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Evidence for B-Lymphocyte Mitogen Activity in *Borrelia burgdorferi*-Infected Mice

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***Borrelia burgdorferi* produces a mitogen for murine B lymphocytes which can be measured in vitro by polyclonal stimulation of proliferation and immunoglobulin production (R. Schoenfeld, B. Araneo, Y. Ma, L. Yang, and J. J. Weis, Infect. Immun. 60:455–464, 1992). Sonicated *B. burgdorferi* cells also stimulated IL-6 production by splenocyte cultures. We have used the murine model for Lyme disease described by Barthold et al. (S. W. Barthold, D. S. Beck, G. M. Hansen, G. A. Terwilliger, and K. D. Moody, J. Infect. Dis. 162:133–138, 1990) to determine whether the *B. burgdorferi* B-cell mitogen is expressed during active infection. To correlate arthritic changes with immune events, we have studied two strains of mice injected with *B. burgdorferi*; one of them, C3H/HeJ, developed severe disease, and the other, BALB/c, developed only mild disease. C3H/HeJ mice displayed a persistent 10-fold increase in circulating immunoglobulin G (IgG) levels, a 2-fold increase in IgM levels, and a 15-fold increase in peripheral lymph node B-cell numbers, providing evidence of mitogenic activity. Infected BALB/c mice also had evidence for mitogen activity, since the IgG level in serum increased three- to fourfold. The bulk of the increase in circulating IgG levels was not directed against *B. burgdorferi* antigens, supporting the occurrence of polyclonal B-cell activation. Analysis of IgG isotypes pointed out a contrast between C3H/HeJ and BALB/c mice in that levels of all isotypes were elevated somewhat in both strains of infected mice but IgG2a levels were much more dramatically increased in the C3H/HeJ mice (28-fold) than in the BALB/c mice (4-fold). In this study, interleukin-6 levels were found to be persistently elevated in the serum of infected C3H/HeJ mice. Interestingly, interleukin-6 levels in serum were much lower in the infected BALB/c mice. These findings indicate that the *B. burgdorferi* mitogen is active in infected animals and may contribute to the inflammatory and immune response to infection.**

Human Lyme disease is a tick-borne infectious disease caused by the spirochete *Borrelia burgdorferi* (32). The human disease has been divided into three stages, based on the time of onset following the tick bite and the tissue involved (32). Stage 1 is an enlarging skin lesion surrounding the site of the tick bite (erythema chronicum migrans) and is associated with flulike symptoms. Stage 2 manifestations occur weeks to months after the tick bite and include cardiac and neurological involvement. The most common symptom of stage 3 disease is arthritis, beginning months to years after the initial infection. Typically, the arthritis is transient and intermittent; however, chronic arthritis is observed in 10% of these individuals (32). Most patients who experience symptoms of all three stages respond to antibiotic treatment, indicating that arthritis and other stage 2 and 3 symptoms are the results of spirochete persistence. The enigma of Lyme disease is the persistence of the organism in the presence of a demonstrable specific immune response. Some investigators have speculated that this may result from abnormalities in immune function associated with stage 2 and 3 disease (10, 16, 29, 30, 32). Previous studies have indicated that the spirochetes cross endothelial monolayers in vitro (9, 33), suggesting that in the infected animal the spirochetes cross vascular endothelium to gain access to many tissues, including the central nervous system, joints, and heart (5, 15, 32). Spirochetes can also gain entrance into endothelial cells, suggesting that an intracellular location could provide a protective niche from the immune defenses of the host (10, 23).

It is possible that bacterial products are directly responsible for arthritis development, although the strong immune response in stage 3 disease suggests that the host immune or inflammatory responses may also participate in the development of arthritis (29). We have recently demonstrated that *B. burgdorferi* produces a molecule that is mitogenic for murine B lymphocytes (27). This mitogen stimulates proliferation of and immunoglobulin production by B cells from naive mice and stimulates immunoglobulin M (IgM) production by the B-cell tumor line CH12.LX (21, 27). Several reports have demonstrated that patients with Lyme disease have an increased level of IgM in serum and a high proportion of constitutively activated B cells. This suggests that the *B. burgdorferi* mitogen could be involved in the pathogenesis of human Lyme disease (30). Furthermore, human lymphocytes have been found to proliferate when incubated with *B. burgdorferi* (38). In this study we analyzed mice infected with *B. burgdorferi* to determine whether mitogenic stimulation of B lymphocytes occurred in vivo. We further analyzed levels of interleukin-6 (IL-6) in the serum of these infected mice, because this cytokine has been implicated in human rheumatoid arthritis (24, 25) and we and others have demonstrated that *B. burgdorferi* stimulates its production by various cell types (19, 27).

MATERIALS AND METHODS

Bacteria. S. Barthold, Yale University, provided the N40 isolate of *B. burgdorferi* at passage 3 from an infected mouse

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(4). Cultures were maintained as 0.5-ml frozen stocks at −70°C. Fresh aliquots were seeded in 15 ml of Barbour Stoenner Kelly II (BSKII) medium (1) and cultured at 32°C.

Mice. C3H/HeJ ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), C57BL/10 ($H\text{-}2^b$), C3H.SW ($H\text{-}2^b$), and B10.BR ($H\text{-}2^k$) mice were obtained from Jackson Laboratories at 5 to 6 weeks of age. They were housed in the Animal Resource Center of the University of Utah Medical Center.

Infection of mice with *B. burgdorferi*. Mice (5 to 6 weeks old) were infected with *B. burgdorferi* by intradermal injection on the back to closely mimic infection via the tick. Control mice were injected with an equal volume of sterile BSKII culture medium.

Measurement of the ankle joints. Rear ankle joints of mice anesthetized with Methoxyflurane (Pitman-Moore) were measured with a metric caliper (Mitutoyo, Tokyo, Japan). Ankle joint measurements provided an indication of the histological severity of arthritis. C3H/HeJ mice displayed severe arthritis in the presence of measurable joint swelling, whereas BALB/c mice displayed minimal swelling and did not develop severe arthritis.

