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Progression from Acute to Chronic Disease in a Murine Parent-into-F_1 Model of Graft-Versus-Host Disease

Jolynne R. Tschetter,* Edna Mozes,† and Gene M. Shearer*[1]

The parent-into-immunocompetent-F_1 model of graft-vs-host disease (GVHD) induces immune dysregulation, resulting in acute or chronic GVHD. The disease outcome is thought to be determined by the number of parental anti-F_1 CTL precursor cells present in the inoculum. Injection of C57BL/6 (B6) splenocytes into (B6 × DBA/2)F_1 (B6D2F_1) mice (acute model) leads to extensive parental cell engraftment and early death, whereas injection of DBA/2 cells (chronic model) results in little parental cell engraftment and a lupus-like disease. This study demonstrated that injection of BALB/c splenocytes into (BALB/c × B6)F_1 (CB6F_1) mice resulted in little engraftment of parental lymphocytes and the development of lupus as expected. Injection of B6 splenocytes into CB6F_1 initiated an initial burst of parental cell engraftment similar to that of B6 into B6D2F_1. However, the acute disease resolved, and the CB6F_1 mice went on to develop chronic GVHD with detectable Abs to ssDNA, dsDNA, and extractable nuclear Ags. Limiting dilution CTL assays determined that B6 splenocytes have CTL precursor frequencies of 1/1000 against both CB6F_1 and B6D2F_1, whereas DBA/2 and BALB/c splenocytes have a CTL precursor frequency of 1/20,000 for their respective F_1s. The Th cell precursor frequency for B6 anti-DBA/2 was 3-fold higher than that for B6 anti-BALB/c determined by limiting dilution proliferation assays. These results indicate the importance of adequate allospecific helper as well as effector T cells for the induction and maintenance of acute GVHD in this model, and presents an unexpected model in which initial acute GVHD is replaced by the chronic form of disease. *The Journal of Immunology,* **2000, 165: 5987–5994.**

The parent-into-F_1 model of graft-vs-host disease (GVHD)[2] provides an example of induced immune dysregulation that involves both T and B cell effector mechanisms. This model is unique in that immunocompetent adult F_1 mice inoculated with spleen or lymph node cells from one or the other parental strains develop very distinct forms of disease. C57BL/6 (B6) × DBA/2 F_1 (B6D2F_1) mice injected with B6 splenocytes resulted in an acute disease associated with the replacement of the B6D2F_1 splenic lymphocytes with lymphocytes of B6 origin (1, 2). Activated $CD8^+$ T cells (2, 3) and Th1 cytokines predominate in this reaction (3–5). Also, CTL can be isolated from the spleens of mice suffering from acute GVHD that specifically recognize B6D2F_1 cells and cells expressing H-2^d (2, 3, 5, 6). Acute GVHD is also associated with sensitivity to endotoxin, and most animals die within 4 to 8 wk (7, 8). In contrast, B6D2F_1 mice injected with DBA/2 splenocytes result in a chronic disease with a low level of parental cell engraftment consisting primarily of $CD4^+$ cells (1) and a Th2 cytokine profile (4, 9, 10). As a result, B6D2F_1 B cells show elevated levels of activity and the production of autoantibodies recognizing ssDNA (11, 12), dsDNA (11–15), and extractable nuclear Ag (ENA) (11, 15, 16). This B cell hyperactivity and autoantibody production eventually lead to the development of glomerulonephritis and a lupus-like condition.

Earlier work characterizing the B6D2F_1 model of GVHD demonstrated a significant parent anti-F_1 CTL precursor frequency difference between B6 and DBA/2 anti-B6D2F_1. Spleen cells from B6 mice exhibited CTL precursor frequency of 1/1450 against B6D2F_1 cells and induced acute GVHD, whereas DBA/2 spleen cells had a CTL precursor frequency of 1/13,500 against B6D2F_1 cells and induced chronic GVHD (2), indicating a role for $CD8^+$ T cells in the development of acute GVHD. In further support of this idea, depletion of $CD8^+$ T cells from the B6 inoculum ablated the acute form of GVHD and induced chronic GVHD (17, 18). Furthermore, repeated injection of DBA/2 cells into B6D2F_1 mice resulted in a shift from chronic to acute disease (19). When considered together, these results were interpreted to mean that CTL precursor frequencies were responsible for determining the outcome for this model of GVHD.

To test the hypothesis that $CD8^+$ T cell precursor frequencies determined GVHD outcome in the parent-into-immune-competent F_1 model, we compared the disease in B6D2F_1 mice injected with parental cells with the disease in (BALB/c × B6) F_1 (CB6F_1) mice injected with parental spleen cells. Both are H-$2^{d/b}$ F_1 hybrids, involving MHC class I and II disparity, and should result in acute disease following injection of B6 cells if the hypothesis that CTL precursor frequency determines disease outcome is correct. Based on our definitions of acute vs chronic disease by parental T cell repopulation, CTL activity, and autoantibody production, we observed that B6-into-CB6F_1 GVHD was reproducibly detected as acute disease (weeks 1–3), followed by a shift to chronic GVHD.

Materials and Methods

Mice

B6, DBA/2, B6D2F_1, BALB/c, and CB6F_1 female mice, 6–8 wk of age, were purchased from either Animal Production Area (Frederick, MD) or Charles River Breeding Laboratories (Wilmington, MA). Animals were cared for in accordance with the guidelines set up by the Institutional Animal Care and Use Committee. An animal protocol for studying GVHD was reviewed and approved by the National Cancer Institute Animal Care and Use Committee before starting experiments.

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[2] Abbreviations used in this paper: GVHD, graft-vs-host disease; B6, C57BL/6; B6D2F_1, (B6 × DBA/2)F_1; CB6F_1, (BALB/c × B6) F_1; CM, complete medium; ENA, extractable nuclear Ag; DPBS, Dulbecco's PBS.

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Preparation of murine cells

Single-cell suspensions were prepared from the spleens of mice in Dulbecco's PBS (DPBS; Life Technologies, Grand Island, NY). Cell suspensions were filtered through a 70-μm sterile mesh screen (Becton Dickinson Labware, Franklin Lakes, NJ), and the cells were washed. For some applications, erythrocytes were lysed by using ACK Lysis Buffer (BioWhittaker, Walkersville, MD), or erythrocytes and dead cells were removed by using Lympholyte M (Accurate Chemical and Scientific, Westbury, NY). Cells were suspended in either DPBS or complete medium (CM) comprised of RPMI 1640 (Life Technologies) supplemented with 2 mM L-glutamine, penicillin, streptomycin, 5×10^{-5} M 2-ME (Life Technologies), 100 μM nonessential amino acids (Life Technologies), 5 μM HEPES (Life Technologies), and 10% heat-inactivated FBS (HyClone, Logan, UT) after the final wash, depending on use.

