Coinfection with *Borrelia burgdorferi* and the Agent of Human Granulocytic Ehrlichiosis Alters Murine Immune Responses, Pathogen Burden, and Severity of Lyme Arthritis

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Received 4 December 2000/Returned for modification 17 January 2001/Accepted 30 January 2001

Lyme disease and human granulocytic ehrlichiosis (HGE) are tick-borne illnesses caused by *Borrelia burgdorferi* and the agent of HGE, respectively. We investigated the influence of dual infection with *B. burgdorferi* and the HGE agent on the course of murine Lyme arthritis and granulocytic ehrlichiosis. Coinfection resulted in increased levels of both pathogens and more severe Lyme arthritis compared with those in mice infected with *B. burgdorferi* alone. The increase in bacterial burden during dual infection was associated with enhanced acquisition of both organisms by larval ticks that were allowed to engorge upon infected mice. Coinfection also resulted in diminished interleukin-12 (IL-12), gamma interferon (IFN-γ), and tumor necrosis factor alpha levels and elevated IL-6 levels in murine sera. During dual infection, IFN-γ receptor expression on macrophages was also reduced, implying a decrease in phagocyte activation. These results suggest that coinfection of mice with *B. burgdorferi* and the HGE agent modulates host immune responses, resulting in increased bacterial burden, Lyme arthritis, and pathogen transmission to the vector.

*Borrelia burgdorferi*, the spirochete that causes Lyme disease, is the most common arthropod-borne pathogen in the United States (35). Over the past 5 years, it has become apparent that the *Ixodes scapularis* ticks that harbor *B. burgdorferi* also transmit the agent of human granulocytic ehrlichiosis (HGE), among other pathogens (18, 20, 22, 23, 59, 70). The agent of HGE is a newly described obligate intracellular pathogen with a tropism for the neutrophil (7). Coinfection with *B. burgdorferi* and the HGE agent has been documented in humans (1, 7, 48, 71), ticks (20, 59, 70), and mice (49). However, the frequency of dual infection and its effect on the course of disease is not known. Laboratory mice can also be infected with *B. burgdorferi* (64, 65) or HGE bacteria (38, 39, 70), and murine models of Lyme borreliosis and granulocytic ehrlichiosis (11, 13, 16, 39) have facilitated studies on these pathogens.

The pathogenesis of Lyme arthritis has been studied in both humans and mice. In humans, *B. burgdorferi* infection commonly results in a pathognomonic skin rash named erythema migrans, and persistent infection can lead to the development of Lyme arthritis (36, 66, 67). Human Lyme arthritis is associated with CD4+ T-cell helper type 1 (Th1) responses to *B. burgdorferi*, including increased gamma interferon (IFN-γ) production by T cells in affected joints (34, 73, 74). The experimental murine model of Lyme arthritis provides some similarities with human joint disease (9). C3H/He mice, which are susceptible to the development of Lyme arthritis, generate high levels of IFN-γ, consistent with a murine Th1 phenotype (45). In contrast, BALB/c mice, which are relatively resistant to Lyme arthritis, develop higher levels of interleukin-4 (IL-4), indicative of a predominant Th2 response (43, 45). Moreover, neutralization of IFN-γ or IL-12 reduces Lyme arthritis in C3H/He mice and inhibition of IL-4 exacerbates disease in BALB/c mice, further demonstrating the importance of CD4+ Th1-cell differentiation in the genesis of joint inflammation (4, 55).

Antibodies to *B. burgdorferi* can also influence the course of Lyme disease. In humans, the development of high-titer BBK32, also known as P35, antibodies during early-stage Lyme disease is associated with a decreased risk of progression to Lyme arthritis (31–33). Similarly, passive transfer of *B. burgdorferi* immune sera (12, 29) can induce disease regression in mice, and outer surface protein C (OspC) (32), decorin-binding protein A (DbpA) (24), or BBK32 (28) antibodies can partially clear *B. burgdorferi* from an infected animal. Therefore, both the host humoral and cellular responses to *B. burgdorferi* can modify the course of spirochete infection and the severity of arthritis (41).

