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Antibody to the Ligand of CD40, gp39, Blocks the Occurrence of the Acute and Chronic Forms of Graft-vs-Host Disease

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Abstract

Chronic and acute graft-versus-host disease (cGVHD and aGVHD) result from donor cells responding to host disparate MHC alleles. In cGVHD (H-2^d → H-2^{bd}), heightened polyclonal immunoglobulin production is due to the interaction of donor allospecific helper T cells (T_h) and the host B cells. In vivo administration of antibody to the ligand for CD40, gp39, blocked cGVHD-induced serum anti-DNA autoantibodies, IgE production, spontaneous immunoglobulin production in vitro, and associated splenomegaly. Antibody production remained inhibited for extended periods of time after termination of anti-gp39 administration. Antiallogeneic CTL responses induced in aGVHD were also prevented by the in vivo administration of anti-gp39 as was the associated splenomegaly. These data suggest that CD40–gp39 interactions are critical in GVHD and that CD40-gp39 may be a valuable ligand-receptor pair for targeting immunotherapeutic agents to control GVHD. (*J. Clin. Invest.* 1994. 94:1333–1338.) Key words: CD40 • CD40 ligand • graft-versus-host disease • T cells • B cells

Introduction

The chronic and acute forms of graft-versus-host disease (GVHD)[1] (1) are characteristically associated with increased polyclonal and autoimmune immunoglobulin (Ig) production and increased allospecific cytotoxic T cell (CTL) activity, respectively. In humans, GVHD occurs after bone marrow transplantation, and can be found in acute and chronic forms. Acute GVHD (aGVHD) takes place within about 60 d post-transplantation and results in damage to the skin, liver, and gut by the action of cytolytic lymphocytes. Chronic GVHD (cGVHD) occurs later and is a systemic autoimmune disease that affects primarily the skin, resulting in the polyclonal activation of B cells and the hyperproduction of Ig and autoantibodies (1).

GVHD is studied in experimental systems by the transfer of parental lymphocytes into an F_1 host (2, 3), resulting in the donor cells responding to the host disparate MHC antigens (4–6). In cGVHD, it has been shown that the heightened polyclonal Ig production is due to polyclonal B cell activation resulting from cognate interactions between donor, allospecific helper T cells (T_h), and the host B cells (1, 4, 7). It has previously been shown that the host B cells are responsible for the autoantibody production due to excessive help provided by donor-derived T cells (4, 5). Recently it has been shown that normal host B cells, not the previously thought B1/CD5$^+$ subset of B cells, are responsible for this autoimmune phenomenon (8, 9). The CD40 ligand, gp39, was recently cloned (10, 11) and found to be a type II membrane protein expressed primarily on activated CD4$^+$ T cells (10–13). It is known to be essential in primary and secondary antigen-specific, syngeneic T–B cognate interactions in vivo (14), and has been shown to be an essential molecule in the development of collagen-induced arthritis (15).

There is ample functional and genetic evidence that gp39 is essential for thymus-dependent (TD) immune responses (14, 16–19). gp39 is expressed transiently following the activation of T_h (20). During the course of TD immune responses in vivo, gp39 is transiently expressed at high levels in defined anatomical sites in the immune spleen on T_h cells that are juxtaposed to the responding B cells (21). These observations support the hypothesis that gp39-expressing T_h delineate sites of cognate T_h–B cell interactions. Genetic proof that gp39–CD40 interactions are essential in the development of TD humoral immunity is provided by the fact that defective expression of the gp39 molecule is responsible for the development of X-linked hyper-IgM syndrome (16–19). These patients have impaired TD immunity with low levels of IgG, IgA, and IgE, even though their B cell responses to polyclonal activators in vitro are normal. Taken together, these data conclusively demonstrate that gp39–CD40 interactions are essential for TD B cell activation.

The role of CD40–gp39 interactions in the development of

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1. *Abbreviations used in this paper:* aGVHD, acute graft-versus-host disease; APC, antigen presenting cell; cGVHD, chronic graft-versus-host disease; CTL, cytotoxic T cell; GVHD, graft-versus-host disease; HIg, hamster immunoglobin; TD, thymus-dependent; T_h, helper T cell.

