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Analysis of the Virus-Specific and Nonspecific B Cell Response to a Persistent B-Lymphotropic Gammaherpesvirus

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Respiratory challenge of mice with murine gammaherpesvirus 68 (γHV68) results in acute replication in respiratory epithelial cells and persistent, latent infection of B cells and macrophages. γHV68 elicits virus-specific Ab, and also nonspecifically activates B cells to Ab production through a CD4+ T cell-dependent process. The current analysis characterizes virus-specific and nonspecific Ab production at the single cell level and investigates the requirements and nature of the nonspecific response. Virus-specific Ab-forming cell (AFC) numbers were dwarfed by the increase in total AFC in all sites examined, indicating substantial nonspecific Ab production. Clear increases and decreases in specific and total AFC numbers occurred in the lymph nodes draining the respiratory tract and the spleen, but AFC numbers in the bone marrow (BM) increased to a plateau and remained constant. The longevity of the BM response was reflected in a sustained increase in virus-specific and total serum Ab levels. Generally, the IgG2a and IgG2b isotypes predominated. Analysis of cytokine-deficient mice, CD40 ligand-deficient mice, and radiation BM chimeras lacking MHC class II expression specifically on B cells indicated that nonspecific Ab production is independent of IL-6 or IFN-γ, and dependent on cognate CD4+ T cell help. Several observations were consistent with polyclonal B cell activation by γHV68, including the induction of durable serum levels of IgG reactive with mammalian dsDNA and murine type II collagen. Our findings indicate new directions for studies of this valuable model of γ-herpesvirus pathogenesis. The Journal of Immunology, 2000, 164: 1820–1828.

M urine gammaherpesvirus 68 (γHV68, also called MHV-68), a virus isolated originally from small rodents (1), is a member of the γ-herpesvirus subfamily, which includes EBV, the Kaposi’s sarcoma-associated human herpesvirus 8 (HHV-8), and herpesvirus saimiri. The γHV68 sequence is more similar to HHV-8 (2, 3), though the pathogenesis of γHV68 infection in the laboratory mouse has a number of biological and immunological features in common with human EBV infection. Little, if anything, is known about the acute phase of HHV-8 infection. The initial site of pathology in mice infected with γHV68 by the intranasal (i.n.) route is the lower respiratory tract where alveolar epithelial cells and mononuclear cells support virus replication (4). Hematogenous spread of the virus results in low level, transient replication in distal sites, particularly the thymus and adrenal glands (4, 5). Life-long latent infection of B lymphocytes (6) and macrophages (7) is established concurrently. Resolution of the pulmonary phase begins as infectious virus is cleared from the lungs within 10–12 days of challenge (4). Prominent features of the host response are a transient, CD4+ T cell-dependent splenomegaly peaking after approximately 2 wk (4, 8, 9), and an infectious mononucleosis-like condition characterized by an increased frequency of activated CD8+ T cells in the peripheral blood (10–12).

Control of acute γHV68 infection in the lung can be mediated by CD8+ cytotoxic T cells (8), and also CD4+ effector T cells that function via secretion of IFN-γ (13). A slight delay in the clearance of virus from the lungs of Ig-deficient μMT mice (14) indicates that virus-specific Ab makes only a minor contribution to this process. However, virus-specific Ig may be important in controlling the long-term, persistent phase of infection. This is suggested by experiments in which adult thymectomized mice were depleted of CD4+ and CD8+ T cells 1 mo after γHV68 infection. The mice retained high circulating levels of virus-specific IgG after depletion, and recrudescence pulmonary infection was not detected (15).

In contrast, elimination of the CD4+ and/or CD8+ T cell subsets in persistently infected μMT mice resulted in the reemergence of infectious virus in the lungs and increased numbers of latently infected cells (13, 16). A likely explanation is that Ab neutralization of free virus, and perhaps also Ab-dependent cellular cytotoxicity (17), supplement CTL-mediated surveillance of latently infected cells.

