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),\(^3\) 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.
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)F1 (BDF1) recipients of 10⁸ 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 F₁-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 BDF₁ 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 F₁ 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 → BDF₁ model to further analyze the transition from acute GVHD to 2° chronic GVHD. We found that most of the BDF₁ recipient mice that recovered from acute GVHD were repopulated by lympho-hematopoietic cells of donor (B10) origin. Such mice were termed GVH F₁ chimeras to distinguish them from irradiation chimeras. The takeover in GVH F₁ 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 F₁ host were long-lived; they appeared to mediate the symptoms of 2° chronic GVHD.

Materials and Methods

Mice. C57BL/10ScSn (B10) (H-2b/b, Ig-1b/b), B10.D2n (H-2d/d), BDF₁ (H-2b/d, Ig-1b/c), and B₁₀ × B₁₀.BR)F₁ (H-2b/k) mice were purchased from Olac 1976 Ltd., Bicester, Oxon, England. BALB/c (H-2a) and B10.AKM (H-2m/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 10⁸ live B10 SpC into adult, nonirradiated BDF₁ (GVH F₁) mice.

Typing of Serum Ig-1 Allotypes. The procedure of preparing anti-Ig-1 allotope serum has been described elsewhere (13). Briefly, mice were immunized with pertussis vaccine to provide antipertussis IgG. At weekly intervals, B10 (Ig-1b/b) pertussis/antipertussis agglutinates were injected subcutaneously into DBA/2 (Ig-1c/c) mice, and vice versa. The serum Ig-1 allotopes of individual GVH F₁ mice were determined by immunoprecipitation in agar gel, by applying the anti-Ig-1b and -Ig-1c allotope 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-1b and Ig-1c antigen were found in the sera of normal BDF₁ (Ν F₁) mice. The anti-Ig-1b serum could still detect the Ig-1c antigen provided by one part of Ν F₁ serum admixed to nine parts of B10 serum. The sera obtained from GVH F₁ mice were assayed undiluted.

Histocompatibility Typing. An anti-H-2b serum was prepared in B10.D2 mice, and an
anti-H-2d serum was prepared in B10 mice, by injecting lymphoid cells from B10 and B10.D2, respectively. Cells of GVH F1 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 treatment with 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 F1 SpC were assayed as described by Pickel and Hoffman (15), with some modifications. 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.

**Mixed Lymphocyte Reactions (MLR).** MLR were performed as described elsewhere (16). Briefly, MLR were set up in four replicate cultures with 5 × 10⁵ responder and 7.5 × 10⁵ 1,500 rad-irradiated stimulator cells per well. The MLR were incubated for 4 d, and [³H]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 × 10⁶) were incubated for 5 d together with 3,300 rad-irradiated stimulator cells (12 × 10⁶) 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 10⁵ PLN cells was determined by protein A PFC assay.

**Protein A PFC Assay.** This assay was performed as described elsewhere (19).
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 BDF1 mice, ~3 mo old, were submitted to 950 rad whole-body irradiation and given antibiotics as described above. The mice were then injected with 107 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 F1 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 BDF1 Recipients by B10 SpC. A systemic GVHR was induced in groups of BDF1 recipient mice by injecting 108 B10 SpC. As reported earlier (2, 12), at 2–6 wk after the induction of GVHR, the GVH F1 mice invariably showed symptoms of acute GVHD, including weight loss, cellular depletion of the lympho-hematopoietic tissue, and anemia. The percentage of GVH F1 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 F1 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 F1 mice and typing their erythrocytes for the presence of H-2b and H-2d antigens. In 70–80% of the GVH F1 mice tested (Table I), neither the host-derived Ig-1c allotypic markers in the serum nor the H-2d alloantigens on the erythrocytes were detectable. In contrast, the Ig-1b allotype and H-2b antigens, which are common to both host and donor, were readily detectable in all these GVH F1 mice (Table I). These findings indicate that most of the GVH F1 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-1b, H-2b) in the Majority of GVH F1 Mice Surviving Acute GVHD**