The first case of HGE was described in 1994 (19). The HGE agent is very similar to *Ehrlichia equi* and *Ehrlichia phagocytophilum* and preferentially resides within the neutrophil (19). Fever, myalgia, thrombocytopenia, leukopenia, and anemia often mark infection (17, 22). Morulae containing the HGE agent are present in peripheral neutrophils of some patients in the early stages of infection (22). In addition morulae can be detected during the first weeks of murine infection with the HGE agent, partially resembling human illness (39, 70). In general, immunocompetent mice clear HGE bacteria from the bloodstream within several weeks, while HGE organisms reside within the polymorphonuclear leukocytes of severe combined immunodeficient (SCID) mice for several months (39), suggesting that acquired immune responses help control this pathogen. This is supported by observations that antibodies to...
HGE provide partial protection from infection (46, 69). Moreover, immunocompetent mice develop high levels of IFN-γ after challenge with the HGE agent (3, 54), and organism levels are elevated in mice deficient in IFN-γ (3), indicating that IFN-γ helps control ehrlichial propagation.

Dual infection involving *B. burgdorferi* and the HGE agent has been documented in humans and mice (7, 49, 52, 56). In addition, ticks may be colonized by both pathogens (49, 59). In a number of coinfection scenarios, the influence of one or both organisms on the host immune response has been associated with the inhibition or exacerbation of disease. For example, Santiago et al. reported that coinfection with *Toxoplasma gondii* and *Leishmania major* inhibited the tissue parasitism observed with *L. major* alone (61). Helmby et al. demonstrated higher levels of malaria parasitemia in mouse coinfection with *Schistosoma mansoni* and *Plasmodium chabaudi*, and increased disease was accompanied by lower tumor necrosis factor alpha (TNF-α) responses to *P. chabaudi* and reduced Th2 responses to *S. mansoni* (37). Higher mortality rates have also been demonstrated in rabbits coinfected with enteropathogenic *Escherichia coli* and the obligate intracellular bacterium *Lawsonia intracellularis* (62), and Marshall et al. showed that increased TNF-α production resulted in the death of mice coinfected with *T. gondii* and *S. mansoni* (53). To date, well-documented cases of acute infection with HGE bacteria and *B. burgdorferi* have been more infrequent than serologic evidence.

**FIG. 1.** Levels of *B. burgdorferi* and the HGE agent after 1 week of coinfection. DNAs from specific tissues were pooled from five mice (from each group of animals) and analyzed by PCR with primers specific for either *B. burgdorferi ospA* or hge-44. Bladder (A) and skin (B) DNAs from singly *B. burgdorferi*-infected (lanes 1) and coinfecteda (lanes 2) mice were analyzed for *ospA*. Blood (C), joint (D), heart (E), and splenic (F) DNAs were analyzed by dilutional PCR for hge-44. The numbers represent concentrations of total DNA used in the PCR. PCR for β-actin was performed with 1 μg of total DNA to ensure that equal amounts of DNA were used. These data represent samples from one experiment with five mice in each group. Five separate experiments with different mice yielded similar results.
of exposure to both pathogens. *B. burgdorferi* and HGE bacteria can, however, be transmitted by the same tick bite (63), and coinfection could influence the host immune response and disease outcome. We have now investigated the effect of murine infection with *B. burgdorferi* and the HGE agent on infection, transmission, immune responses, and severity of Lyme arthritis.

**MATERIALS AND METHODS**

Mice, bacteria, and ticks. Six-week-old C3H/HeN (C3H) and C3H/HeN-scid (C3H-scid) mice were obtained from the Frederick Cancer Research Center (Frederick, Md.). Mice were maintained in filter-framed cages and euthanized by saline-buffered saline (PBS). The cell pellet was resuspended in 500 μl of PBS. The percentage of neutrophils with *Ehrlichia* was determined by the method of Bradford (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer’s instructions.

