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Bone Marrow Is a Major Site of Long-Term Antibody Production after Acute Viral Infection

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Antiviral antibody production is often sustained for long periods after resolution of an acute viral infection. Despite extensive documentation of this phenomenon, the mechanisms involved in maintaining long-term antibody production remain poorly defined. As a first step towards understanding the nature of long-term humoral immunity, we examined the anatomical location of antibody-producing cells during acute viral infection. Using the lymphocytic choriomeningitis virus (LCMV) model, we found that after resolution of the acute infection, when antiviral plasma cells in the spleen decline, a population of virus-specific plasma cells appears in the bone marrow and constitutes the major source of long-term antibody production. Following infection of adult mice, LCMV-specific antibody-secreting cells (ASC) peaked in the spleen at 8 days postinfection but were undetectable in the bone marrow at that time. The infection was essentially cleared by 15 days, and the ASC numbers in the spleen rapidly declined while an increasing population of LCMV-specific ASC began to appear in the bone marrow. Compared with the peak response at 8 days postinfection, time points from 30 days to more than 1 year later demonstrated greater-than-10-fold reductions in splenic ASC. In contrast, LCMV-specific plasma cell numbers in the bone marrow remained high and correlated with the high levels of antiviral serum antibody. The presence of LCMV-specific plasma cells in the bone marrow was not due to persistent infection at this site, since the virus was cleared from both the spleen and bone marrow with similar kinetics as determined by infectivity and PCR assays. The immunoglobulin G subclass profile of antibody-secreting cells derived from bone marrow and the spleen correlated with the immunoglobulin G subclass distribution of LCMV-specific antibody in the serum. Upon rechallenge with LCMV, the spleen exhibited a substantial increase in virus-specific plasma cell numbers during the early phase of the secondary response, followed by an equally sharp decline. Bone marrow ASC populations and LCMV-specific antibody levels in the serum did not change during the early phase of the reinfection, but both increased about two-fold by 15 days postchallenge. After both primary and secondary viral infections, LCMV-specific plasma cells were maintained in the bone marrow, showing that the bone marrow is a major site of long-term antibody production after acute viral infection. These results documenting long-term persistence of plasma cells in the bone marrow suggest a reexamination of our current notions regarding the half-life of plasma cells.

Acute viral infections often confer long-term protective immunity (1, 17, 20). In some cases, as documented by Panum's classic epidemiological study of measles in the Faroe Islands, this immunity can be lifelong (39). In addition to measles, long-term immunity is also seen following yellow fever and smallpox infections (11, 43). An important parameter of protective immunity is the presence of preexisting antiviral antibody. In this context, it is noteworthy that antiviral "B-cell memory" is often manifested by continuous antibody production after resolution of the acute infection. For example, infection with measles virus can result in lifelong production of virus-specific antibody (8, 9, 45). Prolonged antibody production following infection or vaccination has also been observed with other human, as well as veterinary, viruses (17). For instance, people infected with poliovirus have been reported to possess circulating antiviral antibodies for 40 to 60 years and in the case of yellow fever, protective antiviral antibody can be isolated from individuals 75 years after infection (40, 43). Despite extensive documentation of long-term antibody production, many questions concerning the mechanisms involved in these responses, as well as the nature and anatomical location of antibody-producing cells, remain to be clarified.

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In this study, we examined the anatomical site of long-term antibody production by using the lymphocytic choriomeningitis virus (LCMV) model system. LCMV infection of mice provides a useful model to study the interaction of a virus with the immune system of its natural host (30, 38). Previous work involving LCMV and vesicular stomatitis virus has identified transient virus-specific plasma cell populations in the spleen after acute infection (36). A peak in the number of antiviral antibody-producing cells in the spleen occurred around 8 days postinfection and was followed by sharp declines in virus-specific plasma cells after recovery from both LCMV and vesicular stomatitis virus infections. This was in contrast to high antiviral serum antibody levels that remained constant. The immunological paradox of declining splenic plasma cell populations versus lifelong serum antibody maintenance was not addressed in these studies. In this report, we show that the initial antibody response against acute LCMV infection results from antibody production in the spleen, but after the viral infection has cleared and for the life of the animal, most antiviral antibody synthesis occurs in the bone marrow.

MATERIALS AND METHODS

Mice. BALB/cByj (*H-2*^{d}), C57BL/6 (*H-2*^{b}), and B6.PL Thy1^a/Cy (*H-2*^{b}) mice were purchased from Jackson Laboratory, Bar Harbor, Maine. No difference was found between C57BL/6 and B6.PL Thy1^a/Cy mice in the response to LCMV infection, and they were therefore used interchangeably in our experiments and

are referred to as B6 mice here. The congenital carrier colony was derived from neonatally infected mice and bred at the University of California at Los Angeles.

Virus. The Armstrong CA 1371 strain of LCMV was used in this study (3). LCMV-immune mice were obtained by injecting 5- to 8-week-old mice intraperitoneally (i.p.) with 2×10^5 PFU of LCMV strain Armstrong. For the secondary LCMV infection, mice were injected i.p. with 10^6 PFU of LCMV strain Armstrong.

Determination of viral titers. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (3).