Histology of the ankle joints. Rear joints were obtained from sacrificed mice and fixed in 10% formalin solution. Fixed specimens were then decalcified and embedded in paraffin, and sections were stained with hematoxylin and eosin stain.

Flow cytometry. Single-cell suspensions were prepared from popliteal, superficial inguinal, and axillary lymph nodes (8). Cell populations were analyzed by flow cytometry after being stained with appropriate antibodies (8). B cells were detected with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Bethesda Research Laboratories). $CD5^+$ cells were detected with *R*-phycoerythrin-conjugated rat anti-mouse Ly-1 monoclonal antibody (53-7.3; Pharmingen) (20). $CD4^+$ and $CD8^+$ cells were detected with phycoerythrin-conjugated rat anti-mouse L3T4 (GK 1.5) (12) and fluorescein isothiocyanate-conjugated rat anti-mouse Lyt2 (53-6.7) (22) monoclonal antibodies (Becton Dickinson), respectively.

Measurement of immunoglobulin level in serum. Serum was obtained at each time point by retroorbital bleeding. Immunoglobulin levels were determined by antibody capture enzyme-linked immunosorbent assay (ELISA) on microtiter plates coated with goat anti-mouse IgG, IgM, and IgA (heavy and light chain specific) (Zymed) at a concentration of 10 μg/ml (8). Dilutions of serum were added to wells, unbound sample was washed off, and the amount of bound murine immunoglobulin was detected by the addition of horseradish peroxidase-conjugated antibodies to murine IgG, IgM (Boehringer Mannheim), and IgA (Southern Biotechnology Associates, Inc.). The plates were developed by the addition of 0.4 mg of *o*-phenylenediamine per ml and 0.01% H_2O_2 and read at an optical density of 492 nm with a V_{max} 96-well microtest plate spectrophotometer (Molecular Devices). Immunoglobulin levels were estimated by using a standard curve with known concentrations of IgG, IgM, and IgA. Levels of IgG isotypes were determined by using horseradish peroxidase-conjugated antibodies to murine IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates, Inc). Standard curves were generated with known concentrations of murine IgG of the indicated isotype.

Quantification of *B. burgdorferi*-specific IgM and IgG. Serum samples from infected and control mice at week 4 were assayed for anti-*B. burgdorferi* antibody by antibody capture ELISA. Eleven columns per plate were coated with 10 μg of *B. burgdorferi* sonicated antigen per ml (8). The 12th column was coated with goat anti-mouse IgG, IgM, and IgA (Zymed) as described above. Dilutions of serum were added to wells coated with *B. burgdorferi* sonicated antigen, and known concentrations of murine IgG and IgM were added to the wells coated with goat anti-mouse IgG, IgM, and IgA. Unbound sample was washed off, and the anti-*B. burgdorferi* antibody IgG and IgM were detected by the addition of horseradish peroxidase-conjugated antibodies to murine IgG or IgM (Boehringer Mannheim), respectively. Comparison of the optical density of *B. burgdorferi* wells with the optical density of the standard curve allowed estimation of antigen-specific antibody levels.

Measurement of the titer of serum anti-ovalbumin antibody. Serum from infected and control mice was assayed for anti-ovalbumin immunoglobulin titer by ELISA. Plates were coated with purified ovalbumin at a concentration of 10 μg/ml (Sigma). Dilutions of serum were added to wells, unbound sample was washed off, and the titer of anti-ovalbumin immunoglobulin was detected by the addition of horseradish peroxidase-conjugated murine IgG antibodies (Boehringer Mannheim).

Detection of antibodies to mouse type II collagen. Serum samples were tested for the presence of IgG antibody to mouse native type II collagen by ELISA (17). Plates were coated with mouse native type II collagen that had been solubilized from cartilage by limited pepsin digestion and purified to homogeneity as described previously (17). Plates were blocked with 1% bovine serum albumin before addition of test serum samples, and incubations with test serum samples and developing antibodies were carried out at 4°C to preserve collagen structure. Positive control serum from mice with collagen-induced adjuvant arthritis were run simultaneously to ensure the quality of the plates (17).

Measurement of the IL-6 level in serum from infected and control mice. The IL-6 level in serum was assayed by antibody capture ELISA with rat monoclonal antibody against mouse IL-6 (MP5-20F3; Pharmingen) as the capture antibody and a second biotin-conjugated rat monoclonal antibody against mouse IL-6 (MP5-32C11; Pharmingen) as the detecting monoclonal antibody (31). Bound IL-6 was detected by incubation with biotin-conjugated MP5-32C11 followed by horseradish peroxidase-conjugated avidin. Plates were developed as described above. IL-6 levels were estimated from a standard curve with known concentrations of murine recombinant IL-6 (R & D Systems).

RESULTS

Analysis of B-cell function during arthritis development in C3H/HeJ mice infected with *B. burgdorferi*. To determine whether the B-cell mitogen that had been demonstrated in vitro was functioning in the infected mice, we performed a detailed kinetic analysis of arthritis development and levels of IgG, IgM, and IL-6 in serum. A group of 24 C3H/HeJ mice, 6 weeks of age, were infected by intradermal injection with 2×10^6 *B. burgdorferi* cells. Another 24 C3H/HeJ mice were injected with sterile medium. Joint swelling was measured at the time points indicated in Fig. 1. All C3H/HeJ mice infected with *B. burgdorferi* developed histologically severe arthritis within a reproducible time frame (Fig. 1A), consistent with the findings of Barthold et al. (3, 5). Serum samples were collected, and IL-6, IgG, and IgM were assayed at each time point (Fig. 1). Joint measurement showed that swelling started at 10 to 14 days, peaked at 3 weeks (Fig. 1A), and gradually diminished after 12 weeks