Analysis of the Ab response to γHV68 infection has been limited to measurements of circulating Ig. Serum levels of virus-specific IgG and neutralizing activity increase sharply from days 10–20 postinfection, and then continue to rise more gradually over several months (18, 19). In addition, γHV68 infection induces a dramatic and sustained CD4+ T cell-dependent increase in total serum IgG, indicating widespread, nonspecific B cell activation.
(18, 19). The infection of B cells in vitro with γHV68 has a direct activating effect, resulting in proliferation and differentiation to IgM-producing cells. However, significant Ig class switching does not occur, even in the presence of CD4 T cells, indicating the importance of in vivo interactions that are not readily reproduced in cell culture (19). The current study was undertaken to characterize the virus-specific and nonspecific Ab response to γHV68 at the single cell level. This defines for the first time the kinetics and isotype profile of the Ab response in different anatomical compartments during the acute and persistent phases of a γ-herpesvirus infection. Some insights were also gained into the requirements and nature of the nonspecific component of the Ab response.

Materials and Methods

Viruses

Clone G2,4 of γHV68 was originally obtained from Dr. A. A. Nash (Edinburgh, UK). The viral stocks were grown in owl monkey kidney cells and titrated on NIH-3T3 cells (5). Infectious γHV68 administered to mice was free of contamination with LPS (concentration <0.005 EU/ml) as determined by the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). Sendai virus administered i.n. (20) was used as a control agent in some experiments. Purified γHV68 for use in the immunoassays was prepared by differential centrifugation and sucrose banding of tissue culture-grown virus. Mouse Encephalitis virus A/HKx31 (H3N2) (21) and γHV68 were purified from stocks grown in the allantoic cavity of embryonated hen’s eggs. Protein concentrations were determined by the method of Bradford (21).

Mice

Wild-type (+/+) C57BL/6J (B6) and (B6 × 129)F1, mice, and mice deficient (−/−) for IL-6 (B6, 129-Il6−/−) (22), IFN-γ (C57BL/6-Jbg−/−) (23), or CD40 ligand (CD40L) (B6, 129-Cd40−/−) (24) were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC class II (H-2-I-A)−deficient B6.CD2 mice (C57BL/6-I-A−/−) (25) and B cell-deficient C57BL/6-lyg-6 mice (μMT) (26) were bred at St. Jude Children’s Research Hospital.

Radiation chimeras deficient in the expression of MHC class II molecules specifically on the B cell compartment were generated by i.v. injection of mixtures of CD4+ and μMT bone marrow (BM) cells into 8- to 10-kw-old B6 mice that had been lethally irradiated (950 rad) 1 day previously. Control chimeras with +/+ B cells were made using a mixture of B6 and μMT donor BM. Recipient mice were given Sulfatrim in their drinking water and held for at least 10 wk to allow full reconstitution before virus challenge. Chimeric mice were analyzed by flow cytometry (27) at the time of sampling to determine the prevalence and characteristics of various lymphocyte subsets. The staining reagents were FITC-conjugated Abs to CD4 (RM4-4), CD8a (53-6.7), or CD19 (1D3), PE-conjugated mAbs to CD4 (RM4-4), CD8a (53-6.7), or CD19 (1D3), and biotinylated mAb s to CD62L (MECA-79) (28), or CD2 (29), and CD8 (2D104) (30). A secondary reaction was with PE- or FITC-conjugated goat anti-mouse Abs. Bound Abs were detected using peroxidase-conjugated goat anti-mouse Abs (Southern Biotechnology Associates). Serum levels of IgG κ and IgG λ were determined using goat anti-mouse κ or goat anti-mouse λ (Southern Biotechnology Associates) as capture Abs, anti-mouse IgG Ab (Southern Biotechnology Associates) as a detection Ab, and goat anti-mouse IgG conjugated Ab (Southern Biotechnology Associates) as a control Ab.

ELISA for virus-specific Ab

Nunc ImmunoMaxiSorp plates (Fisher Scientific, Pittsburgh, PA) were coated with purified, detergent-disrupted γHV68 diluted in PBS and plated at 0.25 μg/well. After overnight incubation at 4°C, plates were washed with PBS-T (0.05% Tween (0.05%)-BSA (0.5%)) and tibiae. RBCs were removed from the spleen and BM preparations by flushing both femurs with PBS, and plated at 1 × 10^5 cells/well. Plates were incubated for 2.5 h at 4°C. After washing, and then infected i.n. with 1 × 10^3 PFU of γHV68 diluted in PBS to give 0.5 μg/well. Plates were similarly coated with goat anti-mouse κ light chain Abs (Southern Biotechnology Associates) to enumerate AFC secreting κ light chain Abs.