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GVHD has not been studied. The data presented in this study provide compelling evidence that gp39–CD40 interactions are critical in the development of both cGVHD and aGVHD. Administration of anti-gp39 prevented the onset of cGVHD as judged by the reduction of hyperimmunoglobulin production, splenomegaly, and anti-DNA antibodies, as well as the CTL responses associated with aGVHD. Moreover, these studies provide the first in vivo evidence that anti-gp39 can impose profound negative effects on cell-mediated immune responses to alloantigen.

Methods

Mice

DBA/2 (H-2^d), C57BL/6 (H-2^b) and B6D2F_1 H-$2^{b/d}$ [(C57BL/6 × DBA/2) F_1 hybrid] mice were obtained from the National Cancer Institute laboratories (Bethesda, MD) and maintained in a viral-free environment in the animal facility at Dartmouth Medical School. All the mice used in this study were female, and aged 6 to 8 wks old.

Induction of chronic GVHD and acute GVHD

cGVHD was induced by the intravenous injection of parental (DBA/2) spleen cells into nonirradiated (C57BL/6 × DBA/2) F_1 hybrid recipients (22). Parental mice were anesthetized and killed by cervical dislocation prior to removal of the spleen. Dissociated spleen cells were washed and resuspended in RPMI 1640 medium (Whittaker Bioproducts, Walkersville, MD) for intravenous injection (one spleen per recipient) into the F_1 recipients. aGVHD was induced by the intravenous injection of parental C57BL/6 spleen cells into nonirradiated (C57BL/6 × DBA/2) F_1 hybrid recipients. Cells were prepared for transfer as for the induction of cGVHD.

Antibodies

Anti-gp39. MR1 was produced in ascites and purified by ion exchange HPLC, as previously described (14, 23).

Polyclonal anti-isotype antibodies. All anti–IgG_1 and anti–IgA antibodies and standard controls were obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Anti–IgE antibodies. All anti–IgE antibodies (BIE3 and Biotin EM95) and IgE standards (A3B1) were a kind gift from Dr. T. Waldschmidt, University of Iowa, (Iowa City, IA) and Daniel Conrad, Medical College of Virginia (Richmond, VA).

Anti–MHC haplotype antibodies. Anti–H-2K^b (AF6-88.5) and anti–H-2D^d (34-2-12) were obtained from PharMingen (San Diego, CA).

Cell lines

All tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cells included P815 (H-2^d, mouse mastocytoma) c118.5 (H-2^b, subclone of AKR H-2^b-SL1; T cell lymphoma), and LB27.4 (H-2^{bxd}, Hybridoma; A20.2 fused to B10.BR spleen cells).

Polyclonal Ig production in vitro

Spleens from control and cGVHD mice were removed and single-cell suspensions prepared. Cells were treated with Tris-buffered ammonium chloride to remove erythrocytes and total white blood cell counts determined by visual hemocytometer counting. Cells were incubated (5×10^6) in 1 ml of complete (c)RPMI-1640 medium (Whittaker) [supplemented with 10% FCS (Hyclone, Logan, UT), 25 mM Hepes, 2 mM L-glutamine, 5,000 U/ml penicillin, and 5,000 U/ml streptomycin (all from Sigma Diagnostics, St Louis, MO)] for 3 d at 37°C, 5% CO_2. Culture supernatants were collected by centrifugation to remove any cells present. Ig was quantified by an isotype-specific ELISA assay.

Isotype-specific and antigen-specific ELISAs

ELISA for the detection of IgG_1 and IgA. 10 μg/ml goat anti–mouse IgG_1 or IgA in PBS was absorbed onto wells of a 96-well polyvinyl microtiter plate for 1 h at 37°C, then overnight at 4°C. Plates were washed and blocked with PBS containing 1% FCS for 1 h at 37°C. Plates were washed again and the appropriate dilutions of supernatants and standard controls, IgG_1 and IgA were added for 2 h at 37°C. After this time, the plates were washed three times and alkaline phosphatase–conjugated goat anti–mouse IgG_1 or IgA (1/500 dilutions) was added for 2 h at 37°C. Plates were thoroughly washed and 1 μg/ml phosphatase substrate (Sigma Diagnostics) was added, resulting in the appropriate color change. Readings were determined by an ELISA reader (Dynatech Laboratories, Inc., Chantilly, VA) at an absorbence of 410 nm. Concentrations of Ig were determined by comparison to the appropriate isotype standard curve and expressed as the mean±SEM ($n = 3$).

