Title

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Permalink

https://escholarship.org/uc/item/8r38300q

Journal

Journal of Experimental Medicine, 166(3)

ISSN

0022-1007

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Publication Date

1987-09-01

DOI

10.1084/jem.166.3.657

Peer reviewed

REGULATION OF AUTOIMMUNITY AND DONOR CELL ENGRAFTMENT BY RECIPIENT Lyt-2+ CELLS DURING THE GRAFT-VERSUS-HOST REACTION

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An autoimmune disease with features of human SLE can be induced in some normal mice during a graft-vs.-host reaction (GVHR),[1] referred to as SLE-like graft-vs.-host disease (SLE-like GVHD). In particular, injection of lymphocytes from DBA/2 mice into (C57BL × DBA/2)F_1 (BDF$_1$) mice causes the appearance of antibodies to dsDNA with consequent immune complex glomerulonephritis (ICGN) (1, 2). The autoantibodies are primarily produced by BDF$_1$ B cells (3–5). Minimum requirements for the production of autoantibodies are the presence of Lyt-1+,2- T cells in the donor innoculum (6) and incompatibility between parent strains at either the I-A (7) or I-E (8) major histocompatibility locus. No requirement for recipient T cells has been demonstrated, although some studies suggest that recipient T cells may suppress autoimmunity in SLE-like GVHD (3, 5). Using this model, we have examined the hypothesis that autoimmunity in SLE-like GVHD is regulated by recipient T helper/inducer (L3T4+) and/or T suppressor/cytotoxic (Lyt-2+) cells. This was possible because of the recent demonstration that these cells can be selectively and permanently deleted in vivo by treatment with mAbs to the lymphocyte subsets, after adult thymectomy (9). Our results demonstrate a requirement for recipient Lyt-2+ cells in the development of autoimmunity in SLE-like GVHD. Moreover, in this model, recipient Lyt-2+ cells regulate the engraftment of donor cells. Recipient L3T4+ cells do not significantly alter the outcome of the disease and have little or no effect on engraftment of donor cells. This is the first direct demonstration that recipient Lyt-2+ cells regulate donor cell engraftment in the parent-into-F_1 GVHR and that they are required for autoimmunity during the GVHR.

Materials and Methods

Mice. 4–6-wk-old female DBA/2 (H-2^d) and (C57BL/6 × DBA/2)F_1 (BDF$_1$) (H-2$^{b/d}$) mice, free of Sendai virus and mouse hepatitis virus, were purchased from Simonsen

[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; BUN, blood urea nitrogen; GVHD, graft-versus-host disease; GVHR, graft-versus-host reaction; ICGN, immune complex glomerulonephritis; LN, lymph node.

Laboratory, Gilroy, CA or Charles River Breeding Laboratories, Inc., Wilmington, MA, and were housed in the animal care facility at the San Francisco Veterans Administration Medical Center. Groups of 8–12 mice were used for each variable examined.

In Vivo Depletion of Lymphocyte Subsets.   Sustained depletion of L3T4$^+$ or Lyt-2$^+$ lymphocyte subsets in vivo was achieved by using the method of Cobbold et al. (9). Briefly, mice were thymectomized under pentobarbitol anesthesia at 4–6 wk of age and were allowed to recover for 3 wk before intraperitoneal injection with anti-L3T4 (GK1.5) or anti-Lyt-2.2 (2.43) rat IgG2b mAbs, two 0.5-mg doses, 1 wk apart. Control groups received either a rat mAb to chicken OVA (2C7, IgG2a) or saline, as indicated. After 4 wk, mice were bled for fluorescence analysis of circulating lymphocytes to quantitate cell depletion. If, at this point, target cells were detectable at >2% of blood lymphocytes, animals were considered to be incompletely thymectomized and were not used. This analysis excluded 0 of 10 mice treated with anti-L3T4 and 2 of 10 mice treated with anti-Lyt-2.