ELISA for total serum Ig

Total serum levels of IgM, IgG, IgG2a, IgG2b, and IgG3 were determined by a sandwich ELISA employing the washing, blocking, and incubation steps described above. Plates were coated with 5 μg/ml of anti-mouse Abs with specificity for IgM, IgA, IgG1, IgG2a, or IgG3 (Southern Biotechnology Associates), or with anti-mouse IgG2b Ab, clone R9-91 (PharMingen). Bound Ig was detected using ALP-goat anti-mouse IgG1, IgA, IgG1, IgG2a, or IgG3 (Southern Biotechnology Associates), or ALP-anti-mouse IgG2b Ab, clone R12-3 (PharMingen) followed by p-nitrophenyl phosphate substrate. Concentrations were calculated from curves constructed using purified murine Ig standards (Southern Biotechnology Associates). Serum levels of IgG κ and IgG λ were determined using goat anti-mouse κ or goat anti-mouse λ (Southern Biotechnology Associates) as capture Abs, ALP-goat anti-mouse IgG κ or IgG λ (Southern Biotechnology Associates) as detection Abs, and goat anti-mouse IgG conjugated Ab (Southern Biotechnology Associates) as control Ab.

ELISA for anti-collagen Ab

Serum Abs to native murine type II collagen (CII) were measured by ELISA (30). Briefly, sera were incubated in collagen-coated plates, and bound Abs were detected using peroxidase-conjugated goat anti-mouse IgG. The level of anti-CII IgG is reported as the absorbance of a 1/200 serum dilution. An inhibition assay was used to confirm the specificity of binding to the anti-CII ELISA. Briefly, serum was incubated with a range of concentrations of murine CII for 4 h at 4°C before being added to collagen-coated plates, and the ELISA was repeated as described above.

ELISA for anti-dsDNA Ab

Serum Abs binding to mammalian dsDNA were measured by ELISA (31). Briefly, a range of serum dilutions starting at 1/50 were incubated in plates coated with native calf thymus DNA, and bound Abs were detected using...
alcohol, acetone, methanol, and ethanol were determined by reference monoclonal IgM and IgG anti-DNA Abs.

Isoelectric focusing

Isoelectric focusing (IEF) of serum proteins (32) was performed in polyacrylamide Ampholine PAGplates, pH range 3.5–9.5 (Pharmacia Biotech, Piscataway, NJ) on a Multiphor II flatbed electrophoresis system (Pharmacia Biotech) set at 10°C. Electrode strips saturated with 1 M NaOH (cathode) or 1 M H3 PO4 (anolyte) were applied to the gel, and 20-μl aliquots of diluted sera were loaded on application strips positioned near the anode. Proteins focused for 90 min at 1500 V were transferred by capillary action to a methanol-treated and distilled water-moistened polyvinylidene difluoride (PVDF) Immobilon-P transfer membrane (Millipore). The membrane was carefully removed from the gel and sequentially treated with blocking buffer, biotinylated anti-mouse IgG2aκ mAb, clone 5.7 (PharMingen), and HRP-conjugated streptavidin (Pierce, Rockford, IL). The blot was developed with the ECL Western blotting analysis system (Amersham, Arlington Heights, IL) and exposed to Biomax film (Sigma) to record the IgG2a spectrototype pattern.

Statistics

Statistical comparisons of mean values were performed using the nonparametric Mann-Whitney U test for unpaired samples.