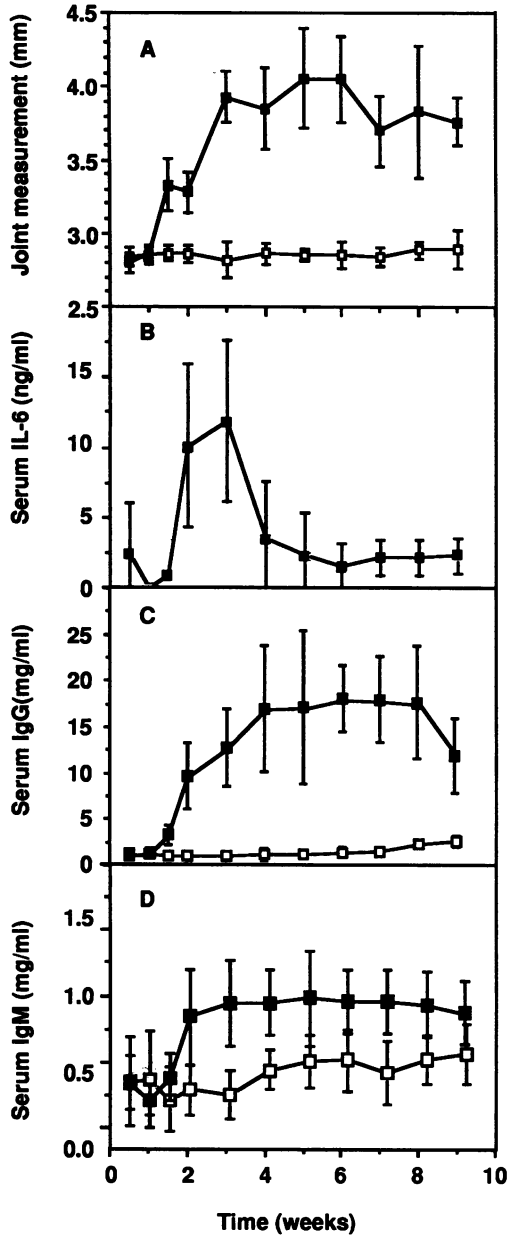

FIG. 1. Measurement of joint swelling and IL-6, IgG, and IgM levels in infected C3H/HeJ mice. C3H/HeJ mice injected with 2×10^6 *B. burgdorferi* cells (■) or sterile medium (□) were studied for ankle joint measurement (A), IL-6 levels in serum (B), IgG levels in serum (C), and IgM levels in serum (D) as described in Materials and Methods. Values represent the mean ± standard deviation of samples from 12 mice at each point. IL-6 was not detectable in serum from control mice.

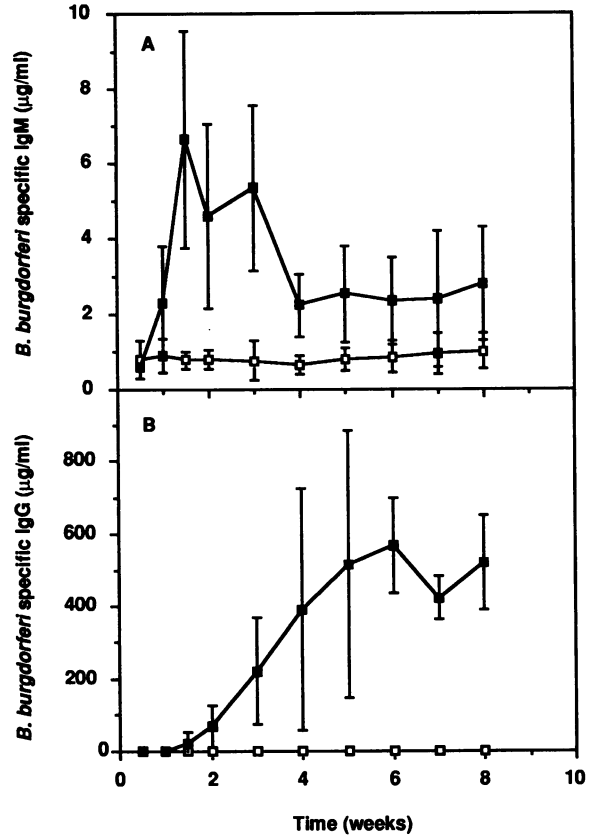

FIG. 2. Measurement of anti-*B. burgdorferi* IgG and IgM levels in serum of C3H/HeJ mice infected with *B. burgdorferi*. Serum samples were collected from three control (□) and three infected (■) C3H/HeJ mice at each time point. The concentration of IgM (A) and IgG (B) bound to the plates coated with *B. burgdorferi* sonicated antigen was estimated as described in Materials and Methods. Values represent the mean ± standard deviation of samples from three mice.

(data not shown). In some animals the IL-6 level in serum was elevated at day 4, but this was not detected in others (Fig. 1B). It is possible that this early spike in IL-6 was missed with the single time point. IL-6 levels returned to baseline at 1 week and were elevated again at 2 to 3 weeks (Fig. 1B). They remained elevated in most animals for the duration of the experiment. At no time was IL-6 detected in the serum of control mice. In infected mice, levels of total IgG in serum were elevated 10-fold and remained elevated for 8 to 9 weeks (Fig. 1C). Total IgM levels increased about twofold, with a similar time course as IgG levels (Fig. 1D), whereas IgA levels were not elevated in the infected mice (data not shown).

To determine the proportion of immunoglobulin in infected mice that was specific for *B. burgdorferi*, we established a quantitative ELISA (described in Materials and Methods). This allowed approximate determination of the concentration of specific antibodies of both IgM and IgG classes in the serum of infected mice. All infected mice produced antigen-specific IgM antibodies in the first 2 weeks after infection. The IgM level peaked at approximately 7 µg/ml and then dropped after the second week of infection (Fig. 2A). Levels of *B. burgdorferi*-specific IgG were much higher than the specific IgM levels, and greater variation among individual mice was observed (Fig. 2B). *B. burgdorferi*-specific IgG was first detected 2 weeks postinfection, and the level gradually increased to about 600 µg/ml by 5 to 6 weeks.

