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Antibody to the Ligand for CD40 (gp39) Inhibits Murine AIDS-Associated Splenomegaly, Hypergammaglobulinemia, and Immunodeficiency in Disease-Susceptible C57BL/6 Mice

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Infection of genetically susceptible C57BL/6 mice with the LP-BM5 isolate of murine retroviruses causes profound splenomegaly, hypergammaglobulinemia, lymphadenopathy, and an immunodeficiency syndrome which includes the development of terminal B-cell lymphomas. Because many of these and the other manifestations of LP-BM5 virus-induced disease are similar to those seen in AIDS, this syndrome has been named murine AIDS, or MAIDS. Previous reports have shown that the onset of MAIDS depends on the presence of both $CD4^+$ T cells and B cells and have suggested that $CD4^+$ T-cell-B-cell interactions are important to disease pathogenesis. Here, we assessed the possibility that interactions between CD40 and its ligand on activated $CD4^+$ T cells, CD40 ligand/gp39, are involved in the development of MAIDS. To test this hypothesis, LP-BM5-infected B6 mice were treated in vivo with anti-gp39 monoclonal antibody. As a result, MAIDS-associated splenomegaly, hypergammaglobulinemia, germinal center formation, and the loss of in vitro responsiveness to the T- and B-cell mitogens concanavalin A and lipopolysaccharide were inhibited. Anti-gp39 monoclonal antibody-treated LP-BM5-infected mice were also able to mount essentially normal alloantigen-specific cytolytic T-lymphocyte responses. These results support the possibility that molecular interactions between CD40 and gp39 are critical to the development of MAIDS.

A murine retrovirus isolate, LP-BM5, causes a murine immunodeficiency syndrome in certain mouse strains, such as the highly susceptible C57BL/6 strain. LP-BM5 is a retrovirus complex containing a mixture of murine leukemia viruses, including ecotropic, recombinant mink cell cytopathic focus-inducing virus and replication-negative defective viruses, with the defective genome serving as the proximal agent causing the immunodeficiency (3, 7, 15, 17, 29). Because many of the disease features of the LP-BM5-induced syndrome resemble those occurring in human AIDS, this retroviral disease has been termed mouse AIDS, or MAIDS. Included among the similarities are hypergammaglobulinemia, splenomegaly, and lymphadenopathy; dependence of the disease on $CD4^+$ T cells; severely depressed T- and B-cell responses; increased susceptibility to infection, disease progression, and death when exposed to environmental pathogens that normally cause limited infections; and the development of terminal B-cell lymphomas (4, 6, 22, 23, 25, 27, 28, 30, 34, 44).

Studies have shown that the induction of MAIDS by LP-BM5 depends on the presence of both $CD4^+$ T cells and B cells (6, 44). Experiments involving in vivo depletion of $CD4^+$ T cells rendered LP-BM5-infected genetically susceptible mice resistant to the development of MAIDS (44). In vivo studies have also shown that mice depleted of B cells from birth by the administration of rabbit antibodies to immunoglobulin M (IgM) and then infected with the LP-BM5 virus failed to develop MAIDS (6). In keeping with the requirement for B cells for disease, B cells have been shown to be the primary cell type for expression of the defective retrovirus after virus infection (18). Furthermore, in vitro studies have shown that the MAIDS B6-1710 terminal B-cell lymphoma expresses an antigen(s) that exhibits the properties of a superantigen in that B6-1710 cells stimulate naive T cells bearing certain Vβ T-cell receptors to proliferate (19). Evidence was provided that was consistent with the possibility that this superantigen-like activity might be encoded by the $Pr60^{gag}$ of the defective virus of the LP-BM5 virus mixture, and it was suggested that defective virus-infected B cells present this superantigen during the course of LP-BM5 infection and that the ensuing response is important to the initiation of MAIDS (19). Although subsequent reports have questioned the superantigen activity of the defective *gag* protein and have suggested a more complex picture for superantigen-like phenomena associated with MAIDS (13, 16, 21, 24, 26, 27, 31, 39), the lack of an agreed upon identity for the superantigen does not compromise support for the requirement for lymphoid cellular interactions in MAIDS. In keeping with the concept of $CD4^+$ T helper (Th) cell-B-cell interactions that are important to disease pathogenesis, in vivo experiments have shown that $CD4^+$ T cells are necessary for the induction of B-cell activation and differentiation to Ig secretion in MAIDS (44). In addition, results from a recent report with mice that are major histocompatibility complex class II negative as a source of B cells, with or without transferred $CD4^+$ T cells, were consistent with a requirement for Th cell recognition of B-cell class II presented antigen for the induction of MAIDS (12).

