)F_1$ recipients all succumbed within 30 d after the injection of GVH F_1 chimera cells (Table VII, group 5). The mixture of GVH F_1 SpC with B10 SpC did not affect the ability of the latter cells to induce LGVHD in BDF_1

TABLE VI

Specific PLN Enlargement and Allohelp Caused by SpC from GVH F_1 Chimeras that Were Injected into the Footpads of Secondary BDF_1 Mice

Cells transferred		Nonirradiated recipient mice*			
				Number of IgG protein A PFC/10^5 PLN cells	
Donor	Number ($\times 10^6$)	Strain	Mean PLN index ± SEM.	Test-side PLN	Contra-lateral PLN
B10	5	BDF_1	2.3 ± 0.4‡	39 ± 15	<5
	10		5.2 ± 0.8§	76 ± 10	<5
	15		5.0 ± 0.8§	158 ± 50	7 ± 3
B10	15	B10	1.4 ± 0.2	<5	<5
GVH F_1 ‖ chimera	5	BDF_1	3.5 ± 0.8§	142 ± 58	<5
	10		3.4 ± 0.7§	161 ± 86	5 ± 2
	15		5.8 ± 0.3§	331 ± 97	18 ± 6
GVH F_1 ‖	15	B10	1.4 ± 0.1	<5	<5
BDF_1	15	BDF_1	1.7 ± 0.1	<5	<5

* Four mice per group.

‡ $P < 0.05$ as compared with the syngeneic control.

§ $P < 0.005$ as compared with the syngeneic control.

‖ Pooled SpC from 20 GVH F_1 chimeras were used; these mice were taken at about day 100 after the induction of the GVHR. Cells from this same pool were used in the mortality assay (Table VII).

recipients (Table VII, group 7). Thus, a lack of relevant alloreactive T cells, rather than the presence of suppressor cells, was responsible for the specific inability of the SpC obtained from GVH F_1 chimeras to induce GVH mortality in lethally irradiated BDF_1 recipients.

Discussion

Recent papers from this laboratory have reported that SLE-like GVHD in nonirradiated F_1 mice is caused by Lyt-1^+-2^- donor Th cells that react against class II (I-A/I-E) alloantigens of the host (3, 11, 23). Induction of acute GVHD, by contrast, required unseparated donor T cells (11). In acute GVHD, apparently there is a sequential alloactivation consisting of first (week 1) class II-reactive Lyt-1^+2^- donor cells that subsequently (weeks 2 to 6) induce class I (K/D)-reactive Lyt-1^+2^+ and Lyt-1^-2^+ allosuppressor effector cells (3, 11, 24, 25).[2] When unseparated SpC of the donor B10 were injected into fully H-2-different BDF_1 mice, the following three stages of GVHR were distinguished: (*a*) In week 1, alloactivated donor Th cells predominated; (*b*) from weeks 2 to 6, donor Ts cells prevailed and caused acute GVHD; and (*c*) in those GVH F_1 mice that survived that stage of acute GVHD, alloreactive donor Ts cells were no longer detectable (Fig. 2) and the stimulatory pathological symptoms of 2° chronic GVHD appeared (8).[2] In the present investigation, we extended the functional analysis of B10 donor T cells obtained from F_1 recipients with 2° chronic GVHD.

TABLE VII
Mortality in Lethally Irradiated Recipients Injected Intravenously with SpC Plus BMC Obtained from Either Normal Donors or GVH F_1 Chimeras

Cells transferred*				950 rad-irradiated recipients			
Group	Donors	Source	Number (× 10^6)	Strain	Cumulative mortality at day: 20	40	80
1	B10	SpC + BMC	20 10	BDF_1	6/10	10/10	10/10
2	B10	SpC + BMC	20 10	$(B10 \times B10.BR)F_1$	7/10	10/10	10/10
3	B10	SpC + BMC	20 10	B10	2/10	2/10	2/10
4	GVH F_1* chimera	SpC + BMC	20 10	BDF_1	1/10	1/10	1/10
5	GVH F_1* chimera	SpC + BMC	20 10	$(B10 \times B10.BR)F_1$	4/10	10/10	10/10
6	GVH F_1* chimera	SpC + BMC	20 10	B10	1/10	1/10	1/10
7	B10 GVH F_1* chimera	SpC + BMC + SplC	20 10 10	BDF_1	5/5	5/5	5/5
8	None	—	—	BDF_1	10/10	10/10	10/10

* Pooled SpC from 20 GVH F_1 chimeras were used; these mice were taken at about day 100 after the induction of GVHR. BMC were pooled from the same mice.