Induction of GVHD

Washed B6, DBA/2, BALB/c, $B6D2F_1$, and $CB6F_1$ splenocytes were suspended at 120×10^6 viable cells/ml in DPBS. GVHD was induced by the injection of 60×10^6 parental cells i.v. into $CB6F_1$ or $B6D2F_1$ mice. Control mice included uninjected age-matched mice and mice injected i.v. with 60×10^6 syngeneic F_1 splenocytes. To maintain as much homogeneity between donor cell populations, both F_1 combinations were injected on the same day using cells processed simultaneously under the same conditions. Both $CB6F_1$ and $B6D2F_1$ mice were injected from the same pool of B6 splenocytes.

Flow cytometric analysis of parental cell engraftment

The spleens of GVHD mice were harvested at 1–4, 6, 8, and 12 wk postinoculation. Single-cell suspensions of splenocytes were prepared, and the number of cells per spleen was counted. Erythrocytes and dead cells were removed using Lympholyte M. Splenocytes were stained with FITC-conjugated anti-H-2^d (clone SF_1-1.1), PE-conjugated anti-H-2^b (clone AF6-88.5) to distinguish parental from F_1 cells in the presence of the FCγRIII clone 2.4G2 to block nonspecific staining. To further identify the splenocyte populations anti-CD3 (clone 145-2C11), anti-CD4 (clone RM4-5 or H129.19), anti-CD8α (clone 53-6.7), or anti-CD19 (clone 1D3) conjugated to CyChrome or biotin plus streptavidin-CyChrome were used. All mAbs were purchased from PharMingen (San Diego, CA). Data were collected on a FACScan flow cytometer and analyzed with CellQuest Software (Becton Dickinson, San Jose, CA).

Autoantibody detection

ELISAs of serum Abs recognizing ssDNA (20) and dsDNA (21, 22) were performed as described previously. Briefly, for ssDNA assays, 96-well Maxisorp plates (Nalge Nunc International, Roskilde, Denmark) were incubated with 10 μg/ml methylated BSA (Sigma, St. Louis, MO) for 90 min at room temperature, followed by incubation of the plates for 2 h with 10 μg/ml calf thymus DNA (Sigma) that had been heated to >80°C for 15 min. Plates were washed and blocked overnight with 5% FBS in DPBS. Mouse serum was incubated on the plates for 2 h in 5-fold serial dilutions from 1:10 to 1:1250. Bound serum Abs were detected using goat anti-mouse IgG conjugated to HRP (Cappel, Aurora, OH) and visualized using 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and measuring the OD at 405 nm. For dsDNA assays, 96-well Maxisorp plates (Nalge Nunc International) were incubated with 5 μg/ml poly-L-lysine for 2 h at room temperature followed by incubation with 5 μg/ml λ phage DNA (Boehringer Mannheim, Indianapolis, IN) for 2 h at room temperature, then 4°C overnight. Other components of this assay were performed as described above.

ELISAs to detect the presence of Ab to ENA were performed using plates from RELISA ENA single-well screening kits (Immuno Concepts, Sacramento, CA). Each microtiter well is coated with Sm, RNP, SS-A, SS-B, Scl-70, and Jo-1 autoantigens. Plates were blocked for 2 h at 37°C. Mouse serum was added to the plates in 5-fold serial dilutions from 1:10 to 1:1250. Bound serum Abs were detected with goat anti-mouse IgG-HRP (Cappel) and 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories) stopped with 2 N H_2SO_4. OD was measured at 450 nm.

Detection of Ig complexes in kidney sections

Detection of Ig complexes in kidney sections were performed as previously described (23). Briefly, kidneys were removed from mice following euthanasia and snap frozen in liquid nitrogen. Six-micrometer sections were cut, air dried, and fixed with acetone. Ig deposits were detected using FITC-labeled goat anti-mouse IgG (Sigma) incubated on slides for 30 min and extensively washed with PBS. A fluorescence microscope was used to visualize specific staining. Immune complexes were evaluated according to density and strength of staining with scores ranging from (−) to (+++). Scores were converted to a numerical value and a mean and SD for each group of mice was determined.

CTL assays

Responding T cells from control and GVHD mice were prepared from pooled spleens, filtered, and washed with DPBS. Stimulator cells were splenocytes from normal F_1 mice treated with ACK lysing buffer and irradiated with 2000 cGy. Final cell concentrations were 2.5×10^6 responding cells/ml and 1.25×10^6 stimulators/ml in CM. Cultures were incubated at 37°C with 5% CO_2 for 5 days. Pooled effector cells were harvested and counted on day 5 for use in the CTL assays. Target cells were P815 (H-2^d) and EL-4 (H-2^b) cell lines labeled with 300 μCi ^{51}Cr for 90 min at 37°C and washed extensively. Target and effector cells were incubated together at 37°C for 4 h at various E:T ratios and compared with target cells and CM for spontaneous release and target cells plus 3% Triton X-100 for maximum release. Supernatants were harvested using Skatron (Sterling, VA) harvesting filters and frames. The percent specific lysis equals [(experimental − spontaneous)/(maximum − spontaneous)] × 100.

Limiting dilution CTL assays

Single-cell suspensions were prepared from the spleens of B6, DBA/2, BALB/c, $B6D2F_1$, and $CB6F_1$ mice. Stimulator cells were $CB6F_1$ or $B6D2F_1$ splenocytes irradiated with 2000 cGy and used at 1×10^6 cells/well in 96-well round-bottom plates (Costar, Corning, NY). CM was supplemented with recombinant murine IL-2 (BioSource International, Camarillo, CA) at a final concentration of 20 U/ml for these assays. Cells were cultured for 8 days at 37°C with 5% CO_2. Target cells were P815 (H-2^d) and EL-4 (H-2^b) cell lines labeled as above. Target cells were added to the limiting dilution wells at 3×10^3 cells/well. After the addition of target cells, plates were briefly spun to collect cells at the bottom of wells. Plates were incubated for 4 h at 37°C.

Limiting dilution assays using B6 anti-$CB6F_1$ and B6 anti-$B6D2F_1$ were performed using 2-fold serial dilutions of responding cells in the range of 1.6×10^4 to 125 cells/well. Assays using DBA/2 anti-$B6D2F_1$ and BALB/c anti-$CB6F_1$ were set up using 2-fold serial dilutions of responding cells in the range of 5×10^4 to 781.25 cells/well. All cell concentrations were tested using 24 replicates. Spontaneous release of ^{51}Cr was determined by incubating target cells for 4 h in wells that contained stimulator cells stimulated with CM and IL-2 for 8 days. All of the supernatant was harvested as above. Wells were scored positive for CTL activity if the cpm for a well was greater than the mean spontaneous release plus 3 SDs.