Induction of GVHR.   Donor lymphocytes from DBA/2 mice were prepared from spleen and lymph node (LN) cells, as follows: The inguinal, mesenteric, and cervical LN were minced in cold RPMI 1640, passed through No. 40 wire mesh, and filtered through loosely packed cotton wool. Spleen cells were passed through No. 40 wire mesh before mixing with LN cells at a spleen cell/LN cell ratio of 2:1. The cells were suspended in PBS at room temperature before injection. BDF$_1$ mice received two injections, 1 wk apart, of 6 × 10^7 live DBA/2 cells, in 0.4 ml, via the lateral tail vein. In the Results, the date of the first injection is day 0; the second injection is day 7.

Assessment of Autoimmunity.   To assess autoimmunity during the GVHR, blood and urine were collected from individual mice every other week after the first injection of parental cells to measure (a) serum antibodies to poly(dA)-(dT), (b) proteinuria, and (c) blood urea nitrogen (BUN). At the same time, mice were weighed and examined for ruffling of fur and for diarrhea, evidence for acute GVHD.

Serum Antibodies to poly(dA)-(dT).   Antibodies to poly(dA)-(dT) were quantified by ELISA by a modification of the method of Fish and Ziff (10), who demonstrated that poly(dA)-(dT) detects antibodies to dsDNA. For the ELISA, Microtest III microtitration plates (Falcon Labware, Oxnard, CA) were sequentially exposed at room temperature to the following reagents (all in 0.1 M Tris-HCl buffer, pH 7.2, with five distilled water washes between each): (a) 75 µl/well of poly-L-lysine, 50 µg/ml, (Sigma Chemical Co., St. Louis, MO) for 1 h; (b) 75 µl/well of poly(dA)-(dT), 20 µg/ml (Sigma Chemical Co.) for 1 h; (c) 300 µl/well of BSA, 20 mg/ml (Sigma Chemical Co.) for 20 min; (d) 75 µl/well of serum samples from individual mice serially diluted in buffer containing 10 mg/ml BSA and 20 mg/ml bovine gamma globulin (BGG) (Sigma Chemical Co.) for 1 h; (e) 300 µl/well of 20 mg/ml BSA for 20 min; (f) 75 µl/well of peroxidase-conjugated goat anti–mouse Ig, 4 µg/ml (Cooper Biomedical, Inc., Malvern, PA) in buffer supplemented with BSA and BGG, as in step d for 1 h. After washing, 75 µl/well of O-phenylenediamine, 1 mg/ml (Zymed Laboratories, San Francisco, CA) and 0.3 µg/ml H$_2$O$_2$ in 0.1 M citrate phosphate buffer, pH 5.0, were then added in the dark at room temperature. The reaction was stopped after 10 min with 75 µl/well of 2 N H$_2$SO$_4$. Absorbance at 490 nm was quantitated by the use of an ELISA reader (model EL 308; Bio-Tek Instruments, Inc., Burlington, VT). Serum from adult MRL-*lpr/lpr* and normal, age-matched, BDF$_1$ mice served as positive and negative controls, respectively.

Proteinuria.   Urine samples were obtained at the same time as serum samples to quantify protein by the use of Albustix (Miles Laboratories Inc., Elkhart, IN). This allows an approximation by colorimetry of proteinuria as follows: trace, 10 mg/dl; 1+, 30 mg/dl; 2+, 100 mg/dl; 3+, 300 mg/dl; 4+, ≥2,000 mg/dl. Normal mice were found to transiently express up to 2+ proteinuria by this method. Therefore, a value of 3+ or 4+ was considered indicative of pathologic proteinuria. To reflect the development of proteinuria in all mice, the values at each time point reflect the level of proteinuria in surviving mice, as well as the last measure of proteinuria in deceased mice.

Blood Urea Nitrogen.   BUN was determined in individual mice using a CentrifiChem System 400 (Union Carbide Corp., Danbury, CT). All mice had values of <30 mg/dl at

Table I

Antibodies Used for Fluorescence Analysis

Antibody	Surface antigen	Cell population identified	Reference
30-H12	Thy-1.2	T cells	11
GK1.5	L3T4	T helper/inducer cells	12
53-6	Lyt-2	T suppressor/cytotoxic cells	13, 14
Ra3-6B2	B220	B cells	15
34-5-8S	H-2D^d	H-2^d cells	16
B8-24-3	H-2K^b	H-2^b cells	17
MAR-18.5	—	Cells bearing rat mAb	18

the time of grafting. A value of ≥ 30 mg/dl was therefore considered abnormal for comparison of groups by χ^2 analysis, using the BUN at the end of the study or, in the event of death, the last measured BUN.