Repopulation of F_1 Recipients by Lympho-hematopoietic Donor Cells. Several groups of investigators have observed that nonirradiated H-2-different F_1 mice undergoing GVHR may be repopulated by parental SpC (26–28). The recent observation (2) that such a repopulation took place in parent → F_1 combinations undergoing acute GVHD, but not as readily in GVH F_1 mice developing a chronic lupus-like GVHD, prompted us to further analyze the relationship between acute GVHD and lympho-hematopoietic repopulation. A likely possibility was that the Lyt-1^+2^+ and Lyt-1^-2^+ F_1-specific donor Ts cells (24, 25, 29),[2] whose presence in GVH F_1 mice shortly preceded and coincided with the pancytopenia of acute GVHD,[2] did indeed mediate the depletion of lympho-hematopoietic host cells. Alternatively, the appearance of the donor Ts cells might be an epiphenomenon secondary to the disappearance of lympho-hematopoietic host cells, a situation analogous to the secondary appearance of Ig allotype-specific Ts cells observed after the preceding deprivation of that allotype (30). Our results favor the former possibility. Repopulation was only achieved by the injection into BDF_1 recipients of unseparated B10 SpC, but not of either Lyt-1^+2^- or Lyt-1^-2^+ B10 SpC alone. Moreover, the unseparated B10 T cells required for repopulation had to be able to react towards the host (Table III). Thus, the complete lympho-hematopoietic repopulation described in the present paper appears to be mediated by the same alloactivated donor Ts cells that cause acute GVHD and the pancytopenia that accompany it (2, 3, 7, 11).[2]

Split Tolerance Towards Host Alloantigens in Survivors of Acute GVHD. Up till now, only limited studies on the anti-F_1 reactivity of donor T cells present in nonirradiated GVH F_1 chimeras have been performed (27, 28). We found that F_1-specific donor Ts cells (Fig. 1) and Tk cells (Table V) were not at all, or were hardly any longer detectable in the spleens of long-term GVH F_1 chimeras. In marked contrast, F_1-specific donor Th cells (Fig. 1), as well as MLR-reactive donor T cells (Table IV), were readily recovered from the GVH F_1 chimeras until at least 5 mo after the induction of GVHR. This split tolerance of the B10 T cells recovered from long-term GVH chimeras was also demonstrable in vivo: whereas the B10 T cells obtained from GVH chimeras had specifically lost their capacity to induce LGVHD in secondary BDF_1 recipients (Table VII), they were still able to induce F_1-specific PLN enlargement and B cell activation (Table VI). These findings conform to other observations showing that PLN enlargement (S. T. Pals, unpublished observation) and B cell activation in vivo (3, 11, 23) can be initiated by alloreactive Th cells alone. In contrast, acute GVHD requires a sequential activation of alloreactive Th and Ts cells (3, 11).[2] An interpretation in immunogenetic terms of the observed split tolerance strongly suggests that alloreactivities, such as CML (31–33), allosuppression (3, 20, 21), and acute GVHD (3), in which class I (K/D) antigens provide the main targets, were seriously impaired in the donor T cells that were recovered from long-term GVH F_1 chimeras. By contrast, alloreactivities, such as MLR (32–34), allohelp (3, 20, 21, 23), and B cell activation (3), that are mainly directed against class II alloantigens, remained unimpaired over several months. According to this interpretation, the stimulatory phenomena observed in 2° chronic GVHD (2)[2] were caused by the long-lived donor Th cells that must have reacted against a minor population of persisting F_1 cells carrying class II alloantigens. Another, not mutually exclusive, explanation for the stimulatory pathological phenomena in 2° chronic GVHD might be that late-acting donor Th cells reacted to class I alloantigens, or yet other antigens, that they recognized in association with syngeneic (donor) class II structures (35).