Both CTL precursor frequencies and Th precursor frequencies described below were determined by the least squares method using a computer program supplied by Dr. Charles Orostz (Ohio State University, Columbus, OH).

Limiting dilution proliferation assays

Single-cell suspensions of splenocytes from B6, $B6D2F_1$, and $CB6F_1$ mice were prepared as described above and used as responder cells. Stimulator cells were B6, $B6D2F_1$, or $CB6F_1$ splenocytes treated with 50 μg/ml mitomycin C (Sigma) per 50×10^6 cells for 30 min at 37°C. Assays were conducted in flat-bottom 96-well plates, using 2.5×10^5 stimulator cells/well and variable numbers of responder cells in CM. Responder cells were used in 0.75-fold serial dilutions from 1×10^5 to 7508.5 cells/well in 48-well replicates. Four milliliters of responder cell suspension at 1×10^6 cells/ml was added to 2 ml of medium in a fresh tube. The new cell suspension was mixed thoroughly, and 4 ml was removed to a fresh tube containing 2 ml of media. This process was continued until eight cell dilutions were ready for use in the assay. One hundred microliters of responder cells and stimulator cells were used per well. Plates were incubated for 4 days at 37°C, pulsed with 1 μCi/well [^{3}H]thymidine, and incubated overnight. Plates were harvested using a Tomtec plate washer (Wallac, Gaithersburg, MD) and counted with a β plate reader (Wallac).

Results

Parental cell engraftment in GVHD mice

It was previously demonstrated that injection of B6 splenocytes into immunocompetent $B6D2F_1$ mice resulted in high levels of parental cell engraftment and a short lethal disease course (2). Injection of DBA/2, the other parent, resulted in a chronic progressive disease with little parental cell engraftment (2). Typical engraftment profiles for acute and chronic GVHD obtained in this study are shown for this strain combination in Fig. 1*A*. Injection of

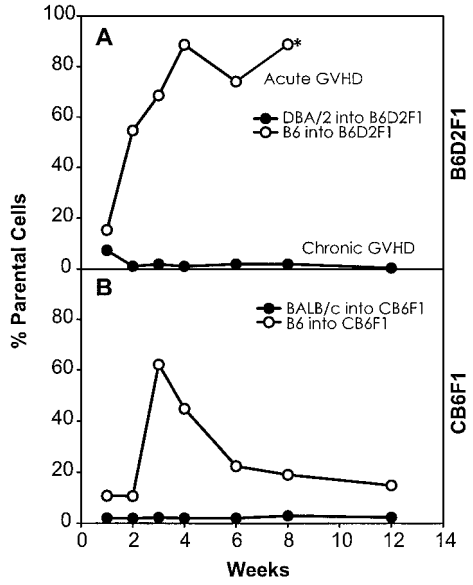

FIGURE 1. Parental cell engraftment profiles of GVHD. Flow cytometry was used to identify F_1 and parental cells in spleens from GVHD mice using H-2^d- and H-2^b-specific mAb. The percentage of parental cells in each GVHD model is plotted as a function of time after parental cell injection. *A*, DBA/2- and B6-into-B6D2F_1 mice. *B*, BALB/c- and B6-into-CB6F_1 mice. Results shown are averages (and SEM) of splenocytes from three different mice. Similar results were seen in two other separate experiments. *, Remaining mice in this group died before data could be collected at week 12.

BALB/c splenocytes into CB6F_1 resulted in the engraftment pattern expected for chronic GVHD, with little engraftment of parental lymphocytes (Fig. 1*B*). Injection of B6 splenocytes into CB6F_1 resulted in an initial burst of parental cell engraftment similar to that of B6-into-B6D2F_1. However, between the third and fourth week of GVHD, the percentage of parental cells unexpectedly began to decrease. This decline in parental B6 cells continued until week 12, at which time the disease mirrored chronic GVHD (Fig. 1*B*). In contrast to the acute GVHD of B6-into-B6D2F_1 mice that die within 4–8 wk (Ref. 7; current study), we have observed B6-into-CB6F_1 GVHD mice surviving for >30 wk.

B6-into-B6D2F_1 mice with acute GVHD exhibited a marked expansion of donor $CD4^+$ and $CD8^+$ lymphocytes with a concomitant decrease in total donor lymphocytes over the 8-wk disease course. By 8 wk, the spleens of acute GVHD consist of ~88% (±1.9%) donor lymphocytes and 11.2% (±5.6%) host lymphocytes. The majority of donor cells in the affected spleens are $CD4^+$, making up 37% of the total cell number (Fig. 2*A*). B6-into-CB6F_1 mice exhibited a similar pattern of donor/host cell changes in the spleens of affected mice. Donor lymphocytes expanded over the first 3 wk of disease, with donor cells peaking at 62.2% (±11.7%). Donor $CD4^+$ T cell numbers increased to 16.5% of the spleen and donor $CD8^+$ T cell numbers increased to 10.6% of the spleen at week 3 (Fig. 2*B*).

Chronic GVHD has a distinctive pattern of donor cell engraftment in the spleen. The only cell population that engrafted was the donor $CD4^+$ T cells at 2–7% of the total spleen cells. This population can be detected throughout the entire disease course and is responsible for the secretion of the cytokines that drive the affected mice to a chronic autoimmune disease (data not shown).

Cellular changes in GVHD mice

Although the percent changes in cells give an overview of the cell populations over time, they do not take into account the dynamics of changes in spleen size and cellularity during the course of disease. To obtain a more complete understanding of the changes within the spleens of the GVHD mice, the number of parental and F_1 splenocytes was determined by multiplying the percentage of each cell type in a given spleen by the total number of cells recovered from that spleen. In acute GVHD there was a drastic decrease in B6D2F_1 cell number by week 2 (Fig. 3*A*). This dramatic decrease seen in host spleen cell numbers was due to the elimination of B6D2F_1 $CD4^+$, $CD8^+$, and $CD19^+$ cells (Fig. 4*A*). Concurrently, the B6 cells that were injected into the F_1 hosts expanded and gradually became the major cell population in the F_1 spleens. At 1 wk post-GVHD, there were 1.56×10^7 B6 $CD4^+$ T cells in spleens of B6-into-B6D2F_1 mice. This number decreased to 3.34×10^6 cells at 4 wk and rebounded to 3.82×10^7 cells at week 8 (Fig. 5*A*). $CD8^+$ T cells of B6 origin followed a similar pattern of expansion, regression, and expansion during acute GVHD (Fig. 5*A*) with cell numbers ranging from 3.96×10^6 to 1.21×10^7.