As an indication of polyclonal B-cell activation, the presence of immunoglobulins directed against non-*B. burgdorferi* antigens was assessed. Serum collected from infected and control C3H/HeJ mice at 4 weeks postinfection was analyzed for anti-ovalbumin antibody. Levels of antibody to ovalbumin were low in control C3H/HeJ mice and were elevated 10- to 15-fold in infected mice (Fig. 3). This is

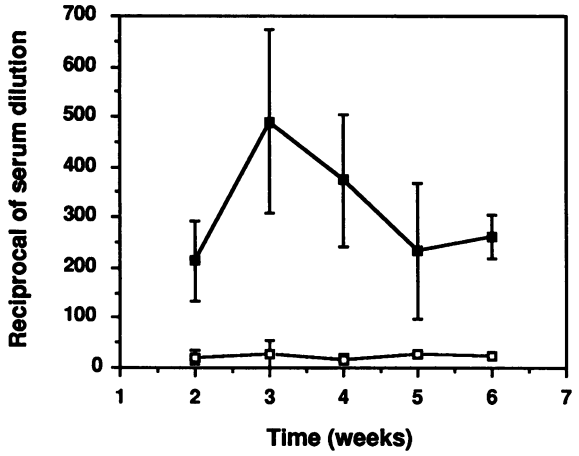

FIG. 3. Anti-ovalbumin titer in the serum of C3H/HeJ mice infected with *B. burgdorferi*. Serum samples were collected from six infected mice and two control mice 4 weeks after injection and assayed for anti-ovalbumin IgG. Values represent the mean ± standard deviation of anti-ovalbumin titers from six infected mice (■) and two control mice (□).

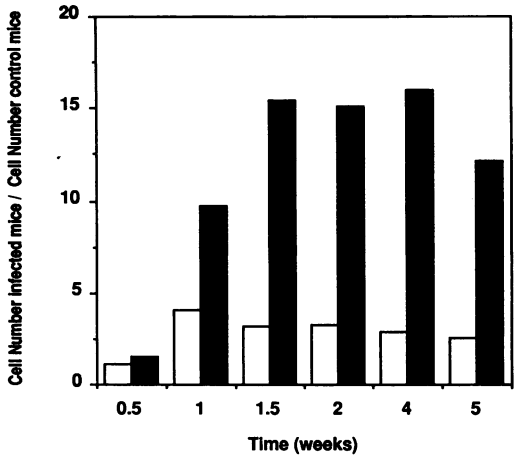

FIG. 4. Expansion of B- and T-lymphocyte populations in infected C3H/HeJ mice. Three control and three infected mice were sacrificed at each time point for population analysis. Popliteal, superficial inguinal, and axillary lymph nodes were pooled for each mouse, and single-cell suspensions were prepared and enumerated. Lymph node cells were stained with fluorescein isothiocyanate-conjugated anti-mouse IgM μ chain antibody to detect B cells and anti-mouse CD4 and CD8 antibody to detect T cells. Samples were analyzed with a FACScan flow cytometer. Open and solid bars represent the fold increase of T-cell and B-cell numbers, respectively, in infected animals compared with control animals.

similar to the elevation in total IgG levels in serum (Fig. 1). The presence of antibodies to collagen in these mice was also assessed, since anti-collagen antibodies are capable of causing arthritis (17, 36). IgG specific for native mouse collagen type II was not detected in serum from control C3H/HeJ mice as determined by ELISA, nor was it found in serum from infected mice (data not shown).

Analysis of lymph node populations and histological abnormalities in infected C3H/HeJ mice. Thirty-six additional 6-week-old C3H/HeJ mice were infected by intradermal injection with 2×10^6 *B. burgdorferi* cells or given sterile medium by injection. Three mice from control and infected groups were sacrificed at days 4, 7, and 10 and weekly thereafter. Major peripheral lymph nodes including popliteal, superficial inguinal, and axillary were collected and determined by weight to be approximately fivefold larger than those from control mice by 7 days of infection. The total cell number of the major draining lymph nodes was also increased about fivefold in infected animals. To identify the expanded population of lymphocytes, cells from these peripheral lymph nodes were pooled, stained with markers for different cell types, and analyzed with a FACScan flow cytometer. The total numbers of B and T cells from each of three control and infected animals were determined for each time point and used to determine the relative increase in the number of each cell type (Fig. 4). Both B- and T-lymphocyte numbers were increased in the infected animals, with the number of B cells increasing 10- to 16-fold and the number of T cells increasing 3- to 4-fold from weeks 1 to 5 (Fig. 4). For example, at 2 weeks postinfection $(1.91 \pm 0.37) \times 10^7$ B cells and $(2.27 \pm 0.44) \times 10^7$ T cells were recovered from the peripheral nodes of infected mice while $(1.26 \pm 0.5) \times 10^6$ B cells and $(6.96 \pm 2.3) \times 10^6$ T cells were recovered from control animals. The B-cell population in the spleen was expanded by about twofold, indicating that there was a systemic increase in the number of B lymphocytes and not a selective sequestering of these cells in the peripheral nodes.

B cells positive for the surface marker CD5, which is present on only a subpopulation of normal B cells, were quantified by double staining with fluorescein isothiocyanate-conjugated goat anti-IgM and phycoerythrin-conjugated anti-CD5. As expected, the overall number of $CD5^+$ B cells rose in infected animals, reflecting the increase in the total B-cell number; however, the percentage of B cells positive for CD5 in spleens remained at 5% during infection. The percentages of T cells positive for CD4 and CD8 were approximately 75 and 25%, respectively, throughout the experiment in both control and infected mice.