| Experiment* | Number of mice with indicated Ig-1 allotype in the serum*| Day 100 | Day 200 | Number of mice with indicated H-2 antigens on their erythrocytes**| Day 100 | Day 200 |
|-------------|----------------------------------------------------------|---------|---------|------------------------------------------------------------------|---------|---------|
|             | Ig-1b | Ig-1c | Ig-1b | Ig-1c | H-2b | H-2d | H-2b | H-2d |
| 1           | 32/32 |     | 31/31 | 7/31  | 32/32 | 8/32 | 31/31 | 7/31 |
| 2           | 29/29 |     |       |       | 29/29 | 8/29 |       |     |
| 3           | 38/38 |     |       |       | 38/38 | 11/38|       |     |

* Each group of BDF1 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-2d serum still detected one part of BDF1 erythrocytes mixed to four parts of B10 erythrocytes.

* The Ig-1b allotype in these mice was still detectable at a serum dilution of 1:10.

* Not tested.
GVH F₁ 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 F₁ 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-2d serum and C were 3, 1, and 2, respectively, those obtained with anti-H-2b serum and C were 98, 94, and 97.

H-2 typing of the spleens of GVH F₁ 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-1c allotype from the sera of another group of GVH F₁ mice (Table II) agrees with this observation. Once established, the repopulation of GVH F₁ mice was a stable condition in that it lasted for at least 200 d (Table I).

**Table II**

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

* Not tested.

* Each number represents the mean cytotoxic index of 6 to 12 individually tested GVH F₁ spleens.
Lympho-hematopoietic Repopulation Requires Unseparated Alloreactive Donor T Cells

| Donor SpC* | Pretreatment | Number of BDF1 recipients† with indicated Ig-1 allotype in the serum/number tested |
|------------|--------------|----------------------------------------------------------------------------------|
|            |              | Ig-1† Ig-1‡                                                                         |
| B10        | NMS + C      | 15/15 2/15                                                                         |
| B10        | Anti-Thy-1.2 + C | 15/15 15/15                                                                          |
| Irradiation chimeras | None | 14/14 14/14                                                                         |
| B10        | Anti-Lyt-1.2 + C | 15/15 15/15                                                                          |
| B10        | Anti-Lyt-2.2 + C | 15/15 15/15                                                                          |

* 10⁸ live SpC were injected.  
† Each group initially consisted of 15 mice; they were tested at about day 100 after the injection of donor cells.

Persistence in Long-term GVH F₁ Chimeras of F₁-specific Donor Th Cells. Next, we analyzed the functional properties of cells derived from the long-term GVH F₁ chimeras. Unless indicated otherwise, the experiments described in this section and all subsequent sections were performed with SpC that were obtained from GVH F₁ 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 F₁ chimeras, could still react against host-type alloantigens.
This was done by measuring the effects of SpC from GVH F₁ chimeras on the primary anti-SE response, in vitro, of B cells syngeneic either to the host (BDF₁) or to the GVH donor (B10). Graded numbers of SpC obtained from GVH F₁ chimeras, normal B10 or BDF₁ mice, or B10 → F₁ 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 F₁ 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 BDF₁ 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 F₁ chimeras to the BDF₁ 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 F₁ chimeras. These findings indicate that host-specific donor Th cells were still present in the spleens of long-term GVH F₁ chimeras, whereas alloreactive Ts cells were no longer detectable. The loss of alloreactive Ts cell activity from the spleens of GVH F₁ 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 F₁ SpC (Fig. 2). For comparison, the suppressive effects resulting from co-culture of N F₁ SpC and GVH F₁ SpC obtained at days 10 and 38 after the induction of GVHR are shown (Fig. 2).

T Cells of Long-term GVH F₁ Chimeras Show Split Tolerance Towards Host Alloantigens. Next, we examined whether the SpC of GVH F₁ chimeras were able to
respond in MLR and CML to host or third-party alloantigens. GVH F1 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 F1 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 F1 chimeras for at least 6 mo. In marked contrast, the T cells obtained from these long-term GVH F1 chimeras showed hardly any CML activity after restimulation with irradiated host-type (BDF1) cells (Table V). Out of the total of seven individually tested GVH F1 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 F1 chimeras did not suppress the anti-BDF1 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 F1 chimeras to generate T killer (Tk) cells against host-type alloantigens.