ELISA for the detection of IgE. Wells of a 96-well polyvinyl microtiter plate were coated with an anti–mouse IgE capture antibody (B1E3 at 2 μg/ml) overnight at 4°C and then blocked with PBS containing 1% FCS for 1 h at 37°C. Plates were washed again and the appropriate dilutions of supernatants and standard controls (A3B1 [IgE]) were added for 2 h at 37°C. Plates were washed thoroughly and the EM95-Biotin (5 μg/ml) was added to each well and incubated for 2 h at 37°C. After this time alkaline phosphatase conjugated to streptavadin was added (1/500 dilution) for a further 2 h. Plates then were developed, as mentioned previously. Concentrations of Ig were determined by comparison to standard curve and expressed as the mean±SEM ($n = 3$).

ELISA for the detection of anti-DNA antibodies. Calf thymus DNA (Sigma Diagnostics) (5 μg/ml) was dissolved in coupling buffer containing 0.1 M sodium carbonate/sodium bicarbonate (pH 9.8). This solution was boiled for 10 min and then incubated on ice for 3 min. The OD_{260} of the DNA then was determined and the concentration was adjusted to obtain the required 5 μg/ml DNA. 100 μl then was added to the wells of a 96-well polyvinyl microtiter plate and incubated overnight at 4°C. The plate then was washed three times and blocked for 1 h at 37°C with PBS containing 1% FCS and 0.02% sodium azide. The plate was washed again and serial dilutions of serum samples were added (100 μl, 1/500 final dilution) and incubated for 2 h at 37°C. The detection antibody, goat anti–mouse IgG_1 alkaline phosphatase, then was added to each well and incubated once again for 2 h at 37°C. Plates were developed as mentioned previously. Antibody titers were compared to a positive serum sample and results were expressed in arbitrary units.

Flow cytometric analysis for the detection of donor derived cells

Spleens were removed from normal BDF_1 and from cGVHD mice treated with and without anti-gp39, and a single-cell suspension was prepared. The cells were layered onto Ficoll-Hypaque (4:1 vol/vol) and then centrifuged at 2,000 rpm for 20 min at room temperature. The resulting lymphocyte layer was removed and washed once with buffered saline solution containing 5% FCS. 10^6 cells per tube were used for staining in a 50 μl final volume. 50 μl of rat serum was added to each tube to prevent nonspecific binding of antibodies. Cells were stained with (*a*) the control antibodies: Rat Ig FITC (10 μg/ml) and PE-Streptavidin (2 μg/ml) and (*b*) FITC H-2K^b (10 μg/ml) and Biotin H-2D^d (10 μg/ml). Cells were incubated for 20 min on ice and then washed twice to remove any unbound antibodies. Finally PE-Streptavidin (2 μg/ml) was added to the appropriate tube to detect the biotin-conjugated antibody for another 20 min on ice. Cells were again washed twice ready for analysis on a FACScan (Becton Dickinson Microbiology Systems, Cockeysville, MD). After positive gating via forward and side scatter, 10,000 events were collected per sample for determination of percent cells positive for the relevant MHC haplotype.

Assessment of CTL activity

Target tumor cell lines were labeled with the isotope by incubating 100 μl of FCS containing $1–5 \times 10^6$ cells for 1 h at 37°C with 0.2 mCi of ^{51}Cr (New England Biolabs, Beverly, MA). Cells then were washed thoroughly and resuspended at 1×10^5 cells/ml in cRPMI. Labeled targets were plated at 1×10^4 per well in 96 well plates with effector cells in E/T ratios of 100:1, 20:1, and 4:1. Target cells used included P815 (H-2^d), LB27.4 (H-2^{bxd}) and c118.5 (H-2^b). The plates were

Table I. Anti-gp39 Treatment Inhibits the Induction of cGVHD-associated Splenonegaly and Spontaneous Antibody Production In Vitro

Group:	Splenomegaly* Fold increase in spleen cell count of the control	Spontaneous in vitro ab production‡ IgA	IgG_1
		ng/ml	*ng/ml*
Control	1	162±54	1±0.7
cGVHD	1.9±0.5	350±60	90±10
cGVHD + αgp39	0.8±0.2	70±3	10±2