Parental cell engraftment in chronic GVHD remained low throughout the disease course, ranging between 2 and 7% (see Fig. 1, *A* and *B*). Converting this percentage to cell numbers resulted in a similar picture to that seen with percentages. Both DBA/2 and BALB/c parental cells were detected at low levels in their respective F_1 hosts throughout all the time points tested (Fig. 3, *B* and *D*). The parental cells that were detected in the spleens of affected mice were predominantly $CD4^+$ T cells (data not shown) as previously reported (1, 16). These $CD4^+$ T cells have been suggested to provide help for driving host B cells and the autoimmune symptoms that are manifested later in the disease (16). F_1 $CD4^+$ and $CD8^+$ T cell levels remained relatively constant over the course of GVHD, whereas $CD19^+$ B cells levels transiently increased during the initial phase of the disease, returned to normal levels, and then began to increase again at the later time points (Fig. 4, *B* and *D*).

B6-into-CB6F_1 GVHD presents complex cellular interaction patterns not typical of either of the other two forms of GVHD. The number of CB6F_1 cells increased during the first 2 wk. A dramatic decrease in the number of CB6F_1 cells was observed between weeks 2 and 3, simultaneous with an increase in the number of parental B6 cells (Fig. 3*C*). The loss of CB6F_1 cells included $CD4^+$, $CD8^+$, and $CD19^+$ cells (Fig. 4*C*), whereas the increase in B6 cells involved $CD4^+$ and $CD8^+$ T cells (Fig. 5*B*). By week 4, these spleens had decreased in size from a peak at week 2 of 200×10^6 to 60×10^6 (Fig. 3*C*). The continued decrease in spleen cell number between weeks 3 and 4 was due to a decrease in B6 cells (Fig. 5*B*). B6-into-CB6F_1 GVHD continued to diverge from acute GVHD as disease progressed. The spleens reached a maximum size of 250×10^6 cells by 6 wk postdisease induction. During this 2-wk period, massive expansion of CB6F_1 cells occurred. Both F_1 $CD4^+$ and $CD19^+$ cells expanded to greater than normal levels, whereas the F_1 $CD8^+$ T cells returned to normal levels (Fig. 4*C*). Concurrently, parental B6 cells underwent a small transitory increase in number (Fig. 5*B*). By 12 wk postdisease induction, the spleens in B6-into-CB6F_1 GVHD mice exhibited a cellular consistency that was similar to that of chronic GVHD mice.

Limiting dilution CTL assays

It was important to compare the CTL precursor frequencies of B6 anti-B6D2F_1 and B6 anti-CB6F_1 because the B6-into-CB6F_1 GVHD resulted in the unexpected sequential acute to chronic pattern of GVHD (on the basis of host spleen repopulation and survival patterns), and acute vs chronic GVHD models were associated with differences in parent anti-F_1 CTL precursor frequencies

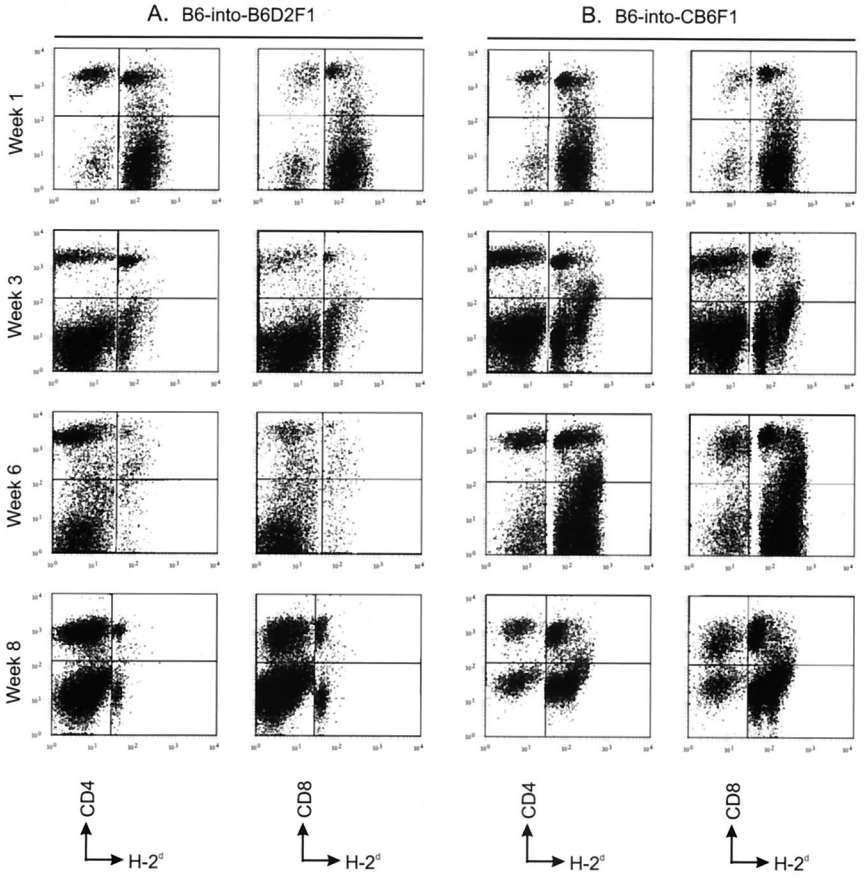

FIGURE 2. Detection of $CD4^+$ and $CD8^+$ cells from mice with B6-into-$B6D2F_1$ GVHD and B6-into-$CB6F_1$ GVHD. Splenocytes from mice with GVHD were stained with mAb to $H\text{-}2^b$ and $H\text{-}2^d$ to determine whether the cells were of host or donor origin. Abs recognizing either CD4 or CD8 were used simultaneously to identify the T cell subsets. *A*, Staining of CD4 and CD8 T cell subsets from B6-into-$B6D2F_1$ mice at weeks 1, 3, 6, and 12. *B*, Staining of CD4 and CD8 T cell subsets from B6-into-$CB6F_1$ mice at weeks 1, 3, 6, and 12. Similar results were seen in two other separate experiments.

(2). Therefore, we repeated and verified the earlier limiting dilution experiments indicating that the acute B6-into-$B6D2F_1$ GVHD vs the chronic DBA/2-into-$B6D2F_1$ GVHD could be accounted for by differences in parent anti-F_1 CTL precursor frequencies. Our frequencies were 1/2,295 for B6 anti-$B6D2F_1$ (compared with the earlier 1/1,450), and 1/19,510 for DBA/2 anti-$B6D2F_1$ (compared with 1/13,500) (2) (Fig. 6). We also simultaneously compared the above parental anti-F_1 CTL precursor frequencies in the B6 anti-$CB6F_1$ (1/2,370) and BALB/c anti-$CB6F_1$ (1/19,230) CTL precursor frequencies (Fig. 6). Despite the differences noted above in the GVHD profiles of B6-into-$B6D2F_1$ and B6-into-$CB6F_1$ (Figs. 1–5), the B6 anti-F_1 CTL precursor frequencies were indistinguishable. Furthermore, the CTL precursor frequencies for DBA/2 anti-$B6D2F_1$ and BALB/c anti-$CB6F_1$ were indistinguishable from each other, although they were 9-fold lower than the B6 anti-F_1 frequencies.