Rear ankle joints taken from these animals for histology studies gave results in agreement with those reported by Barthold et al. (3, 5). The comparison between infected and control C3H/HeJ mice was consistent and dramatic. The rear ankle joints were most severely affected, the abnormalities being observed most consistently in the tendon sheath synovia. Proliferating synovial cells and an influx of neutrophils were usually observed. The joint synovium was also generally thickened, with infiltrating neutrophils. The joint space itself was mildly involved, with evidence of proteinaceous debris and infiltrating neutrophils and plasma cells. The cartilage and bone of the joints was intact, with no evidence of destruction. The involvement of the tendon and tendon sheaths frequently led to the formation of nodules infiltrated with neutrophils (Fig. 5A). Furthermore, foci of proliferating chondrocytes were observed within the tendon sheath (Fig. 5B). Foci of chondrocyte proliferation were detected in joints from all 15 infected animals sacrificed between 10 days and 5 weeks.

Analysis of joint measurement, circulating immunoglobulin, and IL-6 levels in serum in BALB/c mice. Infected mice of a different genetic background were analyzed for evidence of the in vivo expression of the mitogen. BALB/c mice were chosen because they are readily infected with *B. burgdorferi* (2), maintain a persistent infection (3), but develop mild arthritis in contrast to the severe arthritis found in infected C3H/HeJ mice (3). C3H/HeJ mice were included for comparison in this experiment and to ensure that the spirochete cultures used to inoculate the BALB/c mice had arthritic

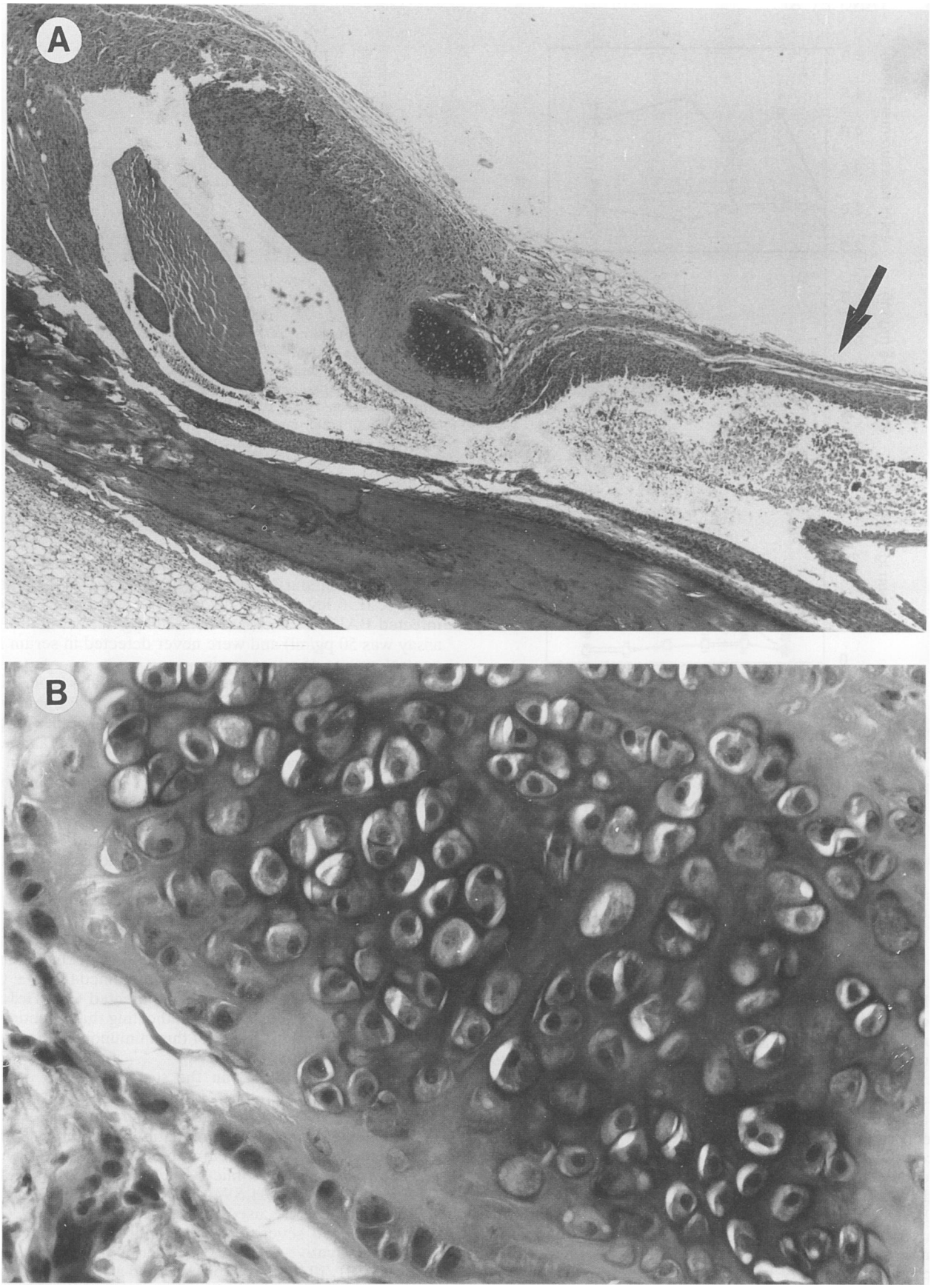

FIG. 5. Tendonitis and chondrocyte proliferation in rear ankle joints from a C3H/HeJ mouse 5 weeks after infection. Pictures were taken with objective lenses of 2.5× (A) and 40× (B). The arrow in panel A points out a nodule filled with neutrophils and other inflammatory cells. Panel B displays an example of a focus of chondrocyte proliferation.