Fluorescence Analysis of Lymphocyte Subpopulations. Peripheral blood was obtained from individual mice by retroorbital bleeding into heparinized pipettes. Mononuclear cells were separated by centrifugation over Lympholyte-M (Cedarlane Laboratories Ltd., Ontario, Canada). Lymphocyte subsets were identified by use of a FACS II (Becton Dickinson & Co., Sunnyvale, CA). For single-color analysis, fluorescein-conjugated mAbs were used. For simultaneous two-color analysis, using a single laser, one mAb was conjugated with fluorescein and the other with biotin, followed by exposure to avidin-conjugated phycoerythrin (Streptavidin; Becton Dickinson & Co.). Analysis was restricted to lymphocytes on the basis of size, as determined by forward-angle light scatter. Fluorescence is expressed in arbitrary units with the use of a logarithmic scale. mAbs used for analysis are listed in Table I. All reagents were centrifuged at 100,000 g for 5 min immediately before use.

Statistics. Data were analyzed using the χ^2 test with the Yates correction, except for comparison of anti-poly(dA)-(dT) antibody titers, which used Student's t test, and for comparison of changes in lymphocyte subpopulations, which used Wilcoxon's rank sum test.

Results

Sustained, Selective Depletion of T Cell Subsets (L3T4$^+$ or Lyt-2$^+$) from BDF$_1$ Mice. When BDF$_1$ mice were thymectomized and treated with two doses of rat mAb to either L3T4 or Lyt-2, there was sustained, selective depletion of target cells 4 wk after the last injection of mAb (Table II). In groups that were not thymectomized before treatment with target-cell mAb (Table II, groups A4 and B4), partial recovery of target cell populations was observed. At this point, no rat mAb could be detected on circulating lymphocytes from any group by FACS staining with mAb to rat κ chain (results not shown). Thus, at the time of donor (parental) cell injection, recipient mice were selectively depleted of target lymphocyte subsets and had no evidence for persistence of antilymphocyte mAbs. As will be discussed below, the selective loss of recipient T cell subsets persisted after donor cell engraftment.

Depletion of Lyt-2$^+$ Cells, but Not L3T4$^+$ Cells, from BDF$_1$ Recipient Mice Prevents Autoimmunity after Injection with Donor DBA/2 Lymphocytes. Injection of DBA/2 cells into nonthymectomized, non–mAb-treated BDF$_1$ recipient mice led to the rapid appearance of antibodies to poly(dA)-(dT) that peaked 4–6 wk after the first injection of donor cells, and were sustained for as long as 12 wk (Fig. 1). Thymectomy alone or thymectomy followed by an irrelevant rat mAb (anti–

TABLE II

Lymphocyte Subpopulations in BDF₁ Mice After Thymectomy and/or Treatment with Antilymphocyte Antibodies

Group	Thymec-tomy	mAb treatment	L3T4	Lyt-2	B220
			%	%	%
A1	+	Anti-Lyt-2	29	1	61
A2	–	—	30	21	38
A3	+	—	22	14	52
A4	–	Anti-Lyt-2	41	4	38
B1	+	Anti-L3T4	1	21	71
B2	–	—	27	17	55
B3	+	—	23	16	56
B4	–	Anti-L3T4	8	30	58

Blood lymphocytes from individual mice (8–10/group) were analyzed 4 wk after the last treatment with mAb, as described in the Materials and Methods. Groups A1–A4 were treated and examined concurrently, as were groups B1–B4.
* Results are the mean percent of circulating lymphocytes, defined on the basis of size, as described in the Materials and Methods.
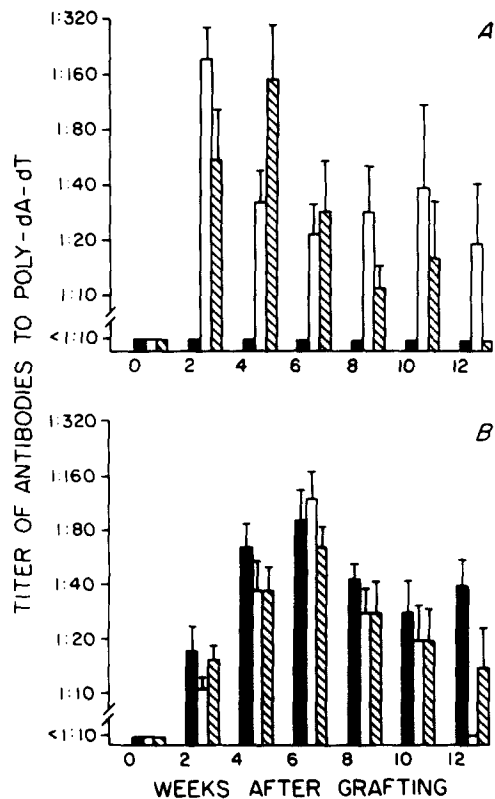