Quantitation of antiviral antibody-secreting cells (ASC). Spleen and bone marrow single-cell suspensions were cleared of erythrocytes by a single round of 0.83% NH_4Cl treatment and resuspended in Iscove's modified Dulbecco's medium supplemented with 5% fetal calf serum (Hyclone, Logan, Utah) and antibiotics (penicillin, streptomycin, and amphotericin B [Fungizone]). LCMV-specific plasma cells were quantitated by a modification of the ELISPOT method of Eriksson et al. (18). Briefly, nitrocellulose-bottom 96-well Multiscreen HA filtration plates (Millipore Corporation, San Francisco, Calif.) were coated either at 50 μl per well with phosphate-buffered saline (PBS) containing 10 μg of purified LCMV per ml or at 100 μl per well with LCMV-infected BHK cell lysate and incubated overnight at 4°C. As an irrelevant-antigen control, wells were coated with uninfected BHK cell lysate. Plates were washed once with PBS containing 0.1% Tween 20 (PBS-T) and three times with PBS. The plates were blocked with 200 μl of Iscove's medium containing 5% fetal calf serum for at least 1 h to decrease the number of remaining protein-binding sites. Blocking medium was replaced with 100 μl of medium containing threefold dilutions of cells and incubated for 4 to 5 h at 37°C in a humid atmosphere with 6% CO_2. Plates were emptied by being flicked and washed three times with PBS and then three times with PBS-T. A 100-μl volume of biotinylated, affinity-purified horse anti-mouse immunoglobulin G (IgG) heavy and light chain (Vector Laboratories, Burlingame, Calif.) diluted 1/1,000 in PBS-T containing 1% fetal calf serum was added to each well and incubated overnight at 4°C. This reagent identifies all isotypes by virtue of its cross-reactivity with Ig light chains. For IgG subclass studies, anti-mouse IgG1, IgG2a, IgG2b, IgG3, and IgGγ antibodies (CALTAG, San Francisco, Calif.) were used for the primary detection reagents. The plates were washed four times with PBS-T, 100 μl of horseradish peroxidase-conjugated avidin D (Vector Laboratories) at a concentration of 5 μg/ml in PBS-T–1% fetal calf serum was added, and the mixture was incubated at room temperature for 1 h. The plates were washed three times with PBS-T and three times with PBS, and detection was carried out by adding 100 μl of horseradish peroxidase-H_2O_2 chromogen substrate. The substrate was prepared by adding 1 ml of a freshly made AEC solution (10 mg of 3-amino-9-ethylcarbazole [Sigma, St. Louis, Mo.] per ml dissolved in dimethylformamide) to 30 ml of 0.1 M sodium acetate buffer (pH 4.8), filtering it through a 0.2-μm-pore-size membrane, and immediately before use adding 150 μl of 3% H_2O_2. Granular red spots appeared in 3 to 5 min, and the reaction was terminated by thorough rinsing with tap water. Spots were enumerated with a stereomicroscope equipped with a vertical white light. ASC and plasma cells are terms used interchangeably in this report when referring to cells that actively secrete antibody.

ELISA. LCMV-specific serum antibody titer was determined by a solid-phase enzyme-linked immunosorbent assay (ELISA) as described previously (3). IgG subclass distribution patterns were determined by serial threefold dilutions of duplicate serum samples.

Determination of total bone marrow cells. For calculation of the total ASC response in bone marrow, we multiplied the response by the marrow cells of two femurs by a coefficient of 7.9, since ^{59}Fe distribution studies have shown that 12.6% of total mouse bone marrow is located in both femurs combined (7). No differences have been detected among the ASC activities of bone marrow cells from the femur, tibia, humerus, rib, or sternum (7). Typically, two adult femurs yield 2.0×10^7 to 2.5×10^7 total bone marrow cells.

PCR amplification of LCMV-specific genomic RNA. LCMV-specific reverse transcriptase (RT) PCR was performed on RNA extracted from bone marrow obtained from both femurs (14). cDNA synthesis and PCR amplification were performed as previously described, with primers specific for the LCMV polymerase gene (5′ primer, 5′-GATTCTCACGTCTTCTTCCA-3′; 3′ primer, 5′-CTCTCCGTAATTTGACTCAC-3′) (28, 42). PCR was performed with a DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.) for 30 cycles (90 s of denaturation at 94°C, 90 s of annealing at 58°C, and 120 s of extension at 72°C). Positive control primers for mouse glyceraldehyde 3-phosphate dehydrogenase were purchased from Clontech (Palo Alto, Calif.), and RT PCR was performed by following the manufacturer's directions. The reaction products were visualized by electrophoresis in a 1.5% agarose gel in 1× TAE buffer containing 0.5 μg of ethidium bromide per ml. A 1-kb DNA ladder (GIBCO-BRL, Gaithersburg, Md.) was run in parallel as molecular size markers.

Southern blot analysis. Southern blot analysis was performed on the PCR products described above as previously described by using a ^{32}P-end-labeled oligonucleotide specific for the LCMV polymerase gene (5′-ATCGAGGCCA CACTGATCTT-3′) (28).

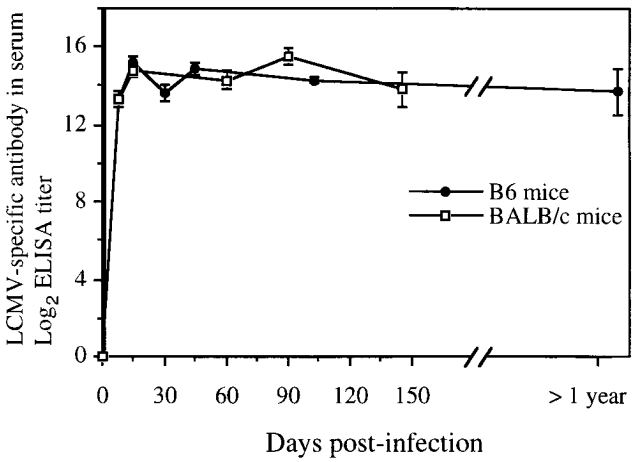

FIG. 1. Acute LCMV infection induces lifelong antiviral serum antibody. BALB/c and B6 mice were infected i.p. with 2×10^5 PFU of LCMV strain Armstrong, and virus-specific serum antibody titers were determined for an average of three mice for each time point by ELISA.