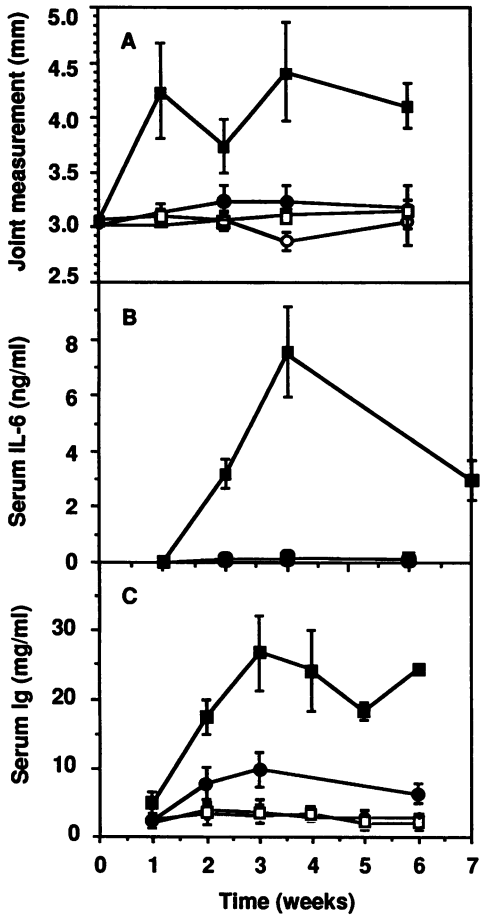

FIG. 6. Detection of ankle joint swelling and IL-6 and immunoglobulin levels in serum in BALB/c and C3H/HeJ mice. Joint measurements (A) represent the mean ± standard deviation from BALB/c control mice (○), BALB/c infected mice (●), C3H/HeJ control mice (□), and C3H/HeJ infected mice (■). Each group consisted of six mice. Serum samples collected from these mice at the indicated time points were assayed for total immunoglobulin (B) and IL-6 (C) levels by antibody capture ELISA. Immunoglobulin and IL-6 levels represent the mean ± standard deviation from three to six mice in each group. IL-6 was not detectable in serum from control mice.

potential. Six mice from each strain were injected intradermally with 2×10^6 spirochetes in 20 μl of BSKII medium. Another six mice from each strain injected with 20 μl of sterile BSKII medium served as controls. Rear ankle joints were measured each week with a metric caliper. Serum from each mouse was collected at the indicated time points and assayed for total immunoglobulin and IL-6 levels. Joint measurement showed that the swelling started 1 week postinfection in C3H/HeJ mice, decreased somewhat in the second week, but remained present from week 3 (Fig. 6A). Infected BALB/c mice displayed very little joint swelling, and swelling was not observed in the control mice. Infected C3H/HeJ mice displayed a more severe joint swelling than BALB/c mice. Histological observations of the joints confirmed that C3H/HeJ mice had severe arthritis while BALB/c mice developed only modest arthritis, consistent with the findings of Barthold et al. (3, 5). Concurrent with the joint swelling, levels of IL-6 in serum and levels of circulating immunoglobulin were elevated (Fig. 6B and C). C3H/HeJ

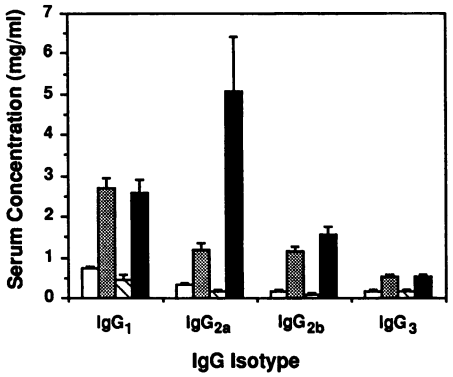

FIG. 7. Detection of IgG isotypes in serum samples from BALB/c mice and C3H/HeJ mice. Serum samples were collected from six control and six infected mice of both strains 5 weeks postinfection. Open bars represent BALB/c controls, stippled bars represent infected BALB/c mice, hatched bars represent C3H/HeJ controls, and solid bars represent infected C3H/HeJ mice. Values represent the mean ± standard deviation of samples from six mice for each isotype of IgG.

mice displayed a 10- to 15-fold increase in the level of total immunoglobulin, whereas in BALB/c mice the increase was fivefold. IL-6 levels in serum were also increased in infected C3H/HeJ mice, starting at week 2 and peaking at 8 ng/ml at week 3. IL-6 levels were detected but were much lower in infected BALB/c mice (less than 300 pg/ml; the limit of the assay was 50 pg/ml) and were never detected in serum from control groups of either strain.

In a separate experiment, serum samples collected 4 weeks postinfection from C3H/HeJ and BALB/c mice were further analyzed for the expression of IgG isotypes (Fig. 7). Serum samples from six control and six infected animals of each strain were analyzed. The IgG1 level in serum was increased fivefold in infected C3H/HeJ mice and fourfold in infected BALB/c mice relative to controls. IgG2a and IgG2b levels were increased 28- and 15-fold, respectively, in infected C3H/HeJ mice. In contrast, IgG2a and IgG2b levels were increased by only four- and sevenfold, respectively, in infected BALB/c mice. The IgG3 level was increased threefold in both C3H/HeJ and BALB/c mice. Serum samples collected at 4 weeks were also analyzed for IgA. The IgA level in serum in BALB/c mice was 0.182 ± 0.03 mg/ml for control animals and 0.189 ± 0.021 for infected animals. Serum from control C3H/HeJ mice contained 0.221 ± 0.007 mg of IgA per ml, and serum from infected C3H/HeJ mice contained 0.184 ± 0.015 mg/ml, indicating that infection had very little effect on the levels of this immunoglobulin class in serum.