FIGURE 1. Geometric mean titer (× SEM) of antibodies to poly(dA)-(dT) in groups of BDF₁ recipient mice treated by (A) thymectomy followed by anti-Lyt-2 mAb (solid boxes), no thymectomy followed by saline (open bars), or thymectomy followed by saline (hatched bars); and (B) thymectomy followed by anti-L3T4 mAb (solid bars), no thymectomy followed by saline (open bars) or thymectomy followed by rat mAb against chicken OVA (hatched bars). Sera from individual mice in each group were assayed by ELISA, as described in Materials and Methods. Week 0 indicates the time of the first injection of donor DBA/2 lymphocytes. Results of sera from normal mice as negative controls fell in the <1:10 region and are not shown.
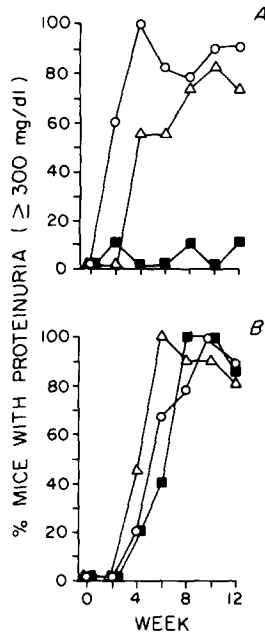
FIGURE 2. Frequency of proteinuria ≥3+ (300 mg/dl) in groups of BDF₁ recipients treated by (A) thymectomy followed by anti-Lyt-2 mAb (squares), no thymectomy followed by saline (circles) or thymectomy followed by saline (triangles); and (B) thymectomy followed by anti-L3T4 mAb (squares), no thymectomy followed by saline (circles) or thymectomy followed by rat mAb to chicken OVA (triangles). Week 0 indicates the time of the first injection of donor DBA/2 lymphocytes. Each point represents proteinuria in surviving mice as well as the last measurement of proteinuria in deceased mice (see Materials and Methods).

chicken OVA) in BDF₁ recipients did not significantly alter levels of autoantibodies (Fig. 1). However, when thymectomized mice were depleted of Lyt-2⁺ cells, no antibodies to poly(dA)-(dT) were detected at any time (Fig. 1A, solid bars; $p < 2.5 \times 10^{-7}$ compared with thymectomy only, by time-adjusted analysis). In contrast, depletion of L3T4⁺ cells from thymectomized BDF₁ recipients had no significant effect on autoantibody production (Fig. 1B, solid bars).

The lack of autoantibodies in BDF₁ mice depleted of Lyt-2⁺ cells was associated with lack of pathologic proteinuria; only an occasional mouse in this group developed transient proteinuria during the 12-wk experiment (Fig. 2A; $p < 0.05$ compared with either control at 8, 10, and 12 wk). In contrast, >80% of mice in all other groups had developed proteinuria by 12 wk (Fig. 2).

Of mice depleted of Lyt-2⁺ cells, only 1 of 8 developed an abnormal BUN during the 12 wk after donor cell injection (Fig. 3A1), compared with 8 of 10 thymectomized BDF₁ recipients depleted of L3T4⁺ cells (Fig. 3B1; $p < 0.025$) and compared with 13 of 18 nonthymectomized, non–mAb-treated BDF₁ recipients (Fig. 3, A2 and B2; $p < 0.025$). A significant rise in BUN was also seen in 10 of 19 recipients subjected to thymectomy only or thymectomy followed by an irrelevant rat mAb (anti–chicken OVA) (Fig. 3, A and B; $0.05 < p < 0.1$). 6 of the 9 mice in these groups that did not achieve a significantly elevated BUN died between 4 and 8 wk after donor cell grafting. Because these mice developed autoantibodies, it is likely that the biweekly sampling interval used for BUN measurement may have missed significantly higher BUN levels immediately before death.