Results

Kinetic analysis of the Ab response in B6 mice

The B cell ELISPOT assay was applied to analyze both the virus-specific and nonspecific AFC response in B6 mice infected i.n. with a sublethal γHV68 dose. At various times after infection, AFC responses were measured in the CLN and MLN, which receive lymphatic drainage from the upper and lower respiratory tract, respectively (33), and in the spleen and BM. The CLN, MLN, and spleen participate in the early CD4+ and CD8+ T cell responses to γHV68 infection (18, 34, 35) and are, in addition, sites where latently infected B cell populations are first established (5). The BM is recognized as a major site for the localization of long-lived AFC (36) and is considered to maintain high circulating levels of virus-specific Ab long after infectious virus has been cleared (37–39).

AFC responses in the CLN, MLN, spleen, and BM. The kinetics of the γHV68-specific and total AFC responses in the CLN, MLN, spleen, and BM are shown in Figs. 1 and 2, respectively. The virus-specific response in the MLN slightly preceded responses in the CLN and spleen. In these sites, an early peak of virus-specific IgM AFC gave way to increased numbers of cells producing IgG isotypes, particularly IgG2a and IgG2b. Peak numbers of virus-specific AFC were attained in the CLN and MLN subsequent to 2 wk after infection, a somewhat delayed response compared with that associated with other respiratory viruses (38). Generally, the numbers of virus-specific IgG AFC in the CLN, MLN, and spleen progressively decreased from 2 to 3 wk after virus infection. Surprisingly, essentially no virus-specific IgA AFC were generated in the CLN, a site where IgA generally contributes substantially to the response following respiratory infection with negative strand RNA viruses (20, 38).

The virus-specific response in the CLN, MLN, and spleen was dwarfed by the increase in total AFC numbers (compare Figs. 1 and 2), measured as the number of lymphocytes producing Ig containing the κ light chain. Such lymphocytes represent ~90–95% of AFC in normal mice (40). The virus-specific assays applied in this study used purified, disrupted virions as target Ags. Responses to γHV68-encoded proteins that are not incorporated into the virion were therefore not detected. Nevertheless, the striking difference between total and specific AFC numbers (for instance, ~250-fold and 49-fold in the CLN and MLN, respectively, on day 10) indicates that the vast majority of AFC induced by γHV68 infection are not virus-specific. Total AFC numbers were maximal in the CLN and MLN from days 8–12 postinfection and preceded the peak of the γHV68-specific response by approximately 5 days, suggesting that there may be subversion of the humoral response to the virus by extensive, nonspecific B cell activation. Interestingly, the isotype profiles of the virus-specific and total Ig responses were remarkably similar, and there was no increase in total IgA AFC numbers. Total AFC numbers in the CLN, MLN, and spleen decreased gradually after 2–3 wk.

The kinetics of the γHV68-specific AFC response in the BM was quite distinct from that in the CLN, MLN, and spleen, where responses clearly peaked and then declined. Virus-specific AFC appeared in the BM between 2 and 3 wk after infection, and numbers thereafter remained roughly constant, with IgG2a and IgG2b predominating. In addition, a significant increase in total IgG2a AFC in the BM (p < 0.05 for day 15 and all subsequent sampling times compared with day 3) followed the same kinetics as the
AFC counts in the CLN (B), MLN (C), spleen (D), and BM (A) 5 individual mice.

measured by ELISPOT assay using plates coated with goat anti-mouse κ light chain Abs. Results are expressed as the number of AFC/10^5 nucleated cells. The mean ± SE is shown for three to seven individual mice.

FIGURE 2. Influence of γHV68 infection on total AFC numbers. Total AFC counts in the CLN (A), MLN (B), spleen (C), and BM (D) were determined for B6 mice infected i.n. with γHV68. Cell suspensions were prepared from individual mice at intervals after infection, and the total number of cells producing IgM, IgA, IgG1, IgG2a, IgG2b, or IgG3 was determined for B6 mice infected i.n. with γHV68 (43). IL-6 is well recognized as a factor acting on B cells to induce Ig secretion (44), and the production of IL-6 by B cells infected in vitro with γHV68 (43) suggests a role for the cytokine as a virus-induced autocrine growth factor. IFN-γ induces Ig production by activated B cells (45) and is also a major cytokine promoting class switching to IgG2a (46). These functions suggest a significant role for IFN-γ in the vigorous, IgG2a-biased, nonspecific Ab response associated with γHV68 infection.