RESULTS

Acute LCMV infection results in lifelong antiviral antibody in the serum. Infection of adult mice with the Armstrong strain of LCMV induces a strong antiviral response that eliminates the virus within 2 weeks. During the course of this systemic infection, several tissues become infected but the virus is cleared by a potent antiviral $CD8^+$ cytotoxic T-lymphocyte response (2, 3, 10, 12, 26, 28, 29, 35, 49). Lifelong T- and B-cell memory is produced, and resistance to rechallenge with LCMV is maintained (10, 28). In our studies, mice were inoculated i.p. at 5 to 8 weeks of age with 2×10^5 PFU of LCMV strain Armstrong and monitored at various time points from 8 days to greater than 1 year postinfection. The data in Fig. 1 show that after acute viral infection, long-term LCMV-specific serum antibody was maintained in both BALB/c and B6 mice. These studies and those of others indicate that after acute LCMV infection, high levels of anti-LCMV antibody are detectable in the serum for the life span of an immune animal (1, 10, 36).

The initial ASC response induced by an acute viral infection is observed in the spleen, but later the bone marrow becomes a major source of ASC. LCMV-infected mice were monitored at various intervals by collecting spleen and bone marrow samples and screening for total LCMV-specific ASC with the ELISPOT assay. Dilutions of cell samples were plated on nitrocellulose-bottom plates coated with LCMV and incubated at 37°C for 4 to 5 h. This incubation period is too short for a memory B cell to differentiate into an ASC, and thus only in vivo-stimulated, actively secreting plasma cells are identified (34). Uninfected mice were included in each experiment, and no virus-specific ASC were detected in either spleen or bone marrow samples. In conducting the ELISPOT assays, three to six mice were studied per time point (unless otherwise noted) and the average and standard deviation were determined from individual spleen and bone marrow samples. In the spleen, the peak ASC response against LCMV occurred at 8 days postinfection (Fig. 2). At day 8, BALB/c mice exhibited an average antiviral plasma cell frequency of 580 ASC per 10^6 spleen cells and B6 mice averaged almost 400 ASC per 10^6 spleen cells. When total plasma cell populations were compared, BALB/c mice averaged 9.2×10^4 LCMV-specific plasma cells per spleen and B6 mice averaged 6.1×10^4 cells per spleen. In