Increased Engraftment of Donor L3T4⁺ and Lyt-2⁺ Lymphocytes Occurs in BDF₁ Mice Depleted of Host Lyt-2⁺ Lymphocytes. 4 wk after the first injection of donor DBA/2 lymphocytes, near the peak of autoantibody production in controls,
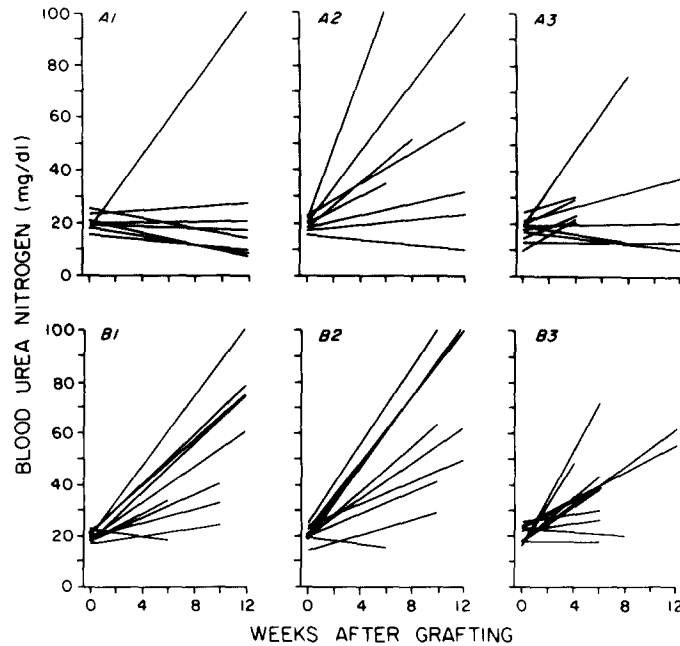
FIGURE 3.   BUN from BDF₁ recipient mice treated with (A1) thymectomy followed by anti-Lyt-2 mAb, (B1) thymectomy followed by anti-L3T4 mAb, (A2 and B2) no thymectomy followed by saline (A3) thymectomy followed by saline or (B3) rat mAb against chicken OVA. Lines begin at week 0, representing the first injection of donor DBA/2 lymphocytes, and end at the last measured BUN (see Materials and Methods).

blood cells from individual mice were analyzed for T cell subsets of both recipient and donor origin. Simultaneous two-color FACS analysis was used, one stain being either anti-H-2K^b or anti-H-2D^d, the other strain being anti-L3T4 or anti-Lyt-2. The use of anti-H-2K^b provided a sensitive distinction between recipient and donor T cell subsets as demonstrated in Fig. 4, while anti-H-2K^d identified the sum of donor and recipient cells. By this analysis, it was demonstrated that BDF₁ recipients depleted of Lyt-2$^+$ cells had a substantial increase in the engraftment of both L3T4$^+$ and Lyt-2$^+$ donor lymphocytes as well as a decrease in recipient L3T4$^+$ cells (Table III). In these mice, virtual repopulation by donor cells occurred, with 95% of circulating T lymphocytes being of parental origin, compared with ≤35% in all other groups ($p < 0.005$). Despite this extensive engraftment of donor T cells, recipient mice depleted of Lyt-2$^+$ cells not only lacked autoimmunity, but also failed to develop signs of acute GVHD, as assessed by loss of weight, ruffling of fur, or development of diarrhea. Moreover, none of the mice in this group died.