Recently, much has been published about $CD4^+$ T-cell interactions with and stimulation of B cells via B-cell CD40 and its ligand (gp39/CD40L) on activated $CD4^+$ Th cells. Ligation of CD40, a 50-kDa membrane protein expressed on both immature and mature B lymphocytes, induces an activation signal in a large number of B cells (41). gp39/CD40L has been shown

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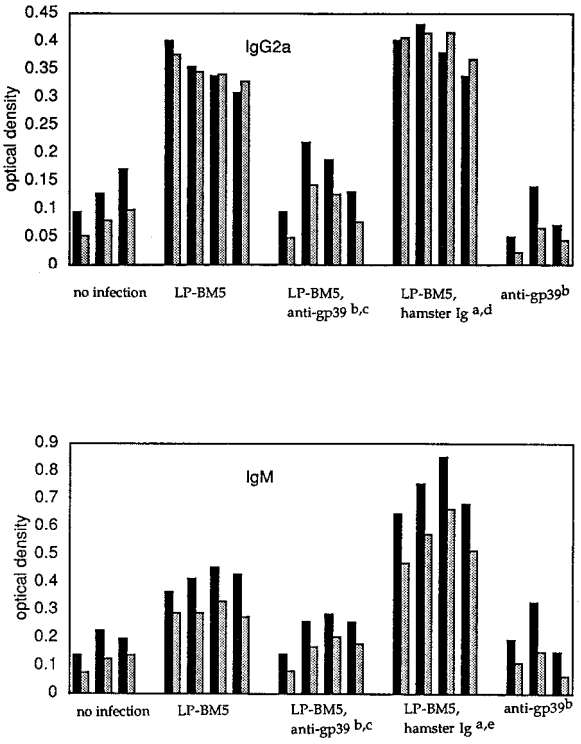

FIG. 1. MAIDS-associated hypergammaglobulinemia is inhibited by anti-gp39 treatment of C57BL/6 mice. LP-BM5 virus and anti-gp39 MAb or HIg were given as indicated in the text. Serum, obtained from mice at 4.5 weeks postinfection with LP-BM5 virus, was diluted 1:5,000 and 1:10,000 in phosphate-buffered saline before analysis. Each pair of solid and shaded bars represents serum values obtained from an individual mouse such that there are four mice per group (experiment 2 as defined in the legend to Fig. 3). This experiment is representative of three other experiments. a, Significantly different from the value for no infection by the Student *t* test ($P < 0.01$); b, not significantly different from the value for no infection ($P > 0.05$); c, significantly different from the values for untreated LP-BM5 infection and LP-BM5 infection treated with HIg ($P < 0.01$); d, not significantly different from the value for LP-BM5 infection ($P > 0.05$); e, significantly different from the value for LP-BM5 infection ($P < 0.01$). However, purified HIg, plated alone as a control, was found to cross-react with the goat anti-mouse IgM capture antibody. Therefore, the serum IgM values obtained from the LP-BM5, HIg-inoculated group of mice appear to be elevated relative to those of mice given LP-BM5 only, but this is artifactual because of the presence of the HIg (bottom panel).

to be transiently expressed on $CD4^+$ T cells after activation through the T-cell receptor for antigen (2, 33). A large number of studies have shown that gp39/CD40 interactions are crucially important to a number of immunologic responses (1, 20, 36). On the basis of requirements for both $CD4^+$ T cells and B cells for LP-BM5-induced MAIDS as described above, it seems possible that gp39/CD40 interactions are also important to the induction of this disease and that by interfering with this molecular interaction, the course of MAIDS might be altered. Here, we addressed this question by treating LP-BM5 virus-infected C57BL/6 mice with anti-gp39 monoclonal antibody (MAb). Various immune parameters were then assessed to determine if the B-cell activation and hypergammaglobulinemia, if not the immunodeficiency per se, of MAIDS were affected by such anti-gp39 therapy.

C57BL/6 mice were infected on day 1 intraperitoneally with 0.2 ml of LP-BM5 virus, prepared as described by Klinken et al. (22) and quantitated by XC plaque assay (38) to contain 4×10^5 PFU/ml. On days 1, 3, 5, and 7, LP-BM5-infected mice received 250 μg of either anti-gp39 MAb or hamster Ig (HIg), utilizing similar peaks purified by ion-exchange high-performance liquid chromatography from ascites obtained from SCID mice given hybridoma cells (33) or from hamster serum (Accurate Chemical and Scientific Corp., Westbury, N.Y.), respectively. Another control group of mice received only anti-gp39 MAb.