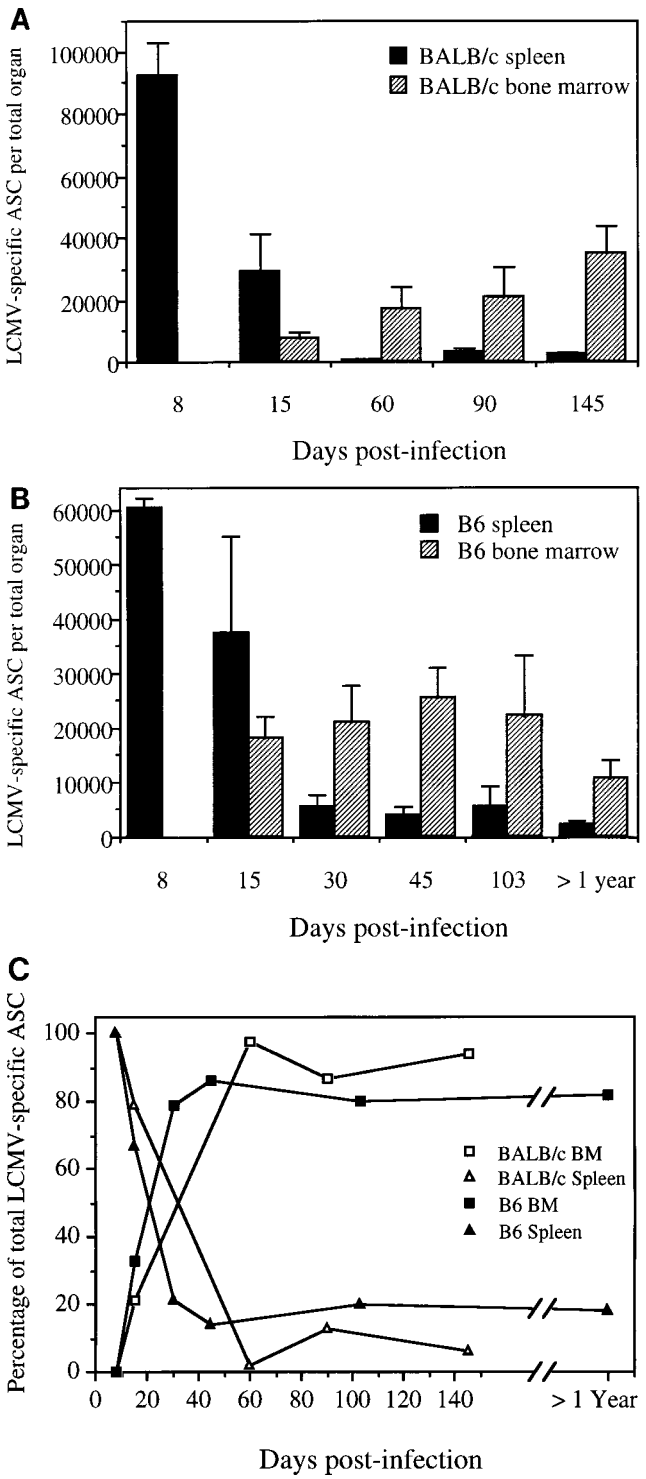

FIG. 2. The initial humoral response to viral infection occurs in the spleen, but long-term antibody synthesis occurs in the bone marrow. BALB/c and B6 mice were infected i.p. with 2×10^5 PFU of LCMV strain Armstrong. The data shown are averages of three to six mice assayed individually at various time points after primary infection. The ELISPOT assay was used to quantitate individual LCMV-specific ASC isolated from the spleen and bone marrow. (A) Kinetics of the ASC response in adult BALB/c mice. (B) Kinetics of the ASC response in adult B6 mice. (C) Percentages of total ASC numbers located in the spleen and bone marrow as a function of time postinfection.

contrast, virus-specific ASC were undetectable (<1 ASC per 2×10^6 cells) in the bone marrow at 8 days postinfection in several independent experiments with both strains of mice (Fig. 2).

By 15 days postinfection, the splenic ASC numbers decreased by approximately 40% in B6 mice and 70% in BALB/c mice. LCMV-specific ASC were below detection in bone marrow at 8 days postinfection, but by day 15, the bone marrow of both strains of mice constituted 20 to 30% of the total ASC. At 15 days postinfection, B6 and BALB/c mice averaged 1.8×10^4 and 7.6×10^3 antiviral plasma cells per total bone marrow, respectively.

Measurements taken at time points after viral clearance (i.e., greater than 2 weeks postinfection) demonstrated the long-term stability of the antiviral ASC response in bone marrow. The BALB/c strain showed no decrease in the LCMV-specific plasma cell populations in the bone marrow at 145 days postinfection, maintaining an average of 2.6×10^4 ASC per total bone marrow (Fig. 2A). Meanwhile, LCMV-specific plasma cells in the spleen dropped over 40-fold, to 2.0×10^3 ASC. Similar kinetics were identified in the B6 strain with stable antiviral ASC populations in the bone marrow maintained at 103 days postinfection and averaging 2.3×10^4 ASC per total bone marrow, followed by a slight twofold drop in aged mice at greater than 1 year postinfection. In contrast, by 30 days postinfection, the splenic ASC populations in B6 mice dropped more than 10-fold, to 5.2×10^3 LCMV-specific plasma cells (Fig. 2B). When the percentage of total antiviral ASC in the spleen and bone marrow compartments were compared (Fig. 2C), we found that virtually 100% of the LCMV-specific ASC were located in the spleen at 8 days postinfection. By day 15, about 20 to 35% of total LCMV-specific plasma cells were located in the bone marrow. At all late time points, the bone marrow constituted approximately 80% of the total antiviral plasma cells in B6 mice and about 90% of the total LCMV-specific ASC in BALB/c mice. The kinetics of the antibody responses of both BALB/c and B6 mice suggest that after the initial peak of the antiviral ASC number in the spleen at day 8 postinfection, the bone marrow soon becomes a major site for long-term antibody synthesis.

IgG2a is the major IgG subclass of bone marrow and splenic ASC and corresponds to the subclass distribution of LCMV-specific Ig in the serum. Previous work has shown that after acute LCMV infection in adult mice, the subclass profile of antiviral serum antibody is skewed in favor of the IgG2a subclass, with some IgG1 also being produced (47, 48). Figure 2 describes the total LCMV-specific ASC response during acute infection, but to further characterize the antibody response to LCMV, we analyzed the IgG subclass profile of LCMV-specific plasma cells in the bone marrow and spleen. After primary LCMV infection, the four subclasses, IgG1, IgG2a, IgG2b, and IgG3, were quantitated by both subclass-specific ELISPOT and ELISA. We found IgG2a to be the most abundantly produced IgG subclass, as shown by the representative ELISPOT experiment results in Fig. 3. The kinetics of the splenic ASC response are shown in Fig. 4A. At 8 days postinfection, IgG2a was the predominant subclass (57%), followed by IgG1 (30%), IgG3 (12%), and IgG2b (1%). This distribution pattern changed slightly by day 15 to 72% IgG2a, 24% IgG1, and 4% IgG2b. LCMV-specific ASC of the IgG3 subclass were not detected in the spleen at 15 or 84 days postinfection. Although there was a greater-than-10-fold drop in total ASC for each IgG subclass between days 15 and 84, the distribution of the IgG subclasses remained essentially the same.

As shown in Fig. 4B, the subclass distribution of the LCMV-specific plasma cells in the bone marrow was similar to that