Total AFC frequencies were determined in the respiratory lymph nodes of IL-6−/−, IFN-γ−/−, and appropriate +/+ control mice at the peak of the response, and serum IgG1, IgG2a, and IgG2b concentrations at this time were compared with preinfection levels.

much smaller virus-specific AFC response in this location. Apparently, the nonspecific B cell activation associated with γHV68 infection generates a long-sustained population of BM AFC secreting non-virus-specific Ab.

Serum Ab responses. Serum levels of virus-specific Ab (Figs. 3, A and B) closely reflected the single cell analysis of the response. Specific IgM levels peaked early and subsided, whereas IgG subclass Ab titers increased rapidly from 1–3 wk after infection to a relatively stable plateau. The kinetic analysis of the AFC response (Fig. 1) indicates that the elevated virus-specific serum Ab levels were maintained in the long-term by AFC in the BM, an observation consistent with studies of other virus systems (37–39). No virus-specific serum IgA was detected during the acute response, reflecting the absence of significant numbers of specific IgA AFC in the respiratory lymph nodes and spleen. Infection with γHV68 induced increases in the total serum levels of a number of Ab isotypes (Fig. 3, C and D), but most striking was a greater than 10-fold increase in the IgG2a concentration by day 10 after infection. Indeed, the increase in serum IgG2a primarily accounts for the previously reported γHV68-associated increase in serum IgG levels (18). The proportion of serum IgG2a that was γHV68 specific was evaluated by absorbing sera from infected mice with purified virus. Treatment of pooled sera collected on day 10 after infection removed ~70% of the virus-specific IgG2a detected by ELISA. However, the total serum IgG2a concentration was unaffected, indicating that most of the Ig produced in the course of infection is not specific for the virus. Substantially elevated serum IgG2a levels were maintained for the duration of sampling, apparently due in large part to the non-virus-specific AFC population that was established in the BM (Fig. 2).

Non-specific Ab production in cytokine-deficient mice

The production of non-virus-specific Ab in vivo following γHV68 infection is dependent on CD4 + T cells (19) and may be driven by cytokines, perhaps acting in concert with virus infection of B cells.

The production of non-virus-specific Ab in vivo following γHV68 infection is dependent on CD4 + T cells (19) and may be driven by cytokines, perhaps acting in concert with virus infection of B cells. Previous studies of other virus systems have identified IL-6 and IFN-γ as factors contributing to the magnitude of the nonspecific Ab response (41, 42), and high levels of these cytokines are generated in vivo by γHV68 (43). IL-6 is well recognized as a factor acting on B cells to induce Ig secretion (44), and the production of IL-6 by B cells infected in vitro with γHV68 (43) suggests a role for the cytokine as a virus-induced autocrine growth factor. IFN-γ induces Ig production by activated B cells (45) and is also the major cytokine promoting class switching to IgG2a (46). These functions suggest a significant role for IFN-γ in the vigorous, IgG2a-biased, nonspecific Ab response associated with γHV68 infection.

Total AFC frequencies were determined in the respiratory lymph nodes of IL-6−/−, IFN-γ−/−, and appropriate +/+ control mice at the peak of the response, and serum IgG1, IgG2a, and IgG2b concentrations at this time were compared with preinfection levels. Neither the isotype profile nor the magnitude of the AFC response (Fig. 4, A–C) were obviously modified in the IL-6−/− mice. Total serum IgG2a and IgG2b levels, but not IgG1 levels, were significantly increased (p < 0.05) in IL-6−/− and IL-6+/+ mice (data not shown). Apparently, IL-6 is not required to drive the nonspecific Ig response. The absence of IFN-γ had a much more profound effect, resulting in significantly increased frequencies (p < 0.05) of IgA, IgG1, and IgG2b AFC in both the CLN and MLN (Fig. 4, D and E) and total AFC in the MLN (Fig. 4, F). Serum IgG2a and IgG2b levels were significantly increased (p < 0.05) in both IFN-γ−/− and IFN-γ+/+ mice, with the rise in IgG2b being particularly marked in the IFN-γ−/− mice. In addition, the serum IgG1 level was significantly increased (p < 0.05) in the IFN-γ−/− mice, but not in the IFN-γ+/+ mice (data not shown). The increase in the nonspecific B cell response in the IFN-γ−/− mice could be thought to reflect that this cytokine is inhibitory, though it is also the case that the lytic phase of infection is controlled less rapidly in the absence of IFN-γ (13, 34), so the magnitude of the antigenic stimulus will be greater.