Chronic GVHD was induced by the intravenous injection of DBA/2 (H-2^d) spleen cells into nonirradiated (C57BL/6 × DBA/2) F_1 hybrid recipients. *At 14 d after cell transfer, spleens of host mice were removed and cell counts were performed to determine the number of cells per spleen. Those mice receiving anti-gp39 received 3 × 250 μg on days 0, 2, 4, and 6. Results are expressed as fold increase compared to the control mice with means SEM±(n = 3). These results are representative of three different experiments. ‡Spleens from mice with or without cGVHD were cultured for 3 d in vitro and the levels of spontaneous IgA and IgG_1 were determined by isotype-specific ELISAs. Results are expressed as the mean concentration (ng/ml)±SEM (n = 3). These results are representative of three different experiments.

briefly centrifuged and then incubated at 37°C in 5% CO_2 for 4 h. The plates were centrifuged once more, and an aliquot of cell-free supernatant was collected from each well for counting by a gamma counter (model LKB 1272; Clinigamma, Wallac Inc., Gaithersburg, MD). Percent-specific lysis is defined as $(a - b)/c$ where a equals cpm released by target cells incubated with effector cells, b equals cpm released by target cells incubated with media only (spontaneous release), and c equals freeze-thaw releasable cpm from target cells (~ 80% of total cpm incorporated).

Results

Anti-gp39 inhibits GVHD-induced splenomegaly and hyper Ig production. In cGVHD, it is primarily the host's own cells that infiltrate and enlarge the spleen, although this is in response to the presence of donor cells (5). In the mouse, one of the consequences of this condition is the enlargement of the spleen. Table I indicates that at 14 d after the initiation of cGVHD, spleens contain almost twice the number of leukocytes compared to mice without cGVHD. Treatment of mice at the onset of cGVHD with anti-gp39 (250 μg/mouse, days 0, 2, 4, and 6) reduced the number of leukocytes per spleen in cGVHD mice to levels that were indistinguishable from mice without disease. It has been reported that hyperproduction of Ig occurs in mice with cGVHD due to cognate interactions between donor T cells and host B cells (4, 8, 24). To determine whether anti-gp39 inhibits hyper-Ig production 14 d after the initiation of disease, spleens were removed from control and cGVHD mice and the B cells assayed for the spontaneous production of IgG_1 and IgA in vitro. Splenocytes from mice with cGVHD produced high levels of IgA and IgG_1 in vitro (Table I). However, splenocytes from mice treated with anti-gp39 (days 0, 2, 4, and 6) produced levels of IgG_1 and IgA similar to mice without disease. The addition of anti-gp39 to cultures of spleen cells from mice with cGVHD did not reduce the levels of in vitro Ig production, suggesting that anti-gp39 was exerting its effects in vivo (data not shown).

In all initial studies, purified hamster Ig (HIg) was used as a control for treatment with the hamster monoclonal anti-gp39 (data not shown). When HIg was used as a control for these experiments, it showed no inhibitory effect on the induction of cGVHD, but actually enhanced the cGVHD in terms of polyclonal Ig production and the resultant splenomegaly. A two- to fourfold increase in in vitro Ig production was detected in the level of Ig produced in cGVHD mice given HIg over that observed in cGVHD mice not given antibodies (data not shown). Consequently it was decided to designate the untreated (receiving no antibodies) F_1 recipient mice as the relevant control group so as not to bias the data due to the enhanced response obtained with HIg.

Anti-gp39 inhibits cGVHD-induced hyper-IgE and anti-DNA autoantibodies. The course of cGVHD can be monitored by the elevation in serum IgE and antibodies to double-stranded DNA (1). Levels of serum IgE were measured using an IgE-specific ELISA. cGVHD-induced mice were treated with anti-gp39 on days 0, 2, 4 and 6, and then no further antibody was administered. Mice were bled at regular intervals and the levels of serum IgE ascertained (Fig. 1 *A*). cGVHD induced a 10–15-fold increase in serum IgE levels. Administration of anti-gp39 for the first 6 d inhibited cGVHD-induced increases in serum IgE throughout the 8-wk time frame of the experiment. In addition to the inhibition of elevated serum IgE, administration of anti-gp39 also blocked the generation of serum anti-DNA autoantibodies. Increases of 5–10-fold in the levels of anti-DNA antibodies were found in cGVHD (Fig. 1 *B*).