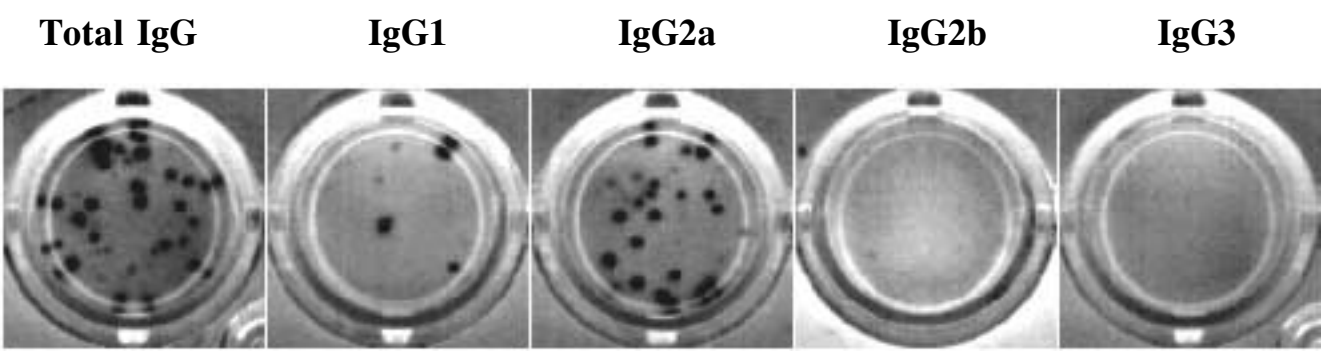

FIG. 3. Representative ELISPOT demonstrating the IgG subclass profile of LCMV-specific plasma cells in the bone marrow at 84 days postinfection. The same number of bone marrow cells was added to each well, and the number of LCMV-specific ASC was quantitated by the ELISPOT assay using reagents specific for total IgG, IgG1, IgG2a, IgG2b, and IgG3.

observed in the spleen. Antiviral ASC were below detection in the bone marrow at 8 days postinfection but were identified in the bone marrow by day 15, and the IgG subclass profile showed IgG2a as the predominant subclass (69%), followed by IgG1 (26%) and IgG2b (5%). LCMV-specific ASC of the IgG3 subclass were not detected in the bone marrow. At 84 days postinfection, the LCMV-specific ASC in the bone marrow were maintained at numbers equal to, or greater than, those found at the peak of the ASC response in the spleen at 8 days postinfection (with the exception of IgG3). This differs from the data in Fig. 2, which shows the total LCMV-specific ASC response and includes both IgG- and IgM-secreting cells. Since the IgM response was transient, the peak response in the spleen at day 8 was larger than the response observed in the bone marrow at later time points. On the other hand, when the late IgG response in the bone marrow at day 84 was studied, it was very similar to the peak IgG-specific ASC response observed in the spleen at day 8 (Fig. 4). This shows that the decline in IgG-specific plasma cells in the spleen is compensated by an equivalent increase in subclass-matched plasma cells in the bone marrow.

The IgG subclass distribution of bone marrow and splenic ASC matched the IgG subclass distribution of LCMV-specific antibody in the serum at all of the time points tested (days 8, 15, and 84). Figure 4C illustrates the data for the day 84 time point. These results show that both the bone marrow and the spleen contribute to serum antibody production, but total virus-specific ASC in the bone marrow outnumber those in the spleen by approximately 10 to 1.

The presence of LCMV-specific ASC in the bone marrow does not appear to be due to prolonged viral infection of the bone marrow. Even though acute LCMV infection is short-lived, it is possible that the migration of LCMV-specific ASC to the bone marrow is due to prolonged viral infection of this anatomical site. We addressed this issue by performing plaque assays of both spleen and bone marrow samples after acute LCMV infection. As shown in Fig. 5, LCMV infection was rapidly cleared from both the spleen and bone marrow, falling below the detection limits of the plaque assay by 8 days postinfection. As a more stringent test for the presence of the virus, LCMV-specific RT PCR was performed on bone marrow samples taken at various time points after infection. LCMV genomic RNA was not detected by RT PCR in the bone marrow at 8, 15, or 84 days postinfection but was identified in LCMV carrier bone marrow (678-bp RT PCR product), as shown in Fig. 6A. The integrity of the RNA extracted from bone marrow was suitable for RT PCR analysis since primers specific for glyceraldehyde 3-phosphate dehydrogenase pro-

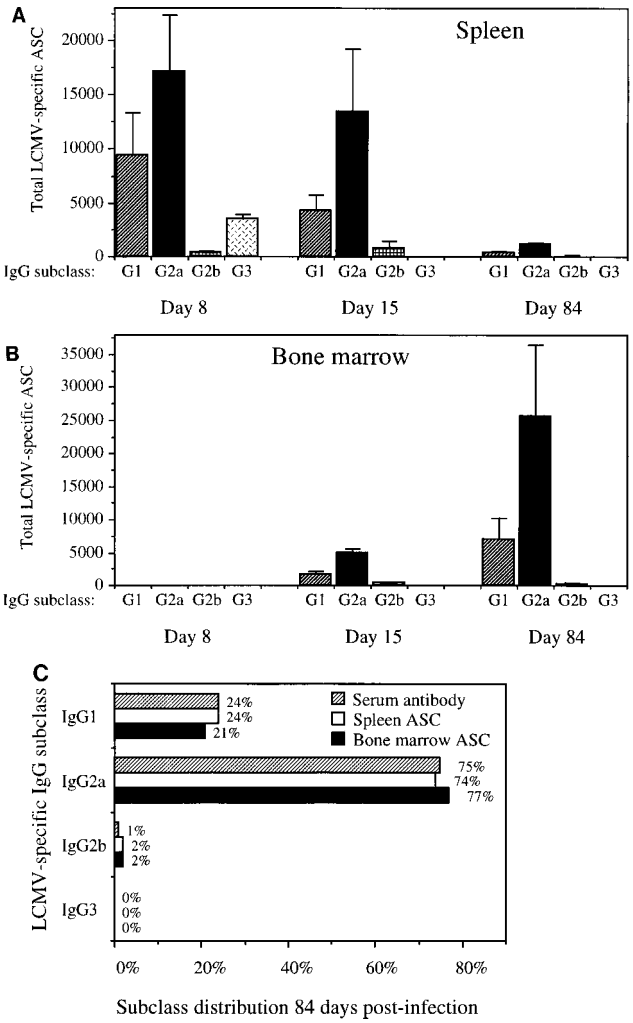

FIG. 4. IgG2a is the predominant subclass produced after acute LCMV infection. BALB/c mice were infected with 2×10^5 PFU of LCMV strain Armstrong i.p., and serum, spleen, and bone marrow samples were collected at 8, 15, and 84 days postinfection. The spleen and bone marrow data are averages of three mice per group analyzed by the ELISPOT assay, and the serum data are averages of two mice analyzed by ELISA. Antibodies specific for IgG1, IgG2a, IgG2b, and IgG3 were used to quantitate plasma cells of each subclass at 8, 15, and 84 days postinfection in spleen (A) and bone marrow (B). (C) The percent subclass distribution of ASC numbers in the spleen and bone marrow corresponds to LCMV-specific serum antibody at 84 days postinfection.