In contrast to the depletion of Lyt-2$^+$ cells, depletion of recipient L3T4$^+$ cells had little or no effect on donor cell engraftment (Table III). Similarly, in BDF₁ mice subjected to thymectomy but not treated with mAb, there was no effect on donor cell engraftment, although host T cells were diminished (Table III). We conclude that depletion of recipient Lyt-2$^+$ cells, but not L3T4$^+$ cells, permits enhanced engraftment of parental cells.
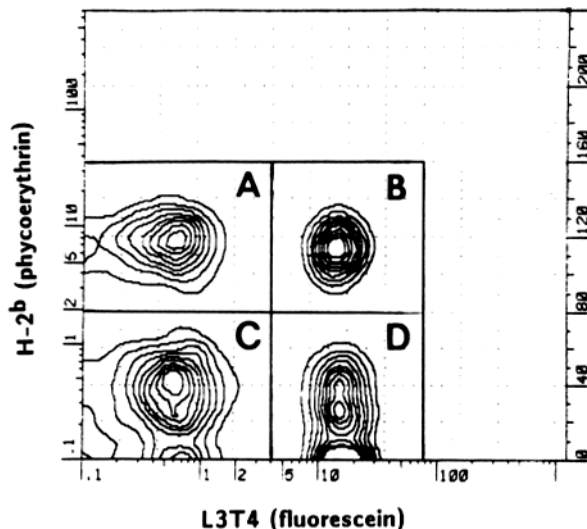
FIGURE 4. Representative contour plot of simultaneous dual-color FACS analysis of blood cells from a BDF₁ recipient of DBA/2 cells. Host cells are identified by mAb to H-2^b (A and B), which donor cells lack (C and D). In this example, host L3T4$^+$ cells (B) are distinguished from donor L3T4$^+$ cells (D).

TABLE III

Host and Donor T Lymphocyte Subpopulations in BDF₁ Mice After Injection with Donor Cells

Group	Thymec-tomy	mAb	Host cells*		Donor cells‡		T cells of donor origin	Auto-immunity
			L3T4	Lyt-2	L3T4	Lyt-2		
			%		%		%	
A1	+	Anti-Lyt-2	1§	1	32	9	95	−
A2	−	None	21	16	8	2	21	+
A3	+	None	8	13	8	3	34	+
B1	+	Anti-L3T4	3	16	6	1	27	+
B2	−	None	19	16	4	1	13	+
B3	+	None	9	12	6	1	25	+

Blood lymphocytes from individual mice (8–10/group) were analyzed 4 wk after the first injection of DBA/2 cells, as described in Materials and Methods.
* Host cells are defined as those reacting with anti-H-2k^b mAb (hybridoma B8-24-3), as shown in Fig. 4.
‡ Donor cells are defined as those reacting with anti-H-2D^d (hybridoma 34-5-8S), but not reacting with anti-H-2K^b mAb (hybridoma B8-24-3).
§ Results are the mean percent of all circulating lymphocytes, defined on the basis of size, as described in Materials and Methods. In recipients treated with anti-Lyt-2, both L3T4$^+$ and Lyt-2$^+$ host cells are diminished, reflecting nearly complete replacement by donor T cells.

Discussion

To test the hypothesis that recipient T cells may regulate the development of autoimmunity in SLE-like GVHD, we permanently depleted F₁ recipient mice of their L3T4$^+$ (helper/inducer) or Lyt-2$^+$ (suppressor/cytotoxic) T cells before the induction of the parent-into-F₁ GVHR. Depletion of recipient Lyt-2$^+$ T cells abrogated autoimmunity and permitted enhanced engraftment of both L3T4$^+$ and Lyt-2$^+$ donor cells. Depletion of recipient L3T4$^+$ cells did not significantly alter autoimmunity and had little or no effect on engraftment of donor cells. Similarly, a rat mAb not reactive with mouse antigens had no effect on autoimmunity or donor cell engraftment.

Previous studies (3, 5) have addressed the role of recipient T cells in the development of autoimmunity in SLE-like GVHD. In these studies, groups of adult-thymectomized, lethally irradiated BDF_1 recipients were reconstituted with fetal liver cells before injection with DBA/2 lymphocytes. In these T cell–depleted mice, a higher percentage developed ICGN and proteinuria than in normal BDF_1 recipients or in adult-thymectomized BDF_1 recipients. In addition, fewer donor cells were required to induce disease. These findings suggest a suppressive role for recipient T cells in SLE-like GVHD. Our studies, however, did not demonstrate a suppressive role for recipient T cells. BDF_1 recipients treated by thymectomy alone did show decreased survival, but they did not have increased levels of autoimmunity.