Non-specific Ab production requires cognate T-B interactions

The CD4 + T cell dependence of non-virus-specific Ab production following γHV68 infection in vivo (19) indicates a requirement for T cell-derived factors and/or cognate CD4 + T cell help to drive B cell activation. A key molecular interaction in T-B collaboration is the binding between CD40L, which is expressed on activated CD4 + T cells, and CD40 on B cells (47). The contribution of CD40-CD40L signaling to nonspecific Ab production induced by γHV68 was assessed in experiments comparing CD40−/− and CD40L−/− mice. Determination of total AFC frequencies at the peak of the response in the respiratory lymph nodes demonstrated absent or relatively weak responses in CD40−/− mice, in contrast to vigorous responses in CD40L−/− mice (Fig. 5, A and B). In a second experiment using a lower virus dose (600 PFU) and sampling the CLN and MLN on days 7, 15, and 35 postinfection, a
B cells in the recipients of C2D demonstrated by flow cytometry (data not shown). As expected, all II-expressing APC required to stimulate CD4 f frequencies remained at low levels in mice with MHC class II, the peak of the response in the respiratory lymph nodes. Total AFC infected with provide a system in which complete CD4 nate T-B help. Radiation BM chimeras were therefore generated to necessarily to the contribution of CD40-CD40L binding for cog-

The proportions of B cells (CD19+) in the two sets of chimeras, as were the proportions of B cells (CD19+) and CD4+ and CD8+ T cells demonstrated by flow cytometry (data not shown). As expected, all B cells in the recipients of CD2−/− + μMT BM were MHC class II−/−. The proportions of activated (CD44high, CD62Llow) CD4+ T cells were also similar in the two groups (data not shown), an observation consistent with recent evidence that the initiation of a CD4+ T cell response does not require B cells expressing class II molecules (49). Thus, even in the face of apparently complete CD4+ T cell activation, γHV68-induced nonspecific B cell activation and Ab production remained dependent on cognate help, perhaps as a prerequisite for the participation of activated B cells in normal germinal center processes. An implication is that the activated T cells are recognizing some antigenic change on substantial numbers of B cells that are not specific for viral components.

Non-specific Ab production reflects polyclonal B cell activation

Vigorous, non-virus-specific Ab production following γHV68 infection is strongly suggestive of polyclonal B cell activation, a feature of infection with a number of herpesviruses (41, 50, 51). The clonality of the nonspecific response to γHV68 was evaluated using isoelectric focusing to generate spectrotype profiles of serum IgG2a Abs, since this isotype dominated the nonspecific response. Spectrotyping of sera collected on day 10 after infection established that the IgG2a increase elicited by γHV68 represented a very diverse molecular population (Fig. 6), indicating polyclonal B cell activation. Indeed, virus infection amplified at least as many bands as could be identified by spectrotyping IgG2a in sera pooled from 10 uninfected mice. Absorption of the day 10 sera with purified virus removed 70% of the virus-specific IgG2a detected by ELISA, but had no effect on the spectrotype, indicating that the bands reflect nonspecific Ab production.

Also, though infection with γHV68 generated an ~10-fold increase in total serum IgG2a levels within 2–3 wk, the ratio of κ- to λ-containing IgG molecules remained constant during this period (data not shown), a result consistent with extensive, nonspecific B cell activation.
Autoantibody induction by γHV68

There is evidence that polyclonal B cell activation results in the production of Abs directed against self components and may lead to the development of systemic autoimmune disease (52). Sera collected at different times following γHV68 infection were thus tested for IgG Abs reactive with mammalian dsDNA and murine CII. Comparisons were made with sera from mice infected with Sendai virus, a negative strand RNA virus that activates B cells nonspecifically (29, 53). Infection with Sendai virus, like γHV68, induced a substantial and sustained increase in total serum IgG2a and IgG2b concentrations, although the maximum IgG2a levels were ~1.5× higher following exposure to γHV68 challenge. The C2D (−/−) + μMT → B6 chimeras have CD4+ T cells, MHC class II−/− B cells (C2D), and MHC class II+/+ macrophages and dendritic cells (μMT). The B6 + μMT → B6 chimeras are the controls. The samples (three from four to five individual mice) were analyzed as described in the legend to Fig. 2.