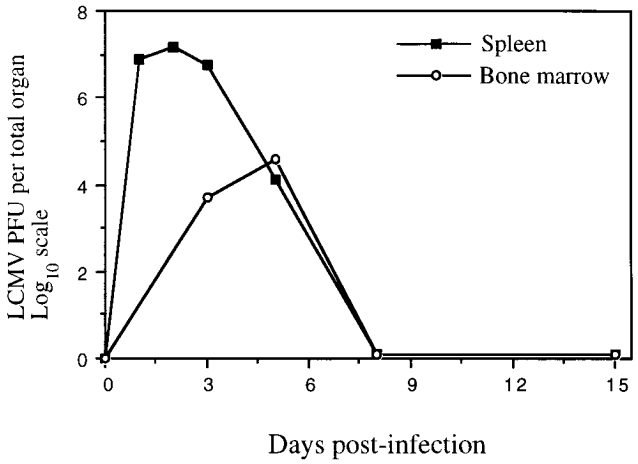

FIG. 5. Infectious virus is rapidly cleared from the spleen and bone marrow. BALB/c mice were infected with LCMV strain Armstrong, and infectious virus in the spleen and bone marrow was determined by plaque assay on Vero cell monolayers. The data shown are average viral titers of two adult mice.

duced the expected 983-bp RT PCR product (Fig. 6B). The specificity of the RT PCR was confirmed by Southern blot analysis of the LCMV PCR products with an end-labeled internal oligonucleotide specific for a region of the LCMV polymerase gene. No signal corresponding to the 678-bp RT PCR product was identified in the samples taken from acutely infected mice (Fig. 6C). This strongly suggests that the presence of LCMV-specific ASC in the bone marrow is not due to viral persistence at this anatomical site.

Secondary infection induced a rapid ASC response in the spleen followed by a delayed increase in ASC numbers in the bone marrow. The memory B-cell response to secondary LCMV infection has previously been shown to rapidly induce a large population of antiviral plasma cells in the spleen, but the humoral response in the bone marrow after secondary LCMV challenge has not been analyzed (36). We therefore examined the antibody response in these organs during a secondary LCMV infection. For the primary infection, mice were inoculated with 2×10^5 PFU of LCMV strain Armstrong. Prechallenge serum was collected 59 days later (to identify individual increases in antiviral antibody levels due to the secondary infection). Sixty days after primary infection, mice were given a secondary challenge of 10^6 PFU of LCMV strain Armstrong and the kinetics of the humoral response were determined. LCMV infection is rapidly cleared in immune mice and is typically below the limit of detection by plaque assay within 3 to 4 days. Infectious virus was below our detection limits in spleen and bone marrow samples by day 3 postchallenge as determined by plaque assay (data not shown).

As shown in Fig. 7, a 10-fold increase in LCMV-specific ASC was observed in the spleen by 3 days postchallenge. The peak antibody response in the spleen was observed at day 5 with an almost 40-fold increase in total antiviral plasma cells. The antiviral antibody response quickly subsides in the spleen, and by day 15, only 30% of the plasma cells observed during the peak response remain.

After secondary viral challenge, the antibody response of immune bone marrow was delayed in comparison with the response of the immune spleen. Bone marrow ASC populations remained almost unchanged at days 3 and 5 postchallenge, but by days 15 and 30 postchallenge, we observed a two-fold increase in LCMV-specific ASC (Fig. 7). After primary infection, we found that greater than 90% of LCMV-specific ASC were located in the bone marrow, but at 5 days post-secondary infection, we observed that the total ASC numbers in the spleen transiently surpassed the number of LCMV-specific ASC found in the bone marrow (Fig. 7). However, after a return to homeostasis (30 days post-secondary infection) we found the profiles of spleen and bone marrow ASC contributions returning to the same ratios observed after primary infection (Fig. 2C), with 80 to 90% of the total antiviral ASC population located in the bone marrow. Therefore, even though LCMV-specific plasma cell populations expanded in both the spleen and bone marrow compartments after secondary infection, the ratio of spleen- and bone marrow-derived ASC remained basically the same. When the pre- and postchallenge LCMV-specific antibody levels in serum were compared, there was little change at day 3 or 5 but by day 15 postchallenge there was an approximately twofold increase in the LCMV-specific serum antibody level (Fig. 7).