The same kind of split tolerance that we observed here in long-term GVH F_1 chimeras was reported by Sprent et al. (22) in lethally irradiated F_1 mice that had been reconstituted with parental BMC pretreated with anti-Thy-1.2 and C. In contrast, we found no evidence for the presence of anti-host–reactive T cells in such irradiation chimeras (Fig. 1, Table IV). Consistent with our observation, complete tolerance of donor T cells towards the host has also been described in fully allogeneic irradiation chimeras (36). The discrepancy between our findings and those of Krown et al. (36) on the one hand, and those of Sprent et al. (22) on the other, might lie in a failure of the latter investigators to remove all the mature T cells from the donor BMC by the conventional anti-Thy 1.2 they used. Our findings and those of Krown et al. (36) both fit the concept that radioresistant host cells determine the self-specificity of immature donor T cells differentiating in an allogeneic host (37, 38). Correspondingly, our findings indicate that the allohelper T cells recovered from the long-term GVH F_1 chimeras were derived from the mature donor T cells injected for the induction of GVHR.

To explain the development of split tolerance of donor T cells in GVH chimeras, at least three possibilities had to be considered: (*a*) There might have been an active suppression of the anti-F_1–reactive Ts/Tk clones. Although it has been observed that T cells obtained from radiation chimeras can specifically suppress the anti-host reactivity of donor T cells (39, 40), there was no evidence for such a mechanism in our GVH F_1 chimeras (Table V and VII), (*b*) the sequential alloactivation in acute GVHD first of donor Th and then of Ts cells follows the rules of the T cell feedback circuit (41). Conceivably, the activation of donor Ts cells might have been quenched because of a reduction of T helper/inducer activity which, in turn, resulted from a numerical decrease in GVH F_1 chimeras of stimulatory (class II) alloantigens. However, when the B10 cells that were recovered from long-term GVH F_1 chimeras were reexposed to BDF_1 stimulator cells, either in vitro or in vivo, no reactivation of Ts/Tk cells ensued, although vigorous allohelper and MLR responses were obtained. (*c*) The early loss of Ts/Tk cell activity may be due to a rather short lifespan of these cells. This possibility is supported by other observations (42, 43; E. Kölsch, personal communication) that the time periods during which activated Ts cells remained detectable were relatively short, whereas the longevity of activated Th cells is well established (44).

Whatever the mechanism of the observed split tolerance may be, our findings clearly indicate that anti-host–reactive donor Th cells can persist in the host for at least 6 mo. These findings challenge the role of donor T cells in chronic GVHD as proposed by Elkins (45). He argues that the role of donor T cells in chronic GVHD might be merely to trigger, during the stage of acute GVHD, a number of pathogenic effects, such as immunosuppression, infection, and activation of latent viruses. These secondary GVH effects would then, independently of donor T cells, cause the symptoms of chornic GVHD. Although we are aware of these GVH effects, our findings suggest that long-lived alloreactive donor Th cells can directly cause and maintain most of the stimulatory pathological symptoms of 2° chronic GVHD. In this context, it is noteworthy that cells obtained from human patients with chronic GVHD, but not those without GVHD, responded to HLA-identical, cryopreserved host cells in an MLR (39, 46).

Summary

We studied the alloreactive properties of donor T cells obtained from F_1 mice that had recovered from the allosuppression of acute graft-vs.-host disease (GVHD) and showed mild symptoms of chronic GVHD, i.e., so-called secondary chronic GVHD. To this end, we used (B10 × DBA/2)F_1 mice that had been injected with 10^8 B10 spleen cells 100–150 d previously. Such GVH F_1 mice were repopulated by lympho-hematopoietic cells of donor (B10) origin, which exhibited split tolerance towards the host: Whereas F_1-specific donor T helper (Th) cells as well as T cells proliferating in the mixed lymphocyte reaction were readily demonstrable, F_1-specific T suppressor (Ts) and T killer (Tk) cells were not, or were hardly, detectable; responses against third-party alloantigens were normal. Upon adoptive transfer to nonirradiated secondary recipients, the B10

cells obtained from the repopulated GVH F_1 mice induced F_1-specific enlargement of the draining popliteal lymph node and enhancement of the IgG formation therein. B10 cells of the same kind were unable, however, to induce lethal GVHD upon transfer to 950 rad-irradiated secondary (B10 × DBA/2)F_1 recipients. We conclude that alloactivated donor Ts/Tk cells disappear from the host at a relatively early stage of GVHD, i.e., at the end of acute GVHD, presumably because they are short-lived. By contrast, the longevity of alloactivated donor Th cells causes the symptoms of secondary chronic GVHD.

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