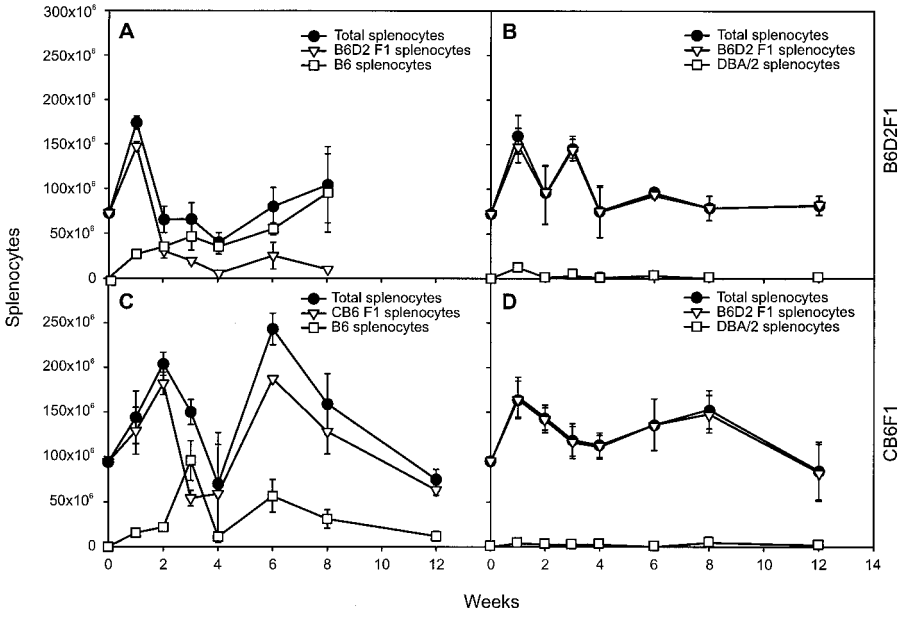

FIGURE 3. Numbers of parental, F_1, and total cells in the spleens of F_1 mice as a function of time after parental cell injection. *A*, B6-into-$B6D2F_1$ (acute) GVHD; *B*, DBA/2-into-$B6D2F_1$ (chronic) GVHD; *C*, B6-into-$CB6F_1$ (sequential acute to chronic) GVHD; *D*, BALB/c-into-$CB6F_1$ (chronic) GVHD. Results shown are averages (and SEM) of splenocytes from three different mice. Similar results were seen in two other separate experiments.

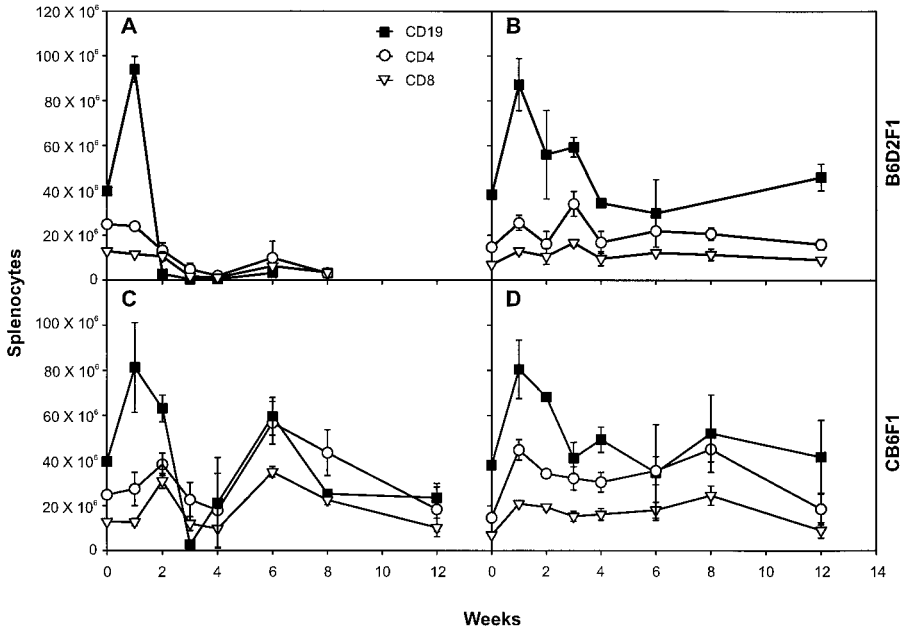

FIGURE 4. Analysis of F_1 cell populations during GVHD. F_1 $CD4^+$, $CD8^+$, and $CD19^+$ cell subsets in the spleens of GVHD mice are plotted as a function of time after parental cell injection. *A*, B6-into-$B6D2F_1$ (acute) GVHD; *B*, DBA/2-into-$B6D2F_1$ (chronic) GVHD; *C*, B6-into-$CB6F_1$ (sequential acute to chronic) GVHD; *D*, BALB/c-into-$CB6F_1$ (chronic) GVHD. Results shown are averages (and SEM) of splenocytes from three different mice. Similar results were seen in two other separate experiments.

Anti-H-2 CTL activity in GVHD mice

Acute GVHD is characterized by the expansion of parental $CD8^+$ T cells and the ability to isolate anti-F_1 and anti-$H\text{-}2^d$ cytotoxicity from the spleens of affected mice (2, 3, 5, 23). To further characterize the B6-into-$CB6F_1$ GVHD, spleens were removed at 2-wk intervals and tested for anti-$H\text{-}2^d$ CTL activity. At 2 and 4 wk post-GVHD induction, similar anti-$H\text{-}2^d$ CTL activity was detected in spleens from both acute and B6-into-$CB6F_1$ GVHD mice (Fig. 7, *A* and *B*). This CTL activity could be detected at all time points tested in mice with acute GVHD (Fig. 7, *C* and *D*). Anti-$H\text{-}2^d$ CTL activity could be detected in B6-into-$CB6F_1$ GVHD during the first two, but not at the later two, time points (Fig. 7, *C* and *D*). $B6D2F_1$ and $CB6F_1$ mice with chronic GVHD were also tested at all four time points for the presence of anti-$H\text{-}2^b$ CTL activity. At no time during the 8 wk was anti-$H\text{-}2^b$ activity detected (data not shown).