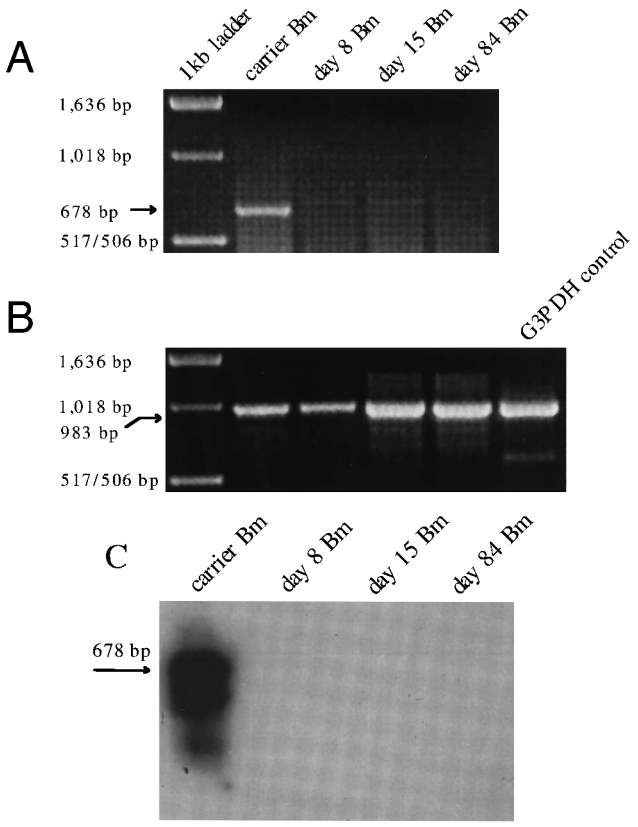

FIG. 6. Viral RNA was not detectable by RT PCR in bone marrow (Bm) following acute LCMV infection. Total RNA was extracted from bone marrow samples at 8, 15, and 84 days postinfection. LCMV-specific primers were used to detect viral RNA by RT PCR. (A) Genomic LCMV RNA, identified as a 678-bp RT PCR product, was not detected in the bone marrow of acutely infected mice by 8 days postinfection but was detectable in LCMV carrier bone marrow. (B) Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was identified as a 983-bp RT PCR product in all RNA samples. (C) The viral RT PCR products were LCMV specific, as shown by Southern blot analysis using an internal LCMV-specific probe.

DISCUSSION

Preexisting antibody is an important defense mechanism against reinfection, and our studies show that the bone marrow is a major site of long-term antibody production after acute

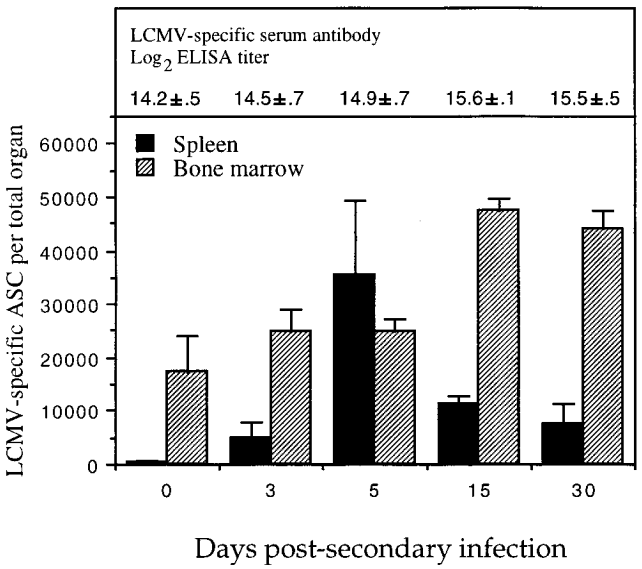

FIG. 7. Secondary LCMV challenge of immune mice results in a rapid yet transient splenic antibody response followed by increased ASC numbers in bone marrow and increased LCMV-specific serum antibody. Adult BALB/c mice were first infected i.p. with 2×10^5 PFU of LCMV strain Armstrong and rechallenged 60 days later with 10^6 PFU of the same virus. Both ELISPOT and ELISA data are averages of three mice per group, except those for day 15, which are averages of two mice. The LCMV-specific ASC responses in the spleen and bone marrow were calculated per total organ. Primary-response serum (prechallenge) was assayed together with the corresponding secondary-response (postchallenge) serum by LCMV-specific ELISA (represented on a $\log_2$ scale above each time point).

viral infection. Mice acutely infected with LCMV maintain lifelong virus-specific serum antibody. We observed that the initial LCMV-specific plasma cells were located in the spleen, but their numbers declined rapidly. Antiviral plasma cells were later found in the bone marrow and maintained at high numbers, even in aged mice almost 1.5 years postinfection. The IgG subclasses of the ASC in the bone marrow and spleen matched the subclasses of LCMV-specific serum antibody, indicating that both organs contribute to circulating antibody in the serum. However, when spleen and bone marrow contributions were compared, we found that bone marrow contributed 80 to 90% of total LCMV-specific ASC. The appearance of ASC in the bone marrow was not due to persistent infection of this organ, since our studies show rapid clearance of infectious virus by plaque assay and genomic viral RNA by LCMV-specific RT PCR. Similar to the results obtained after primary infection, the bone marrow continued to be a major site of antibody production after resolution of acute secondary infection with the same virus. In this case, a rapid B-cell memory response induced a greater-than-40-fold increase in splenic ASC, which transiently outnumbered the LCMV-specific ASC found in the bone marrow. However, as in the primary infection, the splenic ASC numbers rapidly declined after viral clearance, and after a return to homeostasis, the bone marrow contribution to total LCMV-specific ASC remained at 80 to 90%.