T Cells of B10 → F1 Irradiation Chimeras Are Completely Tolerant of the Host. We then asked whether the type of split tolerance found in long-term GVH F1 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.

**Figure 2.** Suppressor activity in the spleens of BDF1 recipients at various times after the induction of GVHR. (O) 10 d, (■) 38 d, or (□) 100 d (GVH F1 chimera) after the induction of GVHR with B10 SpC. Results are expressed as the percentage of PFC in control cultures (1,860 PFC/culture) to which no GVH F1 SpC were added. Results representative of five identical experiments are shown.
TABLE V

| Responder SpC      | Target cells      | Mean number of LU/culture ± SEM |
|-------------------|-------------------|---------------------------------|
| B10*              | BDF₁              | 688 ± 110                       |
| B10*              | (B₁₀ × B₁₀.BR)F₁  | 483 ± 72                        |
| GVH F₁ chimera†   | BDF₁              | 8 ± 5                           |
| GVH F₁ chimera‡   | (B₁₀ × B₁₀.BR)F₁  | 503 ± 114                       |
| B₁₀ + GVH F₁ chimera§ | BDF₁          | 555†                           |
| B₁₀ + GVH F₁ chimera§ | (B₁₀ × B₁₀.BR)F₁ | 555†                           |

* Cells obtained from seven normal B10 mice that were tested individually.  
† Cells obtained from seven GVH F₁ chimeras that were tested individually; these mice were taken at days 100–120 after the induction of the GVHR.  
‡ Mixtures containing 8 × 10⁶ normal B₁₀ SpC and 4 × 10⁶ SpC obtained from a GVH F₁ chimera were co-cultured for 5 d in MLR. Two GVH F₁ chimeras were studied; they had been taken from the group of seven chimeras described in footnote †.  
§ Results of two individual experiments.

GVH Reactivity of Adoptively Transferred SpC Obtained from Long-term GVH F₁ 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 BDF₁ 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 BDF₁ 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 F₁ chimera SpC to induce mortality in lethally irradiated BDF₁ recipients. To avoid mortality due to a hypothetical lack of hematopoietic stem cells in the donor cell inoculum, the SpC obtained from the GVH F₁ chimeras were mixed with BMC from the same mice. The cell suspensions thus obtained did not contain detectable numbers of BDF₁ 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 × 10⁶ GVH F₁ chimera cells into 950 rad-irradiated BDF₁ 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 BDF₁ recipients contained only the Ig-1ᵇ allotype, indicating that they were repopulated by donor (B₁₀) cells. As expected, all BDF₁ recipients of normal B₁₀ SpC died within 40 d after the induction of GVHD (Table VII, group 1). The inability of GVH F₁ chimera cells to induce GVH mortality in irradiated BDF₁ recipients was specific, since (B₁₀ × B₁₀.BR)F₁ recipients all succumbed within 30 d after the injection of GVH F₁ chimera cells (Table VII, group 5). The mixture of GVH F₁ SpC with B₁₀ SpC did not affect the ability of the latter cells to induce LGVHD in BDF₁.
Specific PLN Enlargement and Allohelp Caused by SpC from GVH F₁ Chimeras that Were Injected into the Footpads of Secondary BDF₁ Mice

| Cells transferred | Nonirradiated recipient mice* |
|-------------------|-------------------------------|
|                   | Number | Strain | Mean PLN index ± SEM. | Number of IgG protein A PFC/10⁶ PLN cells |
|                   | (X 10⁶) |       | Test-side PLN | Contra-lateral PLN |
| B10               | 5      | BDF₁  | 2.3 ± 0.4† | 39 ± 15 | <5 |
|                   | 10     | BDF₁  | 5.2 ± 0.8‡ | 76 ± 10 | <5 |
|                   | 15     | BDF₁  | 5.0 ± 0.8‡ | 158 ± 50 | 7 ± 3 |
| B10               | 15     | B10   | 1.4 ± 0.2 | <5 | <5 |
| GVH F₁ chimera    | 5      | BDF₁  | 3.5 ± 0.8‡ | 142 ± 58 | <5 |
|                   | 10     | BDF₁  | 3.4 ± 0.7‡ | 161 ± 86 | 5 ± 2 |
|                   | 15     | BDF₁  | 5.8 ± 0.3§ | 351 ± 97 | 18 ± 6 |
| GVH F₁ chimera    | 15     | B10   | 1.4 ± 0.1 | <5 | <5 |
| BDF₁              | 15     | BDF₁  | 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 F₁ 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 F₁ chimeras to induce GVH mortality in lethally irradiated BDF₁ recipients.