Autoimmune characteristics in GVHD mice

Chronic GVHD is associated with the production of Abs recognizing ssDNA (11, 12), dsDNA (11–15), and ENA (11, 15, 16). GVHD mice were bled at 16 wk post-GVHD induction, and the serum was tested for autoantibody production. $B6D2F_1$ mice injected with DBA/2 cells elicited Abs to all Ags tested (Fig. 8, *A–C*). The BALB/c-into-$CB6F_1$ chronic GVHD mice and the B6-into-$CB6F_1$ GVHD mice resulted in Abs being produced to both ssDNA (Fig. 8*A*) and dsDNA (Fig. 8*B*) at higher titers than seen with DBA/2-into-$B6D2F_1$. At 16 wk post-GVHD induction, B6-into-$CB6F_1$ and both forms of chronic GVHD produced Abs recognizing ENA (Fig. 8*C*). These Abs were not detected in syngeneic injected F_1 controls.

At 24 wk post-GVHD induction, the mice were euthanized and the kidneys tested for the presence of Ig deposits. Control animals

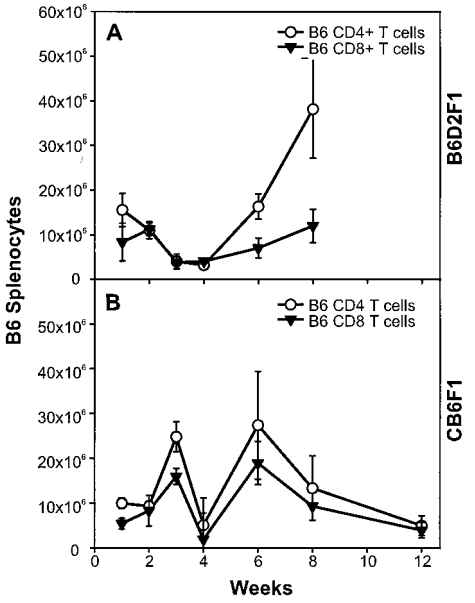

FIGURE 5. Analysis of donor B6 T cell populations during GVHD. $CD4^+$ and $CD8^+$ cell subsets in the spleens of GVHD mice are plotted as a function of time after parental cell injection. *A*, B6-into-$B6D2F_1$ (acute) GVHD; *B*, B6-into-$CB6F_1$ mice (sequential acute to chronic) GVHD. Results shown are averages (and SEM) of splenocytes from three different mice. Similar results were seen in two other separate experiments.

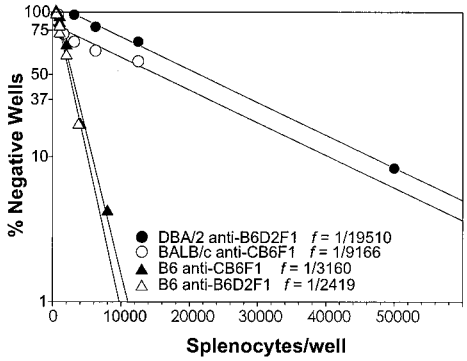

FIGURE 6. Limiting dilution CTL assays. Limiting dilution analysis for B6 anti-$B6D2F_1$, B6 anti-$CB6F_1$, DBA/2 anti-$B6D2F_1$, and BALB/c anti-$CB6F_1$ CTL responses. Similar results were seen in two other separate experiments.

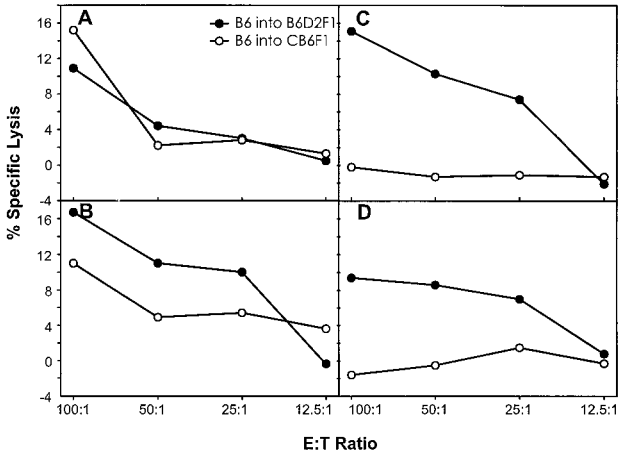

FIGURE 7. Anti-H-2^d CTL activity in the spleens of GVHD mice. $B6D2F_1$ and $CB6F_1$ mice injected with the B6 parental cells were tested for anti-H-2^d CTL activity at 2 (*A*), 4 (*B*), 6 (*C*), and 8 (*D*) wk after injection. The GVHD spleens were stimulated in vitro with irradiated F_1 spleen cells (B6-into-$B6D2F_1$ GVHD spleens stimulated with $B6D2F_1$ cells and B6-into-$CB6F_1$ GVHD spleens stimulated with $CB6F_1$ cells) and assayed on P815 (H-2^d) and EL-4 (H-2^b) target cells 5 days later. Data are shown for lysis of P815 targets. Lysis of EL-4 targets was <3.6% (data not shown) in all assays. Similar results were seen in one other independent experiment.

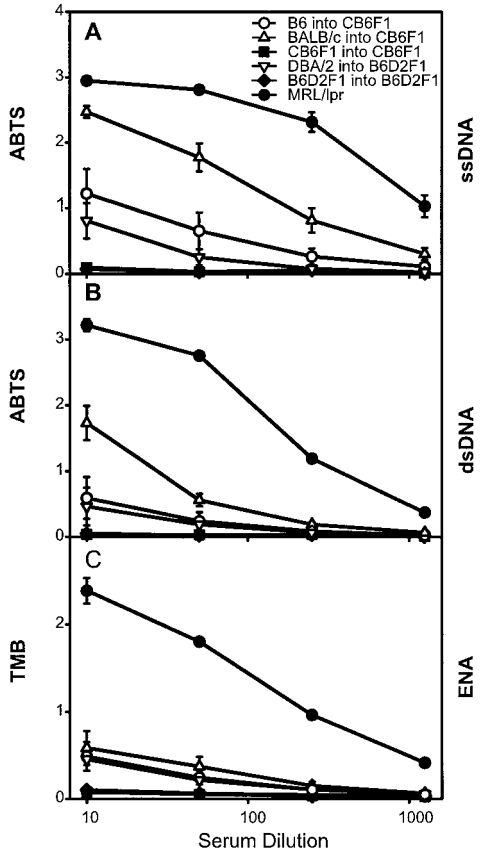

FIGURE 8. Autoantibody production in GVHD mice. Mice were screened at 16 wk following parental cell injection for production of Abs recognizing ssDNA (*A–C*), dsDNA (*D–F*), and ENA (*G–I*) in DBA/2-into-$B6D2F_1$ mice (*A*, *D*, and *G*), BALB/c-into-$CB6F_1$ mice (*B*, *E*, and *H*), and B6-into-$CB6F_1$ mice (*C*, *F*, and *I*). Results were confirmed at other time points in this experiment, and similar results were obtained in four additional independent experiments.