Sera were obtained from these mice at various subsequent time points and were assayed by enzyme-linked immunosorbent assay (ELISA) to assess IgM and IgG2a levels, by use of affinity-purified goat anti-mouse IgM and IgG2a capture antibodies, alkaline phosphatase-labeled goat anti-mouse Ig (Southern Biotechnology Associates), and *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, Mo.), with quantitation at 405 nm by an ELISA reader (Dynatech Laboratories, Inc., Alexandria, Va.). IgM and IgG2a levels have been reported to be considerably elevated as early as 3 weeks post-LP-BM5 infection (29, 34), and thus determination of these serum Ig levels provided a convenient measure of the B-cell activation and hypergammaglobulinemia associated with MAIDS. At 3, 4.5, and 6 weeks post-LP-BM5 infection, IgM and IgG2a levels of the LP-BM5 infected mice were found to be severalfold greater than those of normal mice, whereas mice that received virus and the anti-gp39 MAb had IgM and IgG2a values approximating those of normal controls (Fig. 1 [see the legend for statistical significance]). Infected mice that received HIg, in contrast, displayed elevated serum Ig levels equal to, if not above (in the case of IgM due to a cross-reactivity of HIg with the goat anti-mouse IgM capture antibody), those observed in mice receiving LP-BM5 alone. We found the same pattern of results in three other experiments.

Histological studies have shown that early in the course of infection (2 to 3 weeks), spleens from LP-BM5-infected mice contain enlarged follicles made up of immunoblasts, plasma, and plasmacytoid cells (15, 27). In keeping with this finding, our data above, and the established parameters of B-cell activation and hypergammaglobulinemia in MAIDS, we found in a preliminary experiment that splenic sections obtained from mice that had received LP-BM5 virus 12 days previously stained positive for many germinal centers (data not shown). In further experiments designed to evaluate the effect of treatment with the anti-gp39 MAb on germinal center formation in LP-BM5-infected mice, LP-BM5 virus was given on day 1, followed by the same course of anti-gp39 MAb or HIg described above. On day 13, splenic tissue sections were fixed with acetone, incubated with peanut agglutinin-biotin followed by avidin-horseradish peroxidase, and stained with diaminobenzidine (Sigma). Consistent with our data on serum hypergammaglobulinemia (Fig. 1), tissue sections from virus-infected, anti-gp39-treated mice showed little or no germinal center formation (Fig. 2B). Conversely, splenic sections from mice that received LP-BM5 virus and HIg had many germinal centers (Fig. 2A), defined as containing clusters of peanut agglutinin-binding, activated B cells, in contrast to poorly peanut agglutinin-binding resting follicular B cells (5, 9, 11, 32, 37).

A very prominent feature of MAIDS is splenomegaly (4, 22, 23, 25, 27–30, 34, 44). Thus, the spleen weights of individual mice sacrificed between 8 and 10 weeks after LP-BM5 virus infection from two separate experiments are shown in Fig. 3. Mice from the two regimens that received either LP-BM5 virus alone or LP-BM5 plus HIg, had spleens weighing approximately four to six times those of normal, or anti-gp39 MAb-treated but uninfected B6 mice. In sharp contrast, the LP-BM5-infected and anti-gp39-treated mouse groups had spleen weights approximating the normal values, although there was some mouse-to-mouse variation, with one of seven mice displaying a somewhat enlarged spleen. The spleen weights of