Our studies involved a live systemic viral infection, and one could argue that plasma cell migration to the bone marrow was due to preferential infection of this anatomical compartment. We believe that migration of ASC to the bone marrow due to infection at this site is unlikely for the following reasons. (i) LCMV infection of the bone marrow was readily cleared and fell below our limits of infectious virus detection by 8 days postinfection, and LCMV-specific RT PCR was unable to detect the presence of viral genomes in bone marrow samples. (ii) There is already a precedent for specific antibody synthesis in the bone marrow in which no infectious agent was involved (5–7, 22, 46). More recently, it has been shown that virus-specific ASC are also present in the bone marrow of mice acutely infected with Sendai virus or vesicular stomatitis virus (4, 24, 25). Although viral infection of the bone marrow was not examined in these studies, it is unlikely that either Sendai virus or vesicular stomatitis virus would persist in the bone marrow.

These studies have shown that a primary viral infection induces the appearance of plasma cells in the bone marrow. However, antibody responses to inert antigens often require "boosting" or secondary exposure to have the same effect (5–7, 22, 46). This difference could be due to a threshold of antigenic stimulation and/or lower levels of certain cytokines, both of which may be required for the migration of specific plasma cells to the bone marrow. We are currently studying these questions to determine the mechanisms involved in antibody formation in the bone marrow.

The antibody response after acute viral infection was delayed in the bone marrow with respect to the response observed in the spleen. This may not be due to ASC homing to other organs shown to be capable of antibody production (i.e., lymph nodes or lamina propria), since these have ASC kinetics similar to those of the spleen and produce antibody only transiently after antigenic stimulation (5, 7, 22, 31, 46). Antiviral ASC were not found in the bone marrow at 8 days postinfection, and only low levels of virus-specific plasma cells were identified at 15 days. This delay would allow time for somatic mutation and affinity maturation to occur in the germinal centers prior to the production of bone marrow-destined plasma cells leaving the germinal centers (32). Consistent with this, the distribution of each IgG subclass in the bone marrow corresponded to the day 15 splenic IgG subclass profile and not to the day 8 IgG profile. We also have anecdotal evidence that plasma cells in the bone marrow may produce higher levels of antibody. The spots produced by bone marrow ASC during the ELISPOT assay were consistently larger in diameter and more uniform in size than those produced by splenic ASC under identical assay conditions (data not shown). During the ELISPOT assay, the relative size of the spot produced by an individual plasma cell is proportional to its rate of Ig synthesis (34). This suggests that higher-affinity and/or higher-production plasma cells migrate to the bone marrow. However, analysis of the Ig V region structure of these bone marrow-derived cells is required before any definitive conclusions can be drawn.

It is well established that serum Igs have a short half-life, usually on the order of days to, at most, a few weeks (13, 19). Thus, to maintain high levels of circulating antiviral antibody, there must be a constant pool of plasma cells synthesizing antibody. This brings up the interesting issue of plasma cell longevity. Are plasma cells truly short-lived? The current dogma is that plasma cells have a rapid turnover rate and are replenished by cycling B cells that differentiate within germinal centers of the spleen and lymph nodes (32, 33, 37). While this model is based on convincing experimental data obtained by using inert antigens, it fails to provide a satisfactory explanation for the many examples of prolonged antibody production following acute viral infections (8, 9, 11, 40, 43, 45). In many instances, antibody production continues in the absence of reexposure to the virus (8, 9, 11, 15, 40, 43). Given these observations, one must also consider the possibility that plasma cells may in fact be long-lived. Along these lines, there is some

evidence that plasma cells in the bone marrow may survive longer than plasma cells in the spleen (23). Also, we have preliminary evidence suggesting that bone marrow-derived LCMV-specific plasma cells survive for several months after adoptive transfer into mice with severe combined immunodeficiency (45a). In any case, the prevailing dogma that plasma cells are short-lived needs to be reexamined.

This work brings up several points of interest. For example, what role do $CD4^+$ T cells play in the maintenance of terminally differentiated plasma cells? Are they required to supply factors or possibly cell-to-cell contact to maintain the longevity of these antibody-producing cells? Are they required for the homing of ASC to the bone marrow? On another note, which B-cell subsets migrate to the bone marrow after acute viral infection? Are these mature plasma cells or immature plasma cells that must terminally differentiate once located in the bone marrow microenvironment? Some studies indicate that immature plasma cells migrate to the bone marrow and that specific signals in the bone marrow microenvironment may be necessary for differentiation into mature plasma cells (27, 41). The bone marrow microenvironment may also play a role in maintaining long-term antibody production (16). An important implication of the maintenance of long-term antibody production in bone marrow is the possibility of passively transferring immunity from bone marrow donors. By immunizing bone marrow donors, protective immunity may be transferred to patients who are immunosuppressed and vulnerable to opportunistic infections (21, 44).

Another intriguing question is whether persisting antigen is required for ASC longevity or maintenance. Although our recent studies have shown that antigen is not required for $CD8^+$ T-cell memory, it is possible that persisting antigen may be required for maintenance of B-cell memory and long-term antibody production (20, 28). There are two possible models for explaining chronic antibody production after acute viral infection. According to the currently accepted paradigm, plasma cells are short-lived but their pool is constantly replenished by memory B cells that differentiate into ASC after encountering antigen trapped on follicular-dendritic cells. This model requires the continuous persistence of antigen and/or reexposure to the pathogen. An alternative model is that plasma cells are long-lived and prolonged antibody production can be maintained, at least in some situations, without continuous antigenic stimulation of memory B cells. Experiments are currently in progress to address these critical issues.

Many viral infections in humans lead to prolonged and sometimes life-long antibody production (9, 40, 43, 45). This humoral response is a critical defense against reinfection and applies not only to viral infections but also to microbial infections in general. By learning more about the mechanisms involved in maintaining this chronic antibody production, we should be able to design better adjuvants and vaccines for inducing long-term protective humoral immunity.

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