injected with syngeneic F_1 cells had no detectable Ig deposits in the kidneys ($B6D2F_1$, Fig. 9*A*; $CB6F_1$, Fig. 9*C*). Both models of chronic GVHD, DBA/2-into-$B6D2F_1$ (Fig. 9*B*) and BALB/c-into-$CB6F_1$ (Fig. 9*D*), had significant Ig deposits. The B6-into-$CB6F_1$ GVHD also had significant numbers of Ig deposits within the kidneys (Fig. 9*E*). Eight mice in each group were analyzed for Ig deposits, and numerical values were assigned to each score to compare staining between groups (Table I). These results indicated that both models of chronic GVHD, DBA/2-into-$B6D2F_1$ and BALB/c-into-$CB6F_1$, resulted in very similar kidney-staining patterns and intensity. B6-into-$CB6F_1$ mice also had Ig deposits in the kidney, but the mean score of the group was lower than that seen in either of the chronic models of GVHD. Because the B6-into-$CB6F_1$ mice undergo a period of acute disease before progressing on to chronic disease, differences seen in kidney-staining patterns might be due to a delay in this group in developing renal pathology. Although the initiating events of chronic and B6-into-$CB6F_1$ GVHD are very different, the end result of these two pathways is an autoimmune disease similar to lupus.

Role of Th in determining disease outcome

Because CTL precursor frequencies alone did not account for the differences between acute and B6-into-$CB6F_1$ GVHD, another mechanism must exist. Parental Th cells play a role in driving the F_1 B cell to hyperactivity in chronic GVHD and are presumed to play a supportive role for the CTL in acute GVHD (24). Therefore, it is also possible that differences in the parent anti-F_1 Th cell frequency contribute to determining the final disease outcome. Limiting dilution proliferation assays were performed to test for differences in the B6 response against mitomycin C-treated $B6D2F_1$ and $CB6F_1$. The Th cell limiting dilution curves for B6 anti-$CB6F_1$ and B6 anti-$B6D2F_1$ were identical and had a frequency of 1/79,120 (data not shown). It is possible that these proliferative Th cell frequencies are not different or that the stimulating alloantigens were limiting on F_1 stimulator cells, resulting in the Th frequencies appearing to be indistinguishable. Therefore, these limiting dilution proliferation assays were repeated using mitomycin C-treated homozygous BALB/c and DBA/2 stimulator cells. We observed a >3-fold difference in Th precursor frequency using allogeneic homozygous H-2^d stimulator cells (Fig. 10). B6 anti-DBA/2 exhibited a Th precursor frequency of 1/19,420 spleen cells, whereas B6 anti-BALB/c had a Th precursor frequency of 1/65,900.

Discussion

Previous work in the parent-into-GVHD model of GVHD has characterized acute disease as the loss of F_1 lymphocytes (1, 2), repopulation of the F_1 myeloid and lymphoid cells with cells of donor origin (1, 2), and the presence of anti-F_1 CTL activity in the spleens of affected mice (2, 3, 5, 6). This study demonstrates a model of parent-into-F_1 GVHD that sequentially exhibits characteristics of acute then chronic GVHD. Over the first few weeks of disease, the $CB6F_1$ mice injected with B6 cells exhibit a disease similar to that seen in $B6D2F_1$ mice injected with B6 cells. There are high levels of donor cell engraftment (Figs. 1–3, 5), loss of F_1 lymphocytes (Figs. 2–4), and detectable parent anti-F_1 CTL activity in the spleens of affected mice (Fig. 7). As the disease continues, distinct differences become apparent between B6-into-$CB6F_1$ and B6-into-$B6D2F_1$ mice. In B6-into-$B6D2F_1$ mice, the CTL activity continues to be detectable through week 8 (Fig. 6), and the level of donor cell engraftment remains high (Figs. 1–3, 5), correlating with data published using other acute parent-into-F_1

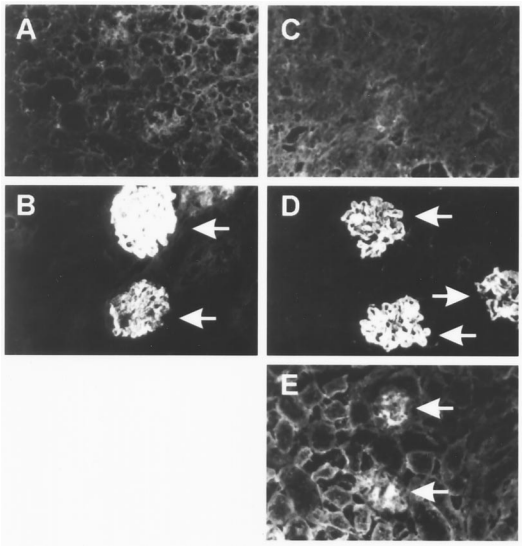

FIGURE 9. Fluorescent Ab detection of murine Ig deposits in kidneys. Chronic and sequential acute-to-chronic GVHD mice were tested for the presence of Ig deposits within the kidneys as a parameter of disease. A representative mouse from each group is shown. Control $B6D2F_1$ injected with syngeneic splenocytes (*A*), DBA/2-into-$B6D2F_1$ mouse (*B*), control $CB6F_1$ injected with syngeneic splenocytes (*C*), BALB/c-into-$CB6F_1$ (*D*), and B6-into-$CB6F_1$ (*E*). Representative results are shown from a single mouse from each group ($n \geq 6$ per group).

combinations (1). In B6-into-$CB6F_1$ mice, the F_1 cells begin to proliferate between weeks 4 and 6 (Figs. 2 and 3), resulting in a drastic decrease in the percentage of parental cells in the spleen (Fig. 1), although the absolute number of B6 cells increases during this same time frame. The number of $CB6F_1$ cells increases from 2.9×10^7 to 1.9×10^8 cells/spleen between weeks 4 and 6, whereas the number of parental cells increases from 1.1×10^7 to 5.7×10^7 cells/spleen. This results in the percentage of parental cells dropping from 44.8 to 22.4%. The percentage of parental cells continues to decline until week 12, when ~12% of the cells are of parental origin.

At later time points, B6-into-$CB6F_1$ GVHD exhibits characteristics of chronic GVHD, a lupus-like illness. This condition manifests itself in the production of autoantibodies recognizing ssDNA, dsDNA, and ENA. But more importantly, the disease continues on to a glomerulonephritis with Ig deposits being readily detectable in kidneys of both models of chronic GVHD and the sequential acute to chronic GVHD, B6-into-$CB6F_1$ (Fig. 9).