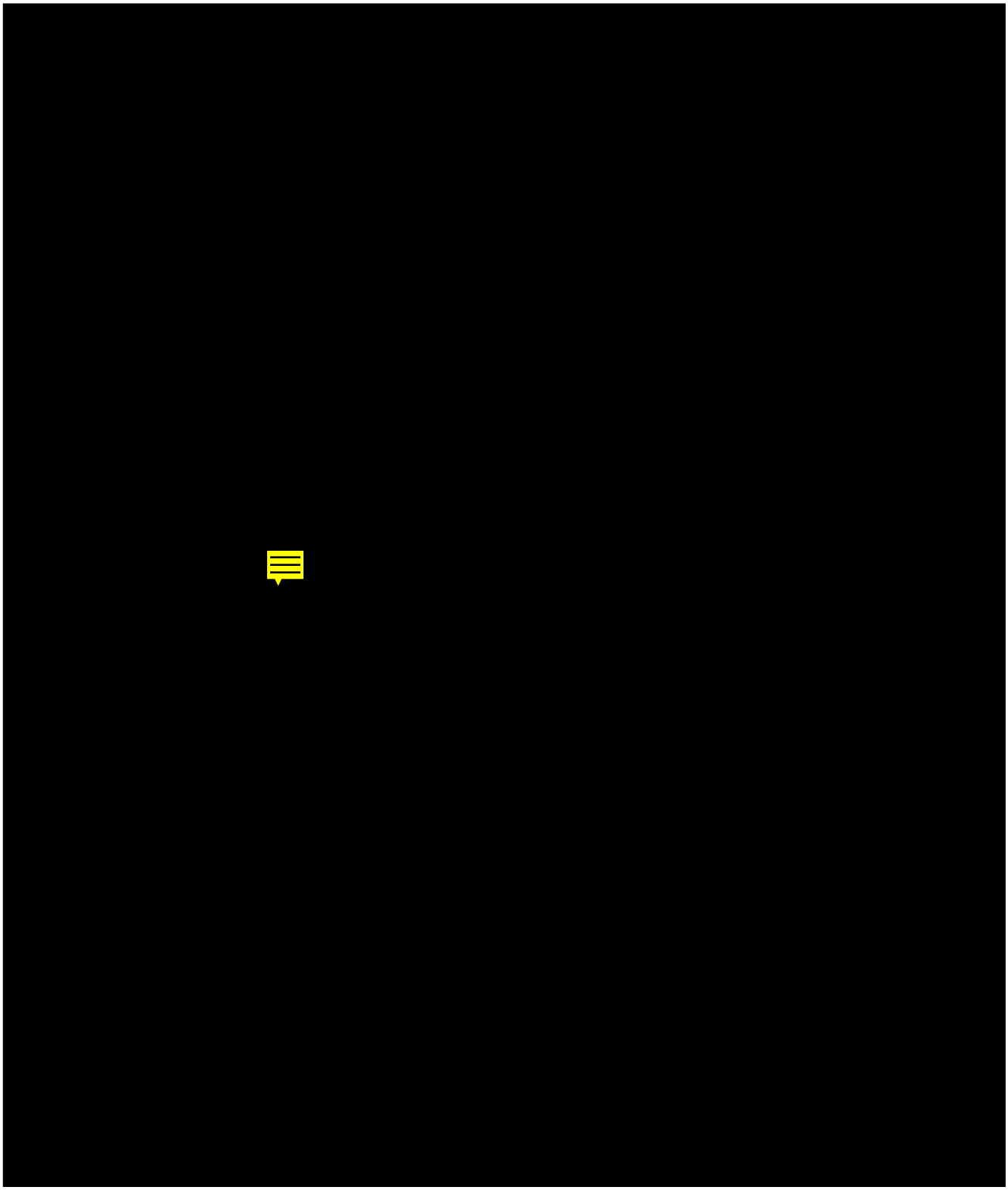

FIG. 2. In vivo treatment of LP-BM5-infected C57BL/6 mice with anti-gp39 MAb prevents the formation of germinal centers (gc). Mice (three per group) were given LP-BM5 virus on day 1 and 250 μg of anti-gp39 MAb or HIg on days 1, 3, 5, and 7. On day 13, immunohistochemistry of splenic sections from LP-BM5-infected mice treated with HIg displayed many germinal centers made up of clusters of peanut agglutinin-staining B cells (A) Tissue sections from LP-BM5-infected mice receiving anti-gp39 therapy showed little or no evidence of germinal center formation (B).

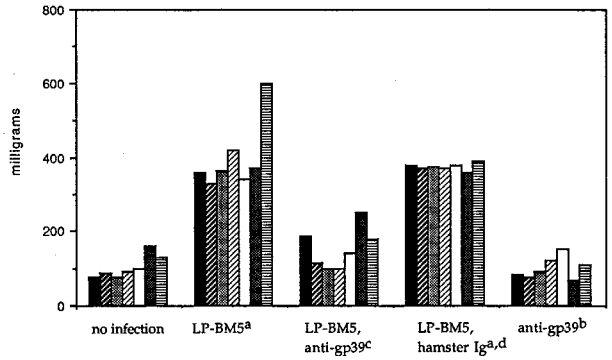

FIG. 3. In vivo treatment of C57BL/6 mice with anti-gp39 antibody inhibits MAIDS-associated splenomegaly. Mice received LP-BM5 virus and anti-gp39 MAb or HIg as described in the text. Values, shown as spleen weight in milligrams, were obtained on day 56 from individual mice from two separate experiments as follows (from left to right): bars 1 to 3, experiment 1; bars 4 to 7, experiment 2. a, Significantly different from the value for uninfected mice by the Student t test ($P < 0.001$); b, not significantly different from the value for no infection ($P > 0.05$); c, significantly different from the values for LP-BM5 infection and LP-BM5 infection treated with HIg ($P < 0.001$); d, not significantly different from the value for LP-BM5 infection ($P > 0.05$).