In our studies, the mAbs used to deplete recipient T cell subsets in vivo, before grafting, did not remain to influence the engraftment of donor cells. First, in control, nonthymectomized, BDF_1 mice treated by the same mAb protocol, there was significant recovery of the target cell populations after 4 wk, the time of the first injection of parent lymphocytes (Table II). This indicates the absence of functional anti–target cell mAb at this time. Second, in every mouse, fluorescence analysis with a mAb to rat κ chain demonstrated a lack of cell-surface rat mAb (results not shown). Third, donor cell engraftment was unaltered in recipients depleted of $L3T4^+$ cells and was enhanced in recipients depleted of $Lyt-2^+$ cells, findings that are inconsistent with the presence of antilymphocyte mAbs at the time of donor cell engraftment.

The enhanced engraftment of parental cells after depletion of recipient $Lyt-2^+$ cells excludes the possibility that the lack of autoimmunity in these mice is due to an insufficiency of donor cells. It is possible either that the increased engraftment suppressed autoimmunity or that recipient $Lyt-2^+$ cells are directly required for the development of autoimmunity. In favor of the former possibility, it has been previously demonstrated (reviewed in reference 19) that the engraftment of specific donor T cell subset(s) during the GVHR correlates with the form of GVHD observed in the BDF_1 recipient. Similarly, the GVHR differs depending on whether there are differences at class I and/or class II major histocompatibility antigens between the donor and host (7, 20–22). With regard to our model, Pals et al. (23, 24) have demonstrated that the development of autoimmunity in BDF_1 recipients of DBA/2 cells is associated with engraftment of donor $L3T4^+$ cells, but very few, if any, donor $Lyt-2^+$ cells, a finding confirmed in our studies of untreated BDF_1 recipients (Table III). In BDF_1 recipients of cells from the C57 parent, both $L3T4^+$ and $Lyt-2^+$ donor cells engraft and there is immune suppression rather than autoimmunity. Removal of $Lyt-2^+$ cells from the C57 parent innoculum converts the disease to an SLE-like GVHD (6). It is therefore possible that donor $Lyt-2^+$ cells kill or suppress autoreactive cells in the host.

Our studies provide the first direct demonstration that $Lyt-2^+$ cells influence the engraftment of parental lymphocytes in F_1 recipients. Several studies (25–27) have demonstrated that F_1 recipients can generate cytotoxic T cells directed against parent cells. In BDF_1 recipients given C57 parent cells, F_1 anti–parent cytotoxic cells have been shown to be $Lyt-2^+$ (28). In light of these findings and ours, it seems likely that F_1 cytolytic $Lyt-2^+$ cells can reduce engraftment of

parental lymphocytes, as they do allogeneic lymphocytes (21, 29). If so, this effect is seemingly independent of recipient L3T4$^+$ cells, because depletion of the latter does not increase donor cell engraftment. The rejection of parental lymphocytes by Lyt-2$^+$ recipient cells is in contrast to rejection of parental hematopoietic cells, which is mediated in F$_1$ mice not by T cells but by NK cells (30–32).

Summary

When lymphocytes from DBA/2 mice are transferred to (C57BL × DBA/2)F$_1$ (BDF$_1$) mice, the ensuing graft-vs.-host reaction (GVHR) causes an autoimmune illness resembling human SLE. To examine the role of recipient T cells in this process, BDF$_1$ mice were depleted of L3T4$^+$ or Lyt-2$^+$ cells by thymectomy followed by treatment with mAbs to L3T4 or Lyt-2. This produced sustained depletion of these T cell subsets. Subsequent grafting with parental DBA/2 lymphocytes produced autoimmune disease in mice depleted of L3T4$^+$ cells and controls but not in mice depleted of Lyt-2$^+$ cells. Analysis of blood lymphocytes 4 wk after donor cell transfer demonstrated that BDF$_1$ recipients depleted of Lyt-2$^+$ cells were virtually repopulated with donor T lymphocytes, compared with ≤35% donor cell engraftment in all other groups. Thus, recipient Lyt-2$^+$ cells influence both host cell engraftment and autoimmunity during the parent-into-F$_1$ GVHR.

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