Influence of the MHC on the susceptibility to arthritis. C3H/HeJ, C57BL/10, and BALB/c mice develop arthritis of different severity after *B. burgdorferi* infection (3). To determine whether major histocompatibility complex (MHC) genes affected the susceptibility to development of arthritis, we compared congenic mice, differing only at the MHC. C3H/HeN ($H\text{-}2^k$), C3H.SW ($H\text{-}2^b$), C57BL/10 ($H\text{-}2^b$), and B10.BR ($H\text{-}2^k$) were used in this study. C3H/HeN ($H\text{-}2^k$) mice developed severe arthritis, similar to the endotoxin-resistant derivative C3H/HeJ as described by Barthold et al. (3, 5). C57BL/10 ($H\text{-}2^b$) mice developed arthritis of moderate severity (3, 5). Histological observations of the rear ankle joints taken at different times postinfection showed that C3H.SW ($H\text{-}2^b$) mice, which share background genes with

TABLE 1. Influence of MHC on the outcome of *B. burgdorferi* infection

Genotype	Arthritis				
	Frequency of joint swelling[a] at week:				Rank severity (week 4)[b]
	1	2	3	4	
C3H/HeN (*H-2*^{k})	9/9	9/9	9/9	9/9	++++
C3H.SW (*H-2*^{b})	9/9	9/9	9/9	9/9	++++
C57BL/10 (*H-2*^{b})	0/5	0/5	2/5	2/5	++
B10.BR (*H-2*^{k})	0/5	0/5	2/5	2/5	++

[a] Fractions represent number of positive mice/total number infected.
[b] Rank severity of arthritis is based on blind ranking of histology of all infected mice of each genotype on a scale of + (least) to ++++ (worst).

C3H/HeN but are of the *H-2*^{b} haplotype, developed arthritis with 100% frequency and the same severity as the C3H/HeN mice (Table 1). Infected B10.BR (*H-2*^{k}) mice, which have the same genetic background as C57BL/10 and the MHC haplotype of C3H/HeN (*H-2*^{k}), developed moderate arthritis with a lower frequency, similar to C57BL/10 (Table 1). These results suggest that the MHC haplotype does not determine the development of arthritis and that genes outside the MHC are involved.

DISCUSSION

Immunological abnormalities, including hyperactive B cells, elevated IgM levels in serum, lymphadenopathy, impaired natural killer function, and delayed development of humoral immunity, have been documented in patients with Lyme disease (11, 16, 29, 30, 32). This has suggested a possible involvement of the specific or innate host responses in the pathogenesis associated with stage 2 and 3 disease (32). Our laboratory has characterized a polymyxin B-insensitive B-cell mitogen produced by *B. burgdorferi* (27). This mitogen stimulates IgM production by splenocytes from naive mice and stimulates the murine B-cell tumor line CH12.LX (21) to produce IgM (27). Because of the persistent nature of infection and the ability of the organism to gain access to the joint and other tissues (5, 15, 29), a B-cell mitogen present during infection could play a role in the pathology of Lyme disease. To support this possibility, it was important to determine whether the mitogen functioned in vivo. This paper provides evidence that a *B. burgdorferi* mitogen is active in vivo in infected animals. Three lines of evidence support the conclusion that B-cell activation in vivo is polyclonal or oligoclonal in addition to being antigen specific. First, the level of IgG in serum in infected mice was elevated about 10- to 15-fold, with the value ranging from 10 to 15 mg/ml (Fig. 1C). In comparison, the amount of IgG specific for *B. burgdorferi* antigens was approximated at 0.6 mg/ml (Fig. 2). Second, the number of B lymphocytes in peripheral lymph nodes of infected animals was increased 10- to 15-fold, with a 5-fold rise in the ratio of B to T cells (Fig. 4). The number of B cells also increased about twofold relative to the number of T cells in spleens from C3H/HeJ-infected mice. Third, the IgG titer in the serum of infected animals to an unrelated antigen, ovalbumin, was increased 10- to 15-fold, which resembles the increase in the total IgG level (Fig. 3). These findings suggest that levels of autoreactive antibodies might be also expanded in infected animals, although anti-collagen antibodies were not identified. Because CD5$^+$ B cells have been shown to produce autoreactive antibodies and are selectively increased in patients with rheumatoid arthritis (7), we determined whether they were expanded in *B. burgdorferi*-infected animals. No selective increase in the number of B cells of this lineage were found in C3H/HeJ animals at any stage of infection. Further studies are required to determine whether autoreactive antibodies are generated during infection.

We previously observed that sonicated *B. burgdorferi* cells stimulate splenocytes to produce IL-6 in vitro (27). IL-6 stimulation could be a property of the mitogen or could reside in a distinct *B. burgdorferi* molecule. In this study we found elevated levels of IL-6 in the serum of infected C3H/HeJ mice, suggesting that this *B. burgdorferi* activity was also expressed in vivo. Because of the potent B-cell-stimulatory effect of IL-6, this activity may serve to enhance immunoglobulin production by mitogen-stimulated B cells and contribute to the magnitude of the increase in total IgG levels seen in infected C3H/HeJ mice. In other studies, excessive production of IL-6 has been linked to hypergammaglobulinemia and autoantibody production (35) and has been found in arthritic synovia (24, 25), suggesting a possible role in the pathology of *B. burgdorferi* infection. Cytokine effects are probably also very important in the local environment of infected tissues. Since spirochetes are able to enter many tissues, cytokine stimulatory products could act directly on resident cells. For example, B cells, synovial cells, or macrophages in affected joints could be stimulated to secrete cytokines, such as IL-1 and IL-6. Histological findings of chondrocyte proliferation in the tendon sheath of affected joints may reflect localized elevation of IL-1 and IL-6 levels, since chondrocytes do respond to these and other cytokines (28, 34). Others have shown that *B. burgdorferi* and purified *B. burgdorferi* LPS stimulate IL-1 production by monocytes and IL-6 production by glial cells (6, 18, 19). The in vitro (19, 27) and, now, in vivo demonstration of IL-6 stimulation by *B. burgdorferi* suggests that the cytokine response of cells of various types residing in invaded tissue could contribute to the pathology.