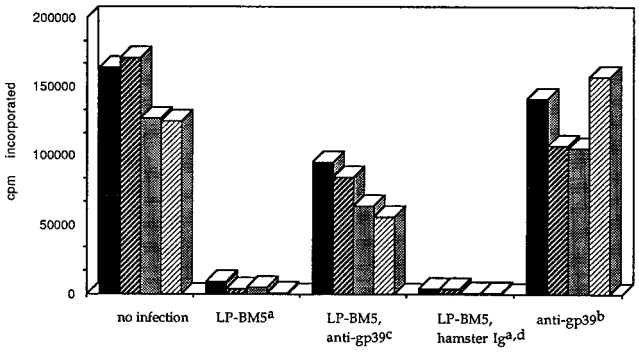

FIG. 4. Response to the mitogen ConA is retained in LP-BM5-infected mice by in vivo treatment with anti-gp39 MAb. Shown are the mice of experiment 2 (Fig. 3) that received LP-BM5 virus and anti-gp39 antibody or HIg as described in the text. Spleen cells from individual mice were obtained on day 56 and assessed for their response to ConA stimulation. All values are shown as delta counts per minute obtained by subtraction of values for responder cells cultured in media alone from values obtained when cells were cultured in media containing ConA. The results of this experiment are representative of a second repeat experiment. a, Significantly different from the value for no infection by the Student t test ($P < 0.001$); b, not significantly different from the values for no infection ($P > 0.05$); c, significantly different from the values for LP-BM5 infection and LP-BM5 infection treated with HIg ($P < 0.01$); d, not significantly different from the value for LP-BM5 infection ($P > 0.05$).

anti-gp39-treated, LP-BM5-infected mice were significantly different from those of both infected, nontreated and infected, HIg-treated mice (Fig. 3, legend), and this pattern of results was also observed in a third experiment.

To measure the effect of anti-gp39 MAb treatment on LP-BM5-induced immunodeficiency, a variety of immune responses were monitored, including reactivity to the mitogens concanavalin A (ConA) and lipopolysaccharide (LPS). Briefly, 4×10^5 spleen cells from individual normal and experimental mice were cultured with either 10 μg of LPS per ml or 2 μg of ConA per ml (Sigma), and proliferation was assessed after 72 h by pulsing of the cells with 1 μci of [^{3}H]thymidine (Dupont, NEN Research Products, Boston, Mass.). In two experiments, it was found that the response of spleen cells to the T-cell mitogen ConA was essentially eliminated in mice receiving only LP-BM5 virus or LP-BM5 plus HIg. In contrast, mice receiving virus and the anti-gp39 MAb were able to mount a response equal to about 50 to 60% of that displayed by either normal C57BL/6 controls or C57BL/6 mice that received only anti-gp39 MAb, but statistically different from the diminished responses exhibited by mice that received LP-BM5 virus only or LP-BM5 plus HIg (Fig. 4 [four mice per group, representing the same mice as those of experiment 2 of Fig. 3]). In the same two experiments, we also evaluated the response to the B-cell mitogen LPS (not shown). As with the response to ConA, LP-BM5 virus infection caused a dramatic loss of response to this mitogen. Again, spleen cells from mice receiving not only LP-BM5 virus but also anti-gp39 MAb demonstrated substantially restored LPS-responsive B cells, compared with those of mice who received only LP-BM5 or LP-BM5 plus HIg. In one experiment, the restoration of the LPS response was complete, reaching the levels of stimulation observed for untreated C57BL/6 mice. In the other experiment, restoration was to the levels of the LPS response of cells from uninfected mice treated only with anti-gp39 MAb, which were somewhat lower than those of unmanipulated control mice. All mice from this anti-gp39 control group displayed normal spleen size, a cellular response to ConA, and the ability to generate an allogeneic cytolytic T-lymphocyte (CTL) response. Thus, anti-gp39 MAb therapy largely restored both T-cell and B-cell mitogen responses in LP-BM5-infected, MAIDS-susceptible C57BL/6 mice.

At 8 weeks post-virus infection, C57BL/6 mice from all inoculation regimens were also tested for their ability to mount allogeneic CTL responses. Spleen cells were cultured with irradiated LB27.4 (*H-2*^{bd}) B-cell hybridoma cells and assayed 6 days later with ^{51}Cr-labeled P815 (*H-2*^{d}) mastocytoma cells as targets. As seen in Fig. 5 (depicting the mice from experiment 2 of Fig. 3), effector cells from all mice who received LP-BM5 virus and anti-gp39 MAb showed allogeneic lytic activity equal to that of either normal mice or of the C57BL/6 mice treated only with the anti-gp39 MAb. The allogeneic CTL response was markedly suppressed in the mice injected with LP-BM5 alone or with LP-BM5 virus and HIg (Fig. 5), although one mouse of the latter group partially retained the ability to