Discussion

The B cell ELISPOT assay has previously been applied in a number of models of virus infection to characterize the production of virus-specific Ab at the single cell level (38, 39, 54, 55). However, the current analysis of Ab production following γHV68 infection takes into account both the generation of virus-specific AFC, and coated with distinct Ags (dsDNA, CII, or the conventional Ag OVA). The induction by γHV68 of IgG Abs reactive with two distinct self Ags further supports the polyclonal nature of B cell activation caused by the virus, and raises the possibility that γ-herpesvirus infections may trigger autoimmune disease.
The nonspecific B cell activation that is a feature of many virus infections (29) and may be particularly relevant to the pathogenesis of the B lymphotropic γ-herpesviruses. The specific response to γHV68 had features in common with the response to other respiratory viruses, namely, the kinetics of Ab production in different anatomical compartments (38), and the general predominance of the IgG2a and IgG2b isotypes (38, 56). There was no indication that the response was modified by potentially continuous antigenic stimulation that may result from reactivation of γHV68 from sites of latency in the lung (14, 16) and lymphoid tissue (5). The γHV68-specific AFC response in the respiratory lymph nodes was somewhat delayed and of a smaller magnitude compared with responses in the same locations following infection with influenza or Sendai virus (20, 38). Although this may reflect differences in the Ag load, a significant factor may be the B cell tropism of γHV68 and the resultant loss of B cells by lytic infection (4), apoptosis (19), or CD8+ T cell-mediated clearance (8). The peak γHV68-specific AFC response in the CLN, MLN, and spleen was slightly preceded by a dramatic increase in nonspecific AFC numbers, suggesting that virus-driven, nonspecific B cell activation may also contribute to a dampening of the specific humoral response. However, numerous viruses including influenza and Sendai have the capacity to activate B cells nonspecifically (29, 57), and factors such as the mechanism and extent of this process may well determine the effect, if any, on virus-specific Ab production.

A surprising feature of the Ab response to γHV68, in marked contrast to the response to other respiratory viruses (20, 28, 38), was the almost complete absence of γHV68-specific IgA AFC in the CLN and MLN. The CLN in particular is recognized as a site biased toward the production of IgA (58), and virus-specific IgA AFC characteristically constitute a significant proportion of the response in the CLN following i.n. immunizations with live or inactivated virus (20). The processes by which IgA responses are generated in the respiratory tract are not well understood, but there is evidence (59) that B cells in close association with the respiratory epithelium (60) are particularly important. Conceivably, lytic infection of these cells at an early stage of γHV68 infection may subvert the induction of specific IgA AFC. The response to γHV68 in the CLN follows approximately the same kinetics as that in the MLN and is well developed before any response is apparent in nonrespiratory lymph nodes such as the axillary and brachial (data not shown). This indicates that γHV68 accesses the CLN from the respiratory tract. The possibility that the virus replicates in epithelial cells in the nasopharynx or other regions of the upper respiratory tract that would be expected to drain to the CLN (60) has not been analyzed. A possible scenario is that γHV68 spreads to the CLN via B cells that are directly infected in the upper respiratory tract. There is recent evidence (61) that EBV enters the human host by B cell infection alone (62).