Anti-gp39 does not alter the frequency of engrafted, donor-derived cells in cGVHD mice. Because the effects of anti-gp39 treatment were apparent for long periods of time (> 8 wks), it was important to assess whether anti-gp39 treatment altered the presence of donor cells in recipient mice during the initial stages of disease induction. Spleens from cGVHD-induced mice had ~ 5–7% single positive (H-2^d) donor-derived cells (Fig. 2) after 7 d of disease progression. The administration of anti-gp39 to cGVHD mice did not alter the frequency of donor-derived cells found in the host.

Effect of anti-gp39 treatment on the induction of aGVHD. aGVHD is associated with the induction of a profound antiallogeneic CTL response. While it is clear that gp39–CD40 interactions are critical for T_h–B cell interactions, it is not clear if the development of cell-mediated immunity may also be altered by anti-gp39 therapy. aGVHD was induced by the administration of C57BL/6 spleens to the F_1 recipients. As shown in Fig. 3, 12 d following the transfer of donor cells, a robust H-2^b anti–H-2^d CTL response was measured. Treatment of mice with anti-gp39, but not HIg, prevented the generation of H-2^b–derived CTL against H-2^d. Controls in this experiment included mice receiving parental cells with no antibody or HIg. The CTL responses of mice receiving no antibody and HIg were indistinguishable.

Discussion

The present study demonstrates that the in vivo administration of anti-gp39 blocks the following GVHD-associated phenomena: splenomegaly, in vitro polyclonal Ig production, elevated levels of serum IgE and anti-DNA antibodies, and the generation of anti-host cytotoxic T cells. The long term prevention

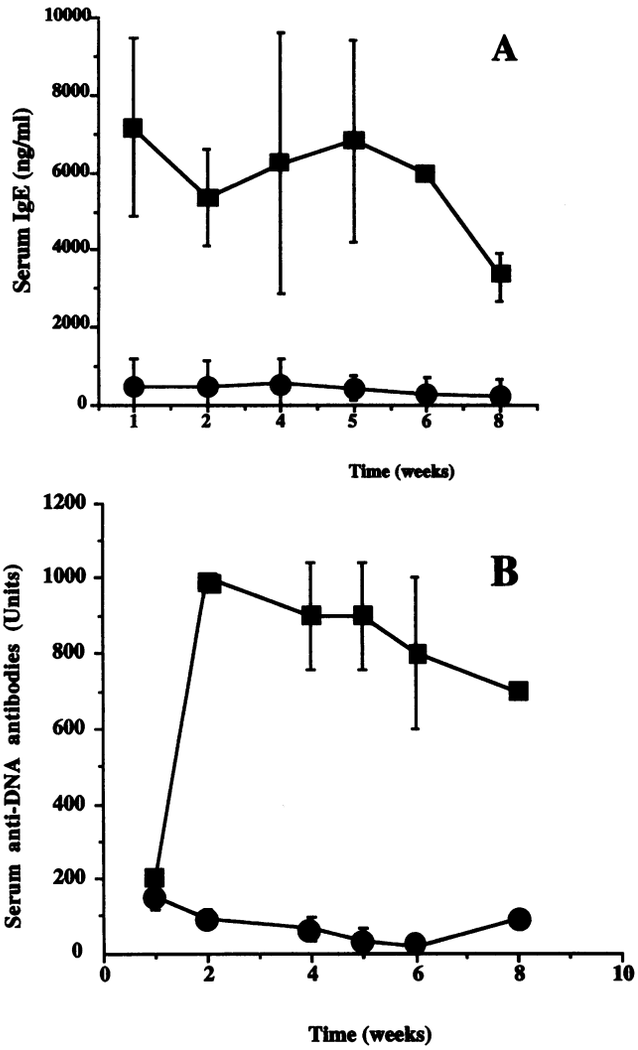

Figure 1. Anti-gp39 treatment inhibits the induction of serum IgE and anti-DNA autoantibody production associated with cGVHD. Levels of (*A*) IgE and (*B*) anti-DNA autoantibody from serum of animals induced with cGVHD either untreated (■) or treated with anti-gp39 (●) were determined by ELISA. All results are compared to IgE standards and expressed in ng/ml±SEM (n = 3) for IgE serum levels, and for anti-DNA antibodies compared to a positive serum, and thus expressed in arbitrary units±SEM (n = 3).

of GVHD was induced by a brief treatment with anti-gp39, suggesting that permanent alterations in host allospecific responses were imposed by blocking gp39–CD40 interactions.