Infection of mice. Mice were infected with either 10,000 spirochetes administered by intradermal injection according to established protocols (27) or 100 μl of HGE-infected C3H-scid blood by intraperitoneal injection (3). In coinfection studies, mice were infected with each organism using a separate inoculation. Mice were sacrificed at 1 week, 2 weeks, and 2 months following infection. Tissues were harvested and stored at −20°C until use.

DNA preparation and PCR. *B. burgdorferi*-specific DNA was amplified from spirochetes cultured for 14 days. Spirochetes were pelleted at 13,000 × g, followed by three washes with phosphate-buffered saline (PBS). The cell pellet was resuspended in 500 μl of H2O and disrupted by sonication with three 10-s pulses. Insoluble material was pelleted at 16,000 × g for 1 min. The supernatant (*B. burgdorferi* lysate) was collected and stored at −20°C until use. HGE-infected HL-60 and uninfected HL-60 cell lysates were obtained by pelleting cells at 4,000 × g, followed by three washes with PBS. The cell pellet was resuspended in 500 μl of H2O and sonicated as indicated above. Insoluble material was removed by centrifugation, and the supernatants (HGE lysate and HL-60 lysate) were stored at −20°C until use. Protein concentrations were determined by the method of Bradford (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer’s instructions.

**RESULTS**

Quantification of *B. burgdorferi* spirochetes and coinfection could influence the host immune response and disease outcome. We have now investigated the effect of murine infection with *B. burgdorferi* and the HGE agent on infection, transmission, immune responses, and severity of Lyme arthritis.

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FIG. 2. Coinfection elevates levels of the HGE agent and *B. burgdorferi* at 2 weeks. DNAs from various tissues of the five mice in each group were pooled and analyzed by PCR with primers specific for either *B. burgdorferi* *ospA* or *hge-44*. Skin (A) and joints (B) were analyzed by competitive PCR with primers specific for *B. burgdorferi* *bbk50* (also known as *p37*) (28, 31). Lanes 1 through 7 contain 10-fold dilutions (87 to 0.000087 fg) of competitor DNA. Primers specific for *ospA* were used to amplify heart (C) and bladder (D) DNAs from singly *B. burgdorferi*-infected (lanes 1) and coinfectected (lanes 2) mice. Joint DNA (E) was analyzed by dilutional PCR for *hge-44*. Splenic DNA (F) from mice either infected with HGE only (lane 1) or coinfectected (lane 2) was analyzed for *hge-44*. Skin DNA (G) from mice infected for 2 months was analyzed by competitive PCR with primers specific for *B. burgdorferi* *bbk50*. Lanes 1 through 9 contain fourfold dilutions (0.137 to 0.000002 fg). PCR for *β-actin* was performed with 1 μg of total DNA to ensure that equal amounts of DNA were used. Five separate experiments were performed, with similar results. Results from one of these five representative experiments are presented.
In vitro stimulation of whole splenocytes. Whole splenocytes were isolated as previously described (5). All assays were performed in duplicate in each study, and at least three separate experiments were performed. Briefly, spleens were mechanically disrupted, followed by depletion of red cells. For B. burgdorferi stimulation, either 10 μg of B. burgdorferi lysate or 4 μg of concanavalin A (ConA) was added to 10^6 cells/ml. For HGE stimulation, the same number of splenocytes were incubated either with 10 μg of lysate from HGE-infected HL-60 cells or uninfected HL-60 cells or with 4 μg of ConA. Supernatants were collected after 48 h of incubation for cytokine determination.