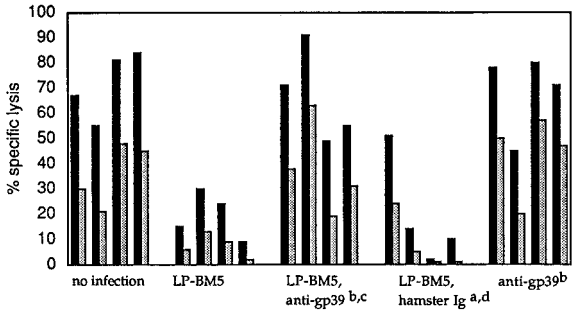

FIG. 5. Allogeneic CTL generation is preserved in LP-BM5-infected C57BL/6 mice treated with anti-gp39 MAb. Shown are the data for the mice of experiment 2 (Fig. 3), who were given LP-BM5 virus and anti-gp39 antibody or HIg as indicated. On day 56, all mice were sacrificed, and spleen cells were cultured with *H-2*-mismatched stimulators (*H-2*^{bd}). Later, a ^{51}Cr release assay was performed with P815 (*H-2*^{d}) targets and effector/target ratios of 100:1 (solid bars) and 20:1 (shaded bars). Each pair of bars represents values for percentage of lysis obtained with effector cells derived from an individual mouse. The percentage of spontaneous release for the P815 targets was 10%. a, Significantly different from the value for no infection by the Student t test ($P < 0.05$); b, not significantly different from the value for no infection ($P > 0.05$); c, significantly different from the values for LP-BM5 infection and infection treated with HIg ($P < 0.05$); d, not significantly different from the value for LP-BM5 infection ($P > 0.05$).

mount an allospecific CTL response. The resistance of one of four HIg-treated control mice to LP-BM5-induced depression of CTL responsiveness is perhaps explainable by the published observation that allogeneic responses are relatively more resistant to disease pathogenesis than, for example, mitogen reactivity and major histocompatibility complex-restricted responses (6, 14, 28). This pattern of substantial restoration of allogeneic CTL responsiveness in LP-BM5-infected mice by anti-gp39 MAb, but not HIg, treatment was observed in two repeat experiments.

These results are consistent with a requirement for an interaction between the B-cell signaling molecule CD40 and its ligand, gp39, on activated Th cells for the development of MAIDS. An alternative explanation that the anti-gp39 MAb simply removes $CD4^+$ Th cells that have been shown to be necessary for MAIDS induction (12, 44) seems unlikely for the following reasons. First, after in vivo anti-gp39 administration, antigen-specific Th cells from anti-gp39-treated immune animals readily transfer helper function to recipients (10). This result indicates that Th cells are neither functionally silenced nor physically deleted as a consequence of in vivo anti-gp39 administration. Second, in situ, the frequencies of interleukin 2-, interleukin 4-, and gamma interferon-producing T cells in immune mice are identical to those in anti-gp39-treated or untreated mice, thus demonstrating that anti-gp39 does not interfere with the frequency of lymphokine-producing cells (42). Third, in vivo administration of anti-gp39 does not diminish the humoral response to T-cell-independent antigens, a response shown to be dependent on endogenous T-cell lymphokine production for maximal responsiveness (10, 42). Fourth, previous observations have shown that in vivo anti-gp39 does not diminish the in vitro proliferative response of lymph node T cells to challenge with antigen, which suggests that anti-gp39 does not exert a negative signal to and does not block the expansion of antigen-specific T cells (32a). Finally, in preliminary experiments, we compared the extent of LP-BM5 virus-induced immunodeficiency in homozygous gp39/CD40L knockout (−/−) mice with that in heterozygous gp39/CD40L knockout (+/−) mice carrying a single copy of the gene by monitoring several immune responses known to be independent of the expression of gp39/CD40L in normal mice. The data from assessment of splenomegaly, ConA responsiveness, LPS responsiveness, and the generation of allospecific ($H\text{-}2^b$ anti-$H\text{-}2^d$) CTLs indicated that +/− but not −/− knockout mice were susceptible to LP-BM5-induced MAIDS. Because the gp39/CD40L molecule is not expressed in the −/− mice, but otherwise functional T and B lymphocytes are present (as evidenced by the positive immune responses seen here and in reference 43), these data were incompatible with a mechanism involving anti-gp39 MAb depletion of $CD4^+$ T cells as an explanation for the MAb therapy experiments discussed above.