Based on data reported in this paper and with the work of others (1, 4, 7), it appears that hyper Ig production, elevated levels of serum IgE, and titers of serum anti-DNA antibodies are induced through a cognate, gp39-CD40–dependent interaction. Published studies have established that host B cells are responsible for autoantibody production (4, 5, 8) in cGVHD. Therefore, expression of gp39 on the donor T cells is likely to be responsible for the heightened levels of Ig. These observations are in accord with other studies (1, 4, 7) indicating that cognate interactions, and not lymphokines, are responsible for the activation of host B cells by allogeneic T cells.

Detection of donor-derived cells in anti-gp39 treated and untreated mice with cGVHD indicates that the allogeneic cells are present but unable to respond. The existence of donor cells has been shown previously up to 10 wks after induction of cGVHD (25). The prolonged negative effect that anti-gp39 imposed on cGVHD was different from its observed effect on syngeneic, antigen-specific humoral immune responses (14). Administration (days 0, 2, and 4) of anti-gp39 inhibited antigen-specific, humoral immune responses for periods up to 3–4 wks, with a return to normal responsiveness at the end of that time interval. Based on a number of factors, the inhibition of antigen-specific immune responses in vivo by anti-gp39 indicated that anti-gp39 mediated its effects by the simple blockade of gp39–CD40 interactions and when the antibodies were cleared, immune responses returned (14). By contrast, in cGVHD, prevention of hyper-IgE and elevated titers of anti-DNA antibodies persisted for at least 8 wks after the termination of anti-gp39 therapy, and none of the mice were observed to relapse with disease (data not shown). These results indicate that allospecific T cell activities are affected either by clonal deletion or anergy of the alloreactive T cells. The induction of allospecific T cell unresponsiveness induced by anti-gp39 is also indicated in aGVHD. High levels of allospecific CTL activity were measured in mice with aGVHD. However, when anti-gp39 was administered at the onset of disease, no anti-host CTL activity was identified.

There are a number of possible mechanisms through which anti-gp39 may have blocked the development of both forms of GVHD. It is unlikely that anti-gp39 blocked T cell responsiveness by a direct cytotoxic effect on T cells because it has been shown that anti-gp39 (MR1 mAb) does not physically delete gp39-bearing T cells in vivo (21). One hypothesis is that the blockade of CD40 signaling by anti-gp39 prevented expression of costimulatory molecules that are required for alloantigen presentation. The most extensively described receptor system that is involved in such costimulation is CD28/CTLA-4 receptor, with its B7.1/B7.2 coreceptor (26–29) (reviewed in 30). Upregulation of B7.1 can be mediated through CD40 signaling as has been shown by Ranheim et al. (31) on normal and leukemic B cells. Therefore, it can be suggested that during cognate interactions between allogeneic donor T cells and host B cells, blockade of gp39 signaling prevented the upregulation of costimulatory molecules (i.e., B7.1/B7.2) on the host B cells. As a consequence, the alloreactive $CD4^+$ T_h cells were deprived of the required costimulatory signals and rendered unresponsive. The requirement for costimulatory molecules in the activation of alloreactive $CD4^+$ has been repeatedly demonstrated in studies employing CTLA-4-Ig and anti-B7 antibodies (28, 32). Since T_h cells and their products (lymphokines) have been shown to be important in the development of CD4-dependent CTL (33–35), induction of tolerance in the CD4 compartment would impair the development of CTL. Perhaps an even more critical factor is that the lack of enhanced costimulatory molecule expression on class I MHC-bearing antigen presenting cells (APC) directly prevented the activation of alloreactive CTL. Therefore, CTL development may have been prevented because of both a lack of T cell help and the inability of APC to effectively activate alloreactive $CD8^+$ T cells.

This hypothesis does not contend that B cells were the preeminent cell for the induction of antiallogeneic responses in GVHD, but suggests that if B cells were prevented from becoming activated, they tolerized the transferred donor cells before those donor T cells were activated by host, professional APC.