The γHV68-specific response in the CLN, MLN, and spleen was accompanied by a dramatic increase in the number of non-virus-specific AFC (Figs. 1 and 2). Both the virus-specific and nonspecific responses displayed similar kinetics and isotype profiles, consistent with speculation (19) that the nonspecific response (like the specific response) requires the participation of activated B cells in normal germinal center processes. Surprisingly, γHV68 infection induced a substantial and long-sustained increase in non-specific AFC numbers in the BM. These cells presumably maintain the elevated levels of circulating, non-virus-specific Ig. To date, the BM as a long-term reservoir of AFC has been described only in the context of specific responses to viral infection (37–39) or immunizations with inert, thymus-dependent Ags (63–65). The observation that γHV68 infection generates increased numbers of non-virus-specific AFC in the BM is further evidence that these cells originate in germinal center reactions, since BM AFC appear to be derived from cells that emerge from germinal centers (65, 66). It will be of interest to determine whether long-lasting, nonspecific AFC populations in the BM also result from infection with the many other viruses that are able to nonspecifically activate B cells (29, 57). These viruses represent a broad range of taxonomic groups, and the process of nonspecific B cell activation is therefore unlikely to have a uniform mechanistic basis.

Experiments were conducted to define more precisely the requirements for the CD4+ T cell-dependent production of non-virus-specific Ab following γHV68 infection (19). Analysis of cytokine-deficient mice (Fig. 4) demonstrated that the nonspecific Ab response is not dependent on IL-6 or IFN-γ. However, nonspecific Ab production induced by γHV68 was substantially diminished in CD40L−/− mice and in chimeric mice lacking MHC class II expression specifically on B cells (Fig. 5). Engagement of the TCR with MHC class II-peptide on B cells cannot take place in the chimeric mice, with consequent disruption of a series of downstream events that normally lead to the participation of B cells in germinal center reactions and the production of thymus-dependent Ab (67). Perhaps pivotal in this process is up-regulation of the transiently expressed CD40L on helper T cells that results from
signaling through the TCR (47). Our results indicate a requirement for cognate CD4+ T cell help to drive the nonspecific B cell activation associated with γHV68 infection, with CD40-CD40L interaction as an essential signaling component. Signaling to B cells through CD40 is a prerequisite for normal germinal center processes (68), and it follows that the production of non-γHV68-specific Ab may depend on the germinal center for B cell proliferation and differentiation. A key question concerns the nature of the CD4+ T cell help that drives the process, which may well be the normal, Ag-specific CD4+ T cell response. Conceivably, a virus-encoded molecule may mediate a non-Ag-specific interaction between MHC class II on the surface of infected B cells and the appropriate TCR on CD4+ T cells (69).

Our observations indicate that the production of nonspecific Abs that is such a prominent feature of γHV68 infection is a consequence of generalized polyclonal B cell activation. In this regard, γHV68 is similar to a number of other herpesviruses including the human pathogens EBV (50, 70) and CMV (51). Both EBV and CMV also induce Abs that bind to host Ags (71, 72), a consequence that may relate to the activity of these viruses as polyclonal B cell activators (52). Herpesvirus-induced polyclonal B cell activation and autoantibody production have been most thoroughly studied in mice infected with murine CMV (MCMV). Although some antigenic cross-reactivity between host and MCMV proteins has been described (73, 74), the production of multiple autoantibodies of different specificities during MCMV infection (75) suggests that generalized polyclonal B cell activation (41, 76) is largely responsible. Autoantibody production in γHV68-infected mice may also derive from nonselective, polyclonal B cell activation. However, isotype-switched Abs reactive with mammalian dsDNA and murine CII were generated in only a proportion of mice infected with γHV68 (Fig. 7, A and B), suggesting the involvement of some stochastic processes. This feature may indicate a role for Ag-driven selection in the production of some autoantibodies, particularly anti-DNA. There is evidence (77) that Abs specific for host dsDNA and non-cross-reactive with viral dsDNA, may be selectively induced by the binding of virus-encoded proteins to cellular DNA. Serum levels of anti-dsDNA and anti-CII Abs remained high in some γHV68-infected mice for the duration of sampling, raising the possibility of autoantibody-mediated pathology, though a role for the virus in the induction of autoimmune disease in the laboratory mouse has not otherwise been investigated. It will be of interest to determine whether the potentially long-lived (36), non-virus-specific AFC in the BM generated by γHV68 infection include autoantibody-secreting cells.

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References