Previous reports have involved models of GVHD that can be manipulated to manifest either signs of acute or chronic GVHD. In a bone marrow transplant model of GVHD, lethally irradiated B6 recipients can develop symptoms of acute or chronic GVHD depending on the number of LP/J cells used to reconstitute the host B6 mouse (25). A second model is the injection of C3H splenocytes into $(B6 \times C3H)F_1$ mice. Cell doses of $>2 \times 10^7$ but $<1 \times 10^8$ cells result in chronic GVHD and the associated production of autoantibodies, whereas injection of $\geq 1 \times 10^8$ cells results in acute GVHD (26). The B6-into-$CB6F_1$ GVHD differs from these models of GVHD because with the same cell dose B6-into-$CB6F_1$ mice exhibit acute and chronic GVHD sequentially. In further support of a model of GVHD that induces symptoms of both acute and chronic GVHD, a mixture of pathogenic and nonpathogenic T cell clones isolated from cyclosporine-induced GVHD in rats when injected into the footpad of naive rats can cause a localized reaction that exhibits chronological pathological changes of acute and chronic GVHD (27).

It should be noted that BALB/c-into-$CB6F_1$ induced the expected chronic GVHD pattern that was indistinguishable from DBA/2-into-$B6D2F_1$. Both of these chronic GVHD models, as well as B6-into-$CB6F_1$ mice, resulted in production of serum Abs to ssDNA, dsDNA, and to ENA. Furthermore, renal Ig deposits were detected in all three of these GVHD combinations. Thus, a lupus-like autoimmune condition was observed in B6-into-$CB6F_1$ GVHD, similar to the two H-2^d-into-H$2^{d/b}$ F_1 models. We cannot exclude the possibility that the acute B6-into-$B6D2F_1$ GVHD would also have resulted in a similar long-term autoimmune disease pattern had they survived their acute disease.

This study demonstrates that similarities in H-2 and in donor anti-host CTL precursor frequencies do not necessarily predict whether parent-into-F_1 GVHD will result in chronic or acute disease. B6-into-$B6D2F_1$ and DBA/2-into-$B6D2F_1$ models that elicit acute (1–8) and chronic GVHD (1, 4, 9–16), respectively, exhibit a 9-fold difference in donor anti-host CTL precursor frequencies. The CTL comparisons also included DBA/2 and BALB/c anti-H-2^b responses on the B6 background. We observed a 9-fold higher frequency in the B6 anti-H-$2^{d/b}$ CTL response, irrespective of whether the H-2^d allele was provided by the DBA/2 (1/2,295) or BALB/c (1/2,370) than in the DBA/2 and BALB/c anti-H-$2^{d/b}$ frequency (1/19,510 and 1/19,230, respectively) (see Fig. 6). This 9-fold difference in CTL precursor frequency was similar to the 9-fold difference reported earlier for B6-anti-$B6D2F_1$ vs DBA/2 anti-$B6D2F_1$ (2). These results indicate that the difference in B6-into-$B6D2F_1$ acute GVHD and B6-into-$CB6F_1$ GVHD cannot be accounted for by parent anti-F_1, H-2 allogeneic CTL precursor frequencies. Nevertheless, the differences between acute GVHD and the two examples of parent-into-F_1 chronic GVHD (anti-H-2^b)

Table I. *Ig deposition in the kidneys of GVHD mice*

Group	Ig Deposits[a]
Classic $B6D2F_1$ GVHD	
$B6D2F_1$ injected with $B6D2F_1$[b]	0 ± 0
$B6D2F_1$ injected with DBA/2 (chronic)[c]	2.3 ± 0.8
$CB6F_1$ GVHD	
$CB6F_1$ injected with $CB6F_1$[b]	0 ± 0
$CB6F_1$ injected with BALB/c (chronic)[b]	2.6 ± 0.5
$CB6F_1$ injected B6 (sequential acute to chronic)[b]	1.2 ± 1.3

[a] Immune complexes were evaluated according to density and strength of staining. Kidneys were given a score of 0–3 depending on the density and strength of staining. The data is reported as the mean ± SD of the scores for each group.
[b] Group had kidneys from eight mice tested for Ig deposits in a blinded fashion.
[c] Group had kidneys from six mice tested for Ig deposits in a blinded fashion.

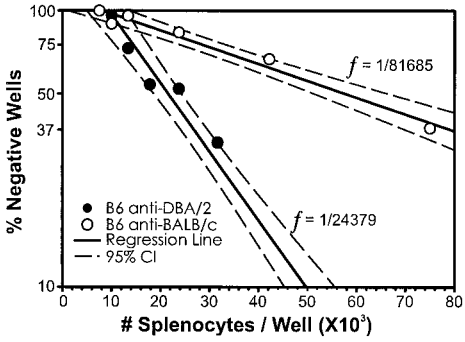

FIGURE 10. Limiting dilution Th assays. Limiting dilution proliferation assay analysis for B6 anti-DBA/2 and B6 anti-BALB/c Th responses. Results were confirmed by two additional independent experiments.

are consistent with the 9-fold difference in parent anti-F_1 CTL precursor frequencies.

When the precursor frequency experiments were extended to include parent anti-F_1 Th cell analysis, a >3-fold difference was observed between B6 anti-DBA/2 and B6 anti-BALB/c (see Fig. 10). It is possible that the 3-fold higher Th precursor frequency of B6 anti-DBA/2 provided an initial advantage that permitted the acute disease to develop and be maintained long enough to result in morbidity by 30 days. Multiple minor histocompatibility differences exist between DBA/2 and BALB/c, including differences in the expression of the Mls Ag.

Mls Ag are superantigens encoded by endogenous murine retroviruses that stimulate a high proportion of T cells bearing a specific TCR Vβ family. DBA/2 cells (and F_1s on this background) bear Mls 1^a, a strong Mls Ag that stimulates the TCR Vβ 6 and 8.1 and is not present on BALB/c cells (and F_1s on this background). The use of Mls 1^a-bearing cells as stimulator cells in vitro results in the production of a Vβ 6^+, $CD4^+$, Th1 cell responder population (28). This was confirmed in vivo; furthermore, the development of a maximal Th1 response was dependent on the presence of endogenously produced IFN-γ (28). Also, $CD8^+$ T cells bearing these same TCR Vβ families can respond to Mls 1^a by producing IFN-γ (29). It is possible that the strong response of B6 anti-Mls 1^a of the DBA/2 is sufficient to initiate and sustain the strong cytokine response necessary for driving CTL, whereas the weaker Mls Ag expressed by the BALB/c (Mls 1^b) is adequate to initiate but not sustain the CTL-effected acute disease. In further support of the possible role of Mls Ag during acute GVHD, studies have shown that Mls 1^a-reactive donor Vβ 6 and 8.1 are expanded during acute GVHD (30). Studies are in progress to determine the possible role of Mls for the acute vs sequential acute-to-chronic in vivo models of GVHD.

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