Histopathology of murine Lyme arthritis. Knees and tibiotarsal joints were fixed with formalin, embedded in paraffin, and examined microscopically for evidence of disease. Arthritis in both the joints and knees of each mouse was assessed as described previously (27). Disease severity was scored on a scale of 0 (no disease), 1 (mild disease), 2 (moderate disease), and 3 (severe disease). The joints, tendons, or ligamentous sheaths were examined for the presence of fibrinoid exudation and necrosis and neutrophil infiltration. The synovium was examined for hypertrophy and hyperplasia. Mild disease consisted of neutrophil infiltration of one ligament, tendon sheath, or joint. Moderate disease was marked by neutrophil infiltration in more than one tendon sheath and one joint and at least some evidence of fibrinoid exudation. Severe disease was marked by neutrophil infiltration of two or more tendon sheaths or joints, with fibrinoid necrosis and synovial hypertrophy. All measurements were made in a double-blinded fashion.

Flow-cytometric analysis. Splenocytes in single-cell suspensions at 10^6 cells in 100 μl were surface stained with a panel of antibodies labeled with either FITC, biotin, phycoerythrin (PE), or Cy-chrome (Pharmingen). CD4^+ T cells, macrophages, and neutrophils were specifically labeled with CD4-Cy-chrome, Mac-3-PE, or Ly6-G-FITC, respectively, for 30 min at room temperature. CD4^+ T cells were stained for CD25 and CD40 ligand. Macrophages were stained for either IFN-γ receptor (CD119a) or major histocompatibility complex (MHC) class II (I-E^k and I-A^k). Neutrophils were stained with Mac-1 and IFN-γ receptor. When necessary, a secondary staining was performed with streptavidin-PE or streptavidin-FITC after washing the samples. After the final incubation, the cells were washed and analyzed with a flow cytometer using the Cell Quest software package (Becton Dickinson, Franklin Lakes, N.J.).

RESULTS

Infection of mice with B. burgdorferi and the HGE agent. Groups of five mice were challenged with both B. burgdorferi and the HGE agent to assess the influence of these two pathogens on the course of infection. As controls, mice were challenged with either HGE bacteria or B. burgdorferi alone. Experiments were repeated three to five times, and in all of the studies, animals were infected with known inocula of each organism, as described in Materials and Methods. Animals were sacrificed at 1, 2, and 8 weeks, time points that represent specific intervals during the course of murine Lyme borreliosis and HGE. B. burgdorferi-infected mice develop acute arthritis and carditis at 2 weeks, and by 8 weeks the disease usually resolves as the animals remain persistently infected (10). Mice challenged with HGE bacteria usually have morulae in the polymorphonuclear leukocytes at 1 to 2 weeks (39, 69). The HGE agent is then generally cleared from the bloodstream (39).

At 1 week, higher levels of B. burgdorferi DNA were detected in the bladders of the coinfected mice than in those of the B. burgdorferi-infected mice (Fig. 1A). Equivalent concentrations of B. burgdorferi DNA were present in the skin of B. burgdorferi-infected and coinfecte (Fig. 1B). Spirochete DNA was not detected in the joints and hearts at this time point (not shown), consistent with the observation that arthritis and carditis are not yet apparent at this interval. Previous reports have demonstrated HGE bacteria in multiple organs, including the lungs, blood, spleen, and liver (22), and the heart and joints are two tissues associated with Lyme disease. Therefore, the presence of HGE bacteria in the blood, spleen, heart, and joints was assessed. At 1 week, the level of HGE bacteria, as demonstrated by PCR, was elevated in the blood of the coinfecte mice compared to animals that were infected only with HGE bacteria (Fig. 1C). In addition, an increased number of neutrophils containing morulae with Ehrlichia were apparent in the blood of the coinfecte mice compared with animals infected only with HGE bacteria (Table 1). Increased levels of the HGE agent were also detected in the joints, hearts, and spleens of the coinfecte mice (Fig. 1D, E, and F). As a typical example, HGE levels in the heart were highest in the coinfecte mice (Table 1). At 8 weeks, the blood of the coinfecte mice was examined by densitometry, and the DNA bands in the coinfecte mice were 3.2 (mean) ± 0.7 (standard deviation) times as intense as the DNA bands in the HGE-infected mice (Student’s t test, P < 0.02).