Discussion

Recent papers from this laboratory have reported that SLE-like GVHD in nonirradiated F₁ 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). When unseparated SpC of the donor B10 were injected into fully H-2-different BDF₁ 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 F₁ 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). In the present investigation, we extended the functional analysis of B10 donor T cells obtained from F₁ 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 F1 Chimeras*

| Group | Donors | Source | Number ($\times 10^9$) | Strain | Cumulative mortality at day: |
|-------|--------|--------|------------------------|--------|----------------------------|
|       |        |        |                        |        | 20 | 40 | 80 |
| 1     | B10    | SpC + 20 BDF<sub>1</sub> | 10 | 6/10 | 10/10 | 10/10 |
| 2     | B10    | SpC + 20 (B10 x B10.BR)F<sub>1</sub> | 10 | 7/10 | 10/10 | 10/10 |
| 3     | B10    | SpC + 10 B10 | 2/10 | 2/10 | 2/10 |
| 4     | GVH F1* chimera | SpC + 20 BDF<sub>1</sub> | 10 | 1/10 | 1/10 | 1/10 |
| 5     | GVH F1* chimera | SpC + 20 (B10 x B10.BR)F<sub>1</sub> | 10 | 4/10 | 10/10 | 10/10 |
| 6     | GVH F1* chimera | SpC + 20 B10 | 10 | 1/10 | 1/10 | 1/10 |
| 7     | B10    | SpC + 20 BDF<sub>1</sub> | 10 | 5/5 | 5/5 | 5/5 |
| 8     | None   | —       | — | 10/10 | 10/10 | 10/10 |

* Pooled SpC from 20 GVH F1 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 F1 Recipients by Lympho-hematopoietic Donor Cells.** Several groups of investigators have observed that nonirradiated H-2-different F1 mice undergoing GVHR may be repopulated by parental SpC (26–28). The recent observation (2) that such a repopulation took place in parent $\rightarrow$ F1 combinations undergoing acute GVHD, but not as readily in GVH F1 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<sup>+</sup>2<sup>+</sup> and Lyt-1<sup>-</sup>2<sup>-</sup> F1-specific donor Ts cells (24, 25, 29), whose presence in GVH F1 mice shortly preceded and coincided with the pancytopenia of acute GVHD, 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<sub>1</sub> recipients of unseparated B10 SpC, but not of either Lyt-1<sup>+</sup>2<sup>-</sup> or Lyt-1<sup>-</sup>2<sup>-</sup> 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).

**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). 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 (5), 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) 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 F₁ 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-F₁-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 F₁ 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 F₁ chimeras of stimulatory (class II) alloantigens. However, when the B10 cells that were recovered from long-term GVH F₁ chimeras were reexposed to BDF₁ 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 chronic 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 F₁ 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)F₁ mice that had been injected with 10⁸ B10 spleen cells 100–150 d previously. Such GVH F₁ mice were repopulated by lympho-hematopoietic cells of donor (B10) origin, which exhibited split tolerance towards the host: Whereas F₁-specific donor T helper (Th) cells as well as T cells proliferating in the mixed lymphocyte reaction were readily demonstrable, F₁-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 F₁ mice induced F₁-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)F₁ 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.

We thank Mr. Deon Kanters for his expert technical assistance and are obliged to Dr. Pavol Iványi for critical reading of the manuscript.

Received for publication 14 June 1983 and in revised form 26 October 1983.

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