As to the precise mechanism by which a disruption of gp39/CD40 interaction inhibits LP-BM5 virus-induced MAIDS pathogenesis, a simple, although unlikely, explanation would be the inhibition of virus infectivity. We feel that this is not the case. First, in the germinal center experiment mentioned above, an additional group of mice received LP-BM5 virus on day 1, followed by MAb on days 14, 15, and 16 (a total of 750 μg)—i.e., MAb therapy after a time at which we have shown extensive germinal centers exist in LP-BM5-infected mice (Fig. 2A). The spleens, sectioned and stained on day 21, from mice receiving LP-BM5 virus with subsequent administration of anti-gp39 antibody showed no or only an occasional remnant of germinal center formation, whereas on day 21, sections taken from mice receiving LP-BM5 on day 1 and treated with HIg still had many germinal centers present. Clearly, at least initially and subsequently through day 13, virus infection occurred in these mice, and yet anti-gp39 MAb therapy inhibited this manifestation of MAIDS. Second, and more incisively, defective *gag* mRNA expression has been semiquantitatively assessed by reverse transcriptase PCR. As shown in a representative experiment (Fig. 6), defective *gag* mRNA was found not to vary significantly in spleen cells from LP-BM5-infected, anti-gp39-treated mice versus that in spleen cells from infected mice that were either untreated or treated with HIg. The defective *gag* mRNA was quantitated by the use of defective *gag*-specific PCR primers (12), with specificity verified by size, differential *Sma*I restriction endonuclease digestion, and sequencing of the PCR product (the latter two properties distinguishing defective versus ecotropic helper *gag*). Quantitation of defective *gag* mRNA included normalization to amplification of hypoxanthine phosphoribosyltransferase message (12, 40). Specific detection of defective *gag* was further confirmed by the failure to amplify mRNA in mice that were uninfected or were infected by the BM5 ecotropic helper virus only (Fig. 6B). With respect to input cDNA and number of PCR cycles, the conditions for both defective *gag* and hypoxanthine phosphoribosyltransferase were experimentally verified to be those that achieved a detectable amount of product that was well below saturating conditions.

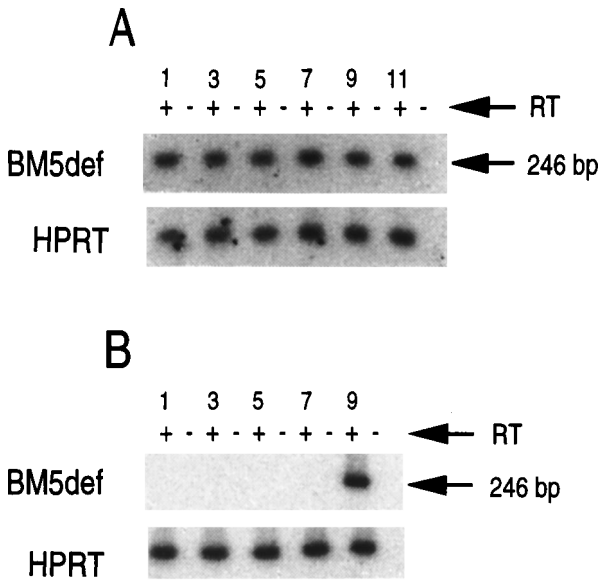

FIG. 6. Anti-gp39 MAb treatment of LP-BM5 virus-infected mice does not cause substantial inhibition of defective Gag mRNA expression. RNA samples (1 μg) from individual mice were either reacted with reverse transcriptase (RT) (odd-numbered lanes) or not reacted (even-numbered lanes) as indicated, with the resulting cDNA product split for amplification by either defective Gag^- or hypoxanthine phosphoribosyltransferase (HPRT)-specific primers for 25 cycles. On the basis of size markers, the bands for defective Gag or HPRT product appeared on a 3% agarose gel at the expected migration points corresponding to 245 and 163 bp, respectively. (A) Lanes 1 to 6 represent samples from three mice infected with LP-BM5 virus and given anti-gp39 MAb treatment; lanes 7 to 12 correspond to three mice receiving LP-BM5 virus plus HIg. Background subtracted PhosphorImager values for defective Gag, normalized to the HPRT signal, were 1.7, 1.6, and 1.4, respectively, for lanes 1, 3, and 5 compared with 1.5, 1.8, and 1.4 for lanes 7, 9, and 11. (B) Specificity controls are shown, with lanes 1 to 4 corresponding to two mice infected with BM5 ecotropic helper virus only, lanes 5 to 8 corresponding to two uninfected mice, and lanes 9 and 10 corresponding to a single LP-BM5-infected mouse. Results are representative of a total of two to eight mice analyzed for each group with the same results.