At 2 weeks, HGE bacteria were also more readily detected in the joints and spleens of the coinfecte mice than in those of the HGE-infected mice (Fig. 2E and F). An elevated percentage of neutrophils with morulae was also detected in the blood of the coinfecte mice (Table 1). At this time point, coinfecte mice had approximately 100-fold more B. burgdorferi-specific DNA in the skin (Fig. 2A). Joints of coinfecte mice had approximately 10-fold more B. burgdorferi DNA than joints of B. burgdorferi-infected mice (Fig. 2B). In addition, the hearts and bladders of the coinfecte mice showed higher levels of spirochete DNA than those of the B. burgdorferi-infected mice (Fig. 2C and D). Histopathologic examination of the murine joints showed more severe arthritis in the coinfecte mice than in the B. burgdorferi-infected mice at this peak phase of infection (Table 2). As expected, mice infected with HGE bacteria did not develop arthritis (not shown).
At 8 weeks the HGE agent was not detected in the blood or spleens of any mice (not shown). B. burgdorferi was present in the skin of both the singly B. burgdorferi-infected and coinfectected mice, and higher spirochete levels were evident in the dually infected animals (Fig. 2G). The joints of both groups of mice contained equivalent levels of spirochete DNA (not shown), and arthritis was resolving in both groups of animals at this interval (Table 2).

Effect of dual infection on the ability of ticks to acquire and transmit each pathogen. The influence of coinfection on transmission of Ehrlichia and B. burgdorferi to ticks was investigated because mixed infection alters the bacterial burden. Approximately 200 uninfected larvae were placed on groups of five infected mice at 1 week postinfection. Engorged larvae were collected, and pools of five ticks were assessed by IFA for spirochetes and by PCR for the HGE bacteria. IFA showed that 95% of the ticks that fed on B. burgdorferi-infected and coinfectected mice contained spirochetes. However, ticks that engorged on coinfectected mice contained significantly higher numbers of spirochetes (mean, 4,189) than ticks that fed on B. burgdorferi-infected mice (mean, 1,740) (Student’s t test, P < 0.02) (Fig. 3A). At 1 week, HGE bacteria were detected by PCR in DNA from groups of five ticks that fed on coinfectected mice but not in ticks fed on mice infected with the HGE agent (Fig. 3B). Three experiments yielded similar results. In addition, when individual ticks were examined by PCR, 50% of the ticks from the coinfection studies had evidence of HGE DNA, while HGE DNA was rarely detected (one tick) in ticks that fed on mice that were infected only with the HGE agent.

Cytokine and antibody profiles during dual infection. Cytokine and antibody responses are potential factors that may be associated with the increase in the bacterial burden observed in the coinfectected animals. Antibody titers were first determined by ELISA with B. burgdorferi and HGE-44 extracts as substrates. HGE-44, an immunodominant Ehrlichia antigen, was used because it exhibits less cross-reactivity with anti-B. burgdorferi sera than HGE lysates (42). Three separate experiments, with five mice in each group, were performed, and the results were averaged. At 8 weeks, B. burgdorferi-specific antibody levels were not statistically different (Student’s t test, P > 0.5) in sera from B. burgdorferi-infected (IgG1, 0.57 ± 0.02; IgG2a, 0.95 ± 0.05; IgG2b, 0.71 ± 0.04; and IgG3, 0.53 ± 0.06) and coinfectected (IgG1, 0.58 ± 0.02; IgG2a, 0.94 ± 0.04; IgG2b, 0.68 ± 0.02; and IgG3, 0.49 ± 0.06) mice. Antibodies specific for HGE-44 were also comparable (Student’s t test, P > 0.5) in the HGE-infected (IgG1, 0.43 ± 0.02; IgG2a, 1.04 ± 0.03; IgG2b, 0.60 ± 0.01; and IgG3, 0.56 ± 0.03) and coinfectected (IgG1, 0.38 ± 0.01; IgG2a, 1.06 ± 0.03; IgG2b, 0.52 ± 0.07; and IgG3, 0.24 ± 0.04) mice, except for the IgG3 antibodies, which were at slightly lower levels (Student’s t test, P < 0.05) in the coinfectected mice.