Analysis of mice with distinct genetic background also provided evidence for the in vivo expression of the *B. burgdorferi* mitogen (Fig. 6). Infected BALB/c mice expressed a three- to fourfold increase in the level of circulating immunoglobulins, which was clearly elevated relative to that of control animals but was significantly lower than that seen with infected C3H/HeJ mice. Serum samples from infected BALB/c mice had low levels of IL-6 (less than 300 pg/ml) compared with samples from infected C3H/HeJ mice, which peaked in the range of 8 to 10 ng/ml at 2 to 3 weeks postinfection (Fig. 1 and 6). BALB/c mice consistently displayed mild arthritis in this study (Fig. 6) and in the previous work of Barthold et al. (3), whereas C3H/HeJ mice consistently displayed severe arthritis (Fig. 1 and 6). The difference in the magnitude of the response to the B-cell mitogen and IL-6 stimulatory activity in infected mice of these strains is correlated with the difference in severity of arthritis and suggests that the response to the mitogen could be involved in arthritis development. It is also possible that these strain-specific differences reflect differences in the number of persistently infecting spirochetes, because even though equivalent numbers of bacteria were injected, this does not ensure equivalent distribution throughout tissues of the mice.

It is also important to consider the influence that other aspects of the host response to *B. burgdorferi* infection could play in the outcome of mitogen stimulation. For example, the *B. burgdorferi* mitogen stimulates mouse splenocytes to

produce IgM in vitro (27), whereas in vivo the elevation is mainly in IgG levels (Fig. 1, 6, and 7). This may reflect the presence of cytokines produced by antigen-specific T cells and their influence on isotype switching (13, 14). Class switching of IgM to IgG induced by cytokines in the infected animals could be important for the development of disease. Our experiment showed that levels of all subclasses of IgG were elevated in the arthritic C3H/HeJ mice, with IgG1, IgG2a, and IgG2b levels predominantly increased (Fig. 7). In infected BALB/c mice, the IgG1 level was elevated as much as in the C3H/HeJ animals, but the elevation in the IgG2a level was much smaller than in C3H/HeJ mice (4-fold for BALB/c versus 28-fold for C3H/HeJ) (Fig. 7). Because IgG2a activates complement and binds to phagocytic cells, it could directly mediate tissue damage (13). Therefore, if autoantibodies are mediating disease in *B. burgdorferi*-infected mice, differences in the IgG isotype may be significant. Importantly, IgA levels were not increased in *B. burgdorferi*-infected mice.

Two types of helper T cells that may be important in the outcome of microbial infections have been characterized. Cells of the Th1 phenotype make the cytokine gamma interferon, which stimulates IgG2a production, whereas cells of the Th2 phenotype produce IL-4, which stimulates IgG1 production. Others have shown that the cytokines produced by antigen-specific T cells influence the isotype of IgG production by both antigen-specific and antigen-unrelated B lymphocytes (14). Our findings suggest that there may be strain-related differences in helper T-cell activity or function, with BALB/c mice displaying primarily a Th2 phenotype response and C3H/HeJ mice developing both Th1 and Th2 responses. The recent isolation of T-cell clones with the Th1 phenotype from humans with Lyme disease is therefore relevant to our findings of IgG isotypes stimulated by cytokines associated with Th1 responses in the arthritic C3H/HeJ mice (13, 37). These findings suggest that there may be a correlation between IgG2a appearance and pathology or between the presence of specific cytokines produced by antigen-specific T cells and disease. The role played by IgG of a particular isotype produced by mitogen-activated B cells will be a subject for further analysis.

The mouse model of Lyme disease offers an opportunity to study host factors involved in the development of disease and to compare inflammatory events in mice which develop severe arthritis with events in mice which develop only mild arthritis. The arthritis observed in the C3H/HeJ mice did not involve loss of cartilage integrity even in the presence of infiltrating neutrophils (Fig. 5), (3, 5), which is consistent with the absence of anti-collagen antibodies. Furthermore, C3H/HeJ mice did not develop collagen-induced arthritis, and this trait has been mapped to MHC haplotype $H\text{-}2^k$ (36). Studies with inbred mice (Fig. 3) indicate that genetically determined traits of the host are responsible for susceptibility to *B. burgdorferi*-induced arthritis (3, 5). We studied the involvement of genes of the MHC by using C3H/HeJ and the congenic C3H.SW ($H\text{-}2^b$) and the congenic C57BL/10 ($H\text{-}2^b$) and B10.BR ($H\text{-}2^k$). With this group of congenic mice, the severity of arthritis was determined not by the MHC but rather by genes outside this locus (Table 1). These findings are not in agreement with those of Schaible et al. (26), and this discrepancy may reflect different choices of congenic strains and different suppliers of inbred mice, a variable we have found to affect arthritis development in our laboratory. Genetic traits involved in arthritis development may be related to cytokine production in response to the bacteria, both by antigen-specific T lymphocytes and by other responsive cell types.

In summary, a number of new findings are reported in this paper. (i) There is polyclonal B-cell activation in *B. burgdorferi*-infected mice, providing evidence that the mitogen is active in vivo; (ii) the isotype of IgG found in C3H/HeJ mice suggests the presence of both Th1 and Th2 phenotype helper T cells, whereas the Th2 type of help is suggested to be predominant in BALB/c mice; and (iii) the level of IL-6 is elevated in the arthritic C3H/HeJ mice but is much lower in infected BALB/c mice. It is attractive to speculate that the interplay among mitogen-activated B cells, IL-6, and cytokines produced by antigen-specific T cells is involved in the development of arthritis. The contrasts between C3H/HeJ and BALB/c mice suggest that the host immune response may influence the response to the mitogen. Whether the degree of severity of arthritis is directly influenced by these responses will require further analysis.

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