The effectiveness of anti-gp39 MAb therapy in preserving the ability of LP-BM5-virus infected mice to exhibit nearly

normal levels of serum Ig, spleen weights, responses to mitogens, and allogeneic CTL generation appears to confirm the earlier conclusions that Th cell-B-cell interactions are critical for MAIDS pathogenesis and provides some mechanistic insight by defining a crucial role for a gp39/CD40 molecular interaction. In that it has been reported that the defective virus of the LP-BM5 complex may also be expressed in infected macrophages (8), it is possible that gp39/CD40 interactions involving Th cells and $CD40^+$ macrophages are important in MAIDS in addition to, or instead of, Th cell-B-cell interactions. Along these lines, a recent report demonstrated the importance of CD40 on dendritic cells for the dendritic cell-dependent infection of $CD4^+$ T cells by human immunodeficiency virus. These findings were interpreted to suggest that human immunodeficiency virus infection in vivo might be dependent in part on interactions between CD40 and CD40L (gp39) (35). The precise nature of the Th cell-B-cell and/or Th cell-macrophage interactions, including whether the initial activation of Th cells required for gp39 expression is due to superantigen recognition, will require additional investigation. A thorough understanding of gp39/CD40 interactions in MAIDS may provide a useful paradigm to be tested in other retrovirus-induced immunodeficiencies, including AIDS.

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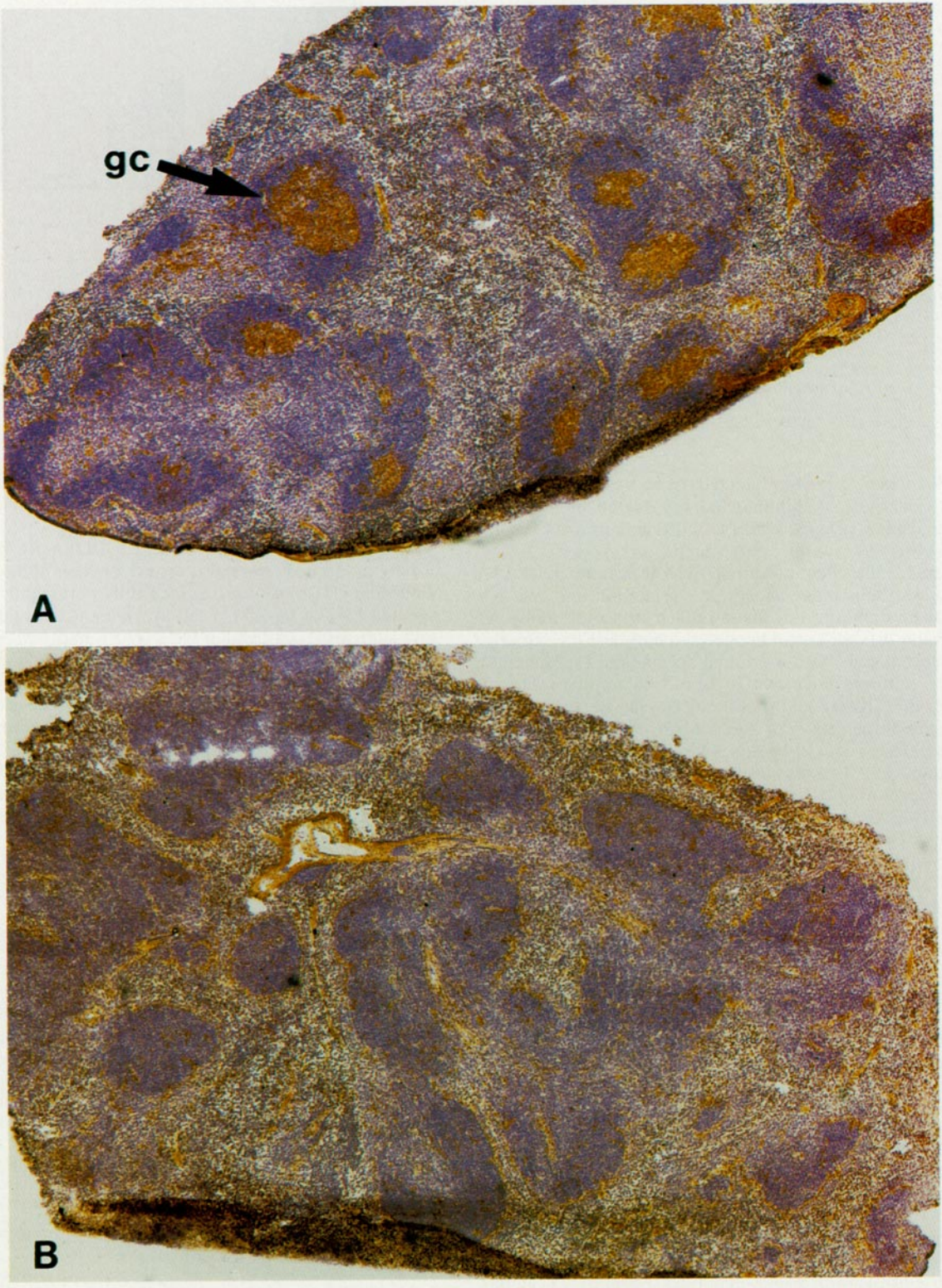
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