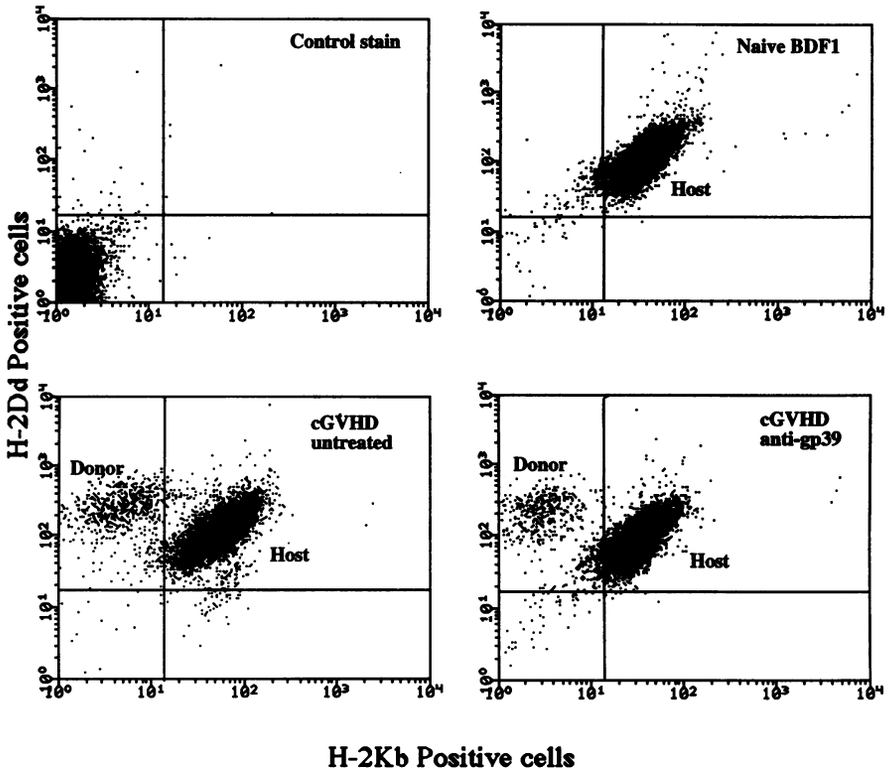

Figure 2. Identification of donor-derived cells within the recipient splenic cell population. Donor-derived cells (DBA/2, H-2^{d}) were identified within the recipient spleen cells (BDF1, H-2^{db}) by two-color flow cytometry. Recipient cells stain positively for both H-2K^{b} and H-2D^{d} antibodies, whereas DBA/2-derived cells only stain H-2D^{d} positively.

This is reasonable given the large number of quiescent B cells that could putatively act as vehicles of tolerance induction compared to the more limited number of professional APC. In addition to B cells, both activated monocytes and dendritic cells express CD40, and it may be that CD40-triggering influenced the ability of these APC to present antigen. Therefore, the current hypothesis contends that there is a CD40-dependent triggering that is essential for some unidentified APC (perhaps B cells) to elicit a positive allogeneic T cell response.

Alternative interpretations of the data presented are also possible. Since T cell receptor signaling, via anti-CD3, of T cells can be augmented by recombinant gp39 (36), it could be argued that anti-gp39 is blocking T-T cell signaling and blocking a critical costimulatory effect required for the generation of CTL. Each of these possible mechanisms is under study to resolve which is operative in the system that has been presented. Irrespective of the mechanisms involved in disease prevention by anti-gp39, anti-gp39 therapy in humans may provide a novel strategy to prevent GVHD.

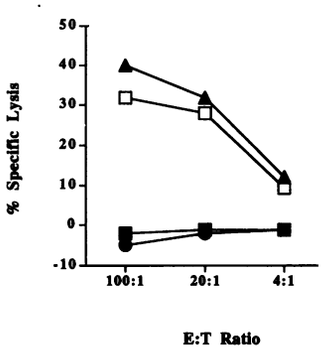

Figure 3. Anti-gp39 inhibits CTL formation associated with aGVHD induction. CTL formation was determined for spleens derived from naive BDF_1 animals (■), and BDF_1 animals induced with aGVHD and either untreated (▲), treated with HIg (□), or administered anti-gp39 (●). Data represents responses derived using P815 (H-2^{d}) cells as targets. When LB27.4 (H-2^{bxd}) targets were used, percent specific lysis was approximately half of that obtained for P815 at all E/T ratios (data not shown) and no CTL activity was obtained using the c118.5 (H-2^{b}) cell line as targets (data not shown).

Acknowledgments

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