Lyme disease severity is partially dependent on the number of spirochetes that invade the joints and on the immune response generated during infection (4, 55, 72). Our results show that coinfection increased the bacterial burden. Therefore, we investigated the effect of dual infection on cytokines that are associated with Lyme arthritis. In each experiment, sera were pooled from five mice in each group. Three separate experiments were performed, and the means and standard deviations are presented. Sera were first analyzed for IL-12, IFN-γ, and TNF-α, cytokines that are known to be expressed during B. burgdorferi infection and that correlate with increased acute inflammation (4, 21, 34, 45, 68). IL-5 and IL-6, indicative of Th2 responses that have been associated with resistance to Lyme arthritis, were also analyzed (5, 44, 47, 57, 58). At 2 weeks postinfection, capture ELISA revealed diminished IFN-γ, IL-12, and TNF-α levels and increased IL-6 levels in the sera of the coinfectected mice compared to B. burgdorferi-infected mice (Fig. 4). Three separate experiments showed that these results were statistically significant (TNF-α, P < 0.005; IFN-γ, P < 0.05; IL-12, P < 0.001; IL-6, P < 0.05 [Student’s t
FIG. 4. IL-12, IFN-γ, TNF-α, IL-5, and IL-6 levels in murine sera. Sera were pooled from groups of five control (uninfected), B. burgdorferi-infected, or B. burgdorferi- and HGE-infected mice. IFN-γ (A), IL-12 (B), TNF-α (C), IL-6 (D), and IL-5 (E) levels were analyzed by capture ELISA. Results from one of three comparable experiments are presented. *, P < 0.05 using Student’s t test, as stated in the text. Error bars indicate standard deviations.
IL-5 levels were not appreciably different between the groups (IL-5, \( P > 0.5 \)).

Restimulation assays were then performed to examine responses directed towards either *B. burgdorferi* or *Ehrlichia* antigens. At 2 weeks, splenocytes from infected mice were stimulated in vitro with either 10 \( \mu \)g of *B. burgdorferi* lysates or 4 \( \mu \)g of ConA per ml for 48 h. Culture supernatants were collected and analyzed for IFN-\( \gamma \) (A), IL-2 (B), IL-6 (C), and IL-10 (D) by capture ELISA. Black and white bars, supernatants from *B. burgdorferi*-infected and coinfectd mice, respectively. Results from one of three similar experiments are presented. \( * P < 0.05 \) using Student’s \( t \) test, as stated in the text. Error bars indicate standard deviations.

The reduced levels of IL-2 produced by splenocytes of coinfectd mice upon stimulation with *B. burgdorferi* lysates suggested that these CD4\(^+\) T cells may not exhibit the same degree of activation as cells from mice infected with *B. burgdorferi* alone. The expression of T-cell activation markers such as CD40 ligand and IL-2 receptor (CD25) was therefore investigated by fluorescence-activated cell sorter analysis. One experiment using five mice in each infection group is presented in Fig. 7. CD4\(^+\) T cells from coinfectd mice showed a reduction in expression of the CD40 ligand (Fig. 7A). Twenty-four percent of the cells from *B. burgdorferi*-infected mice showed expression of CD40 ligand, while HGE-infected and coinfectd mice showed 8 and 5% expression, respectively (Fig. 7A).
RESULTS

Results from three separate experiments demonstrated that the average reduction in CD40 ligand in mixed infection was 84% ± 9% compared with *B. burgdorferi* infection and 59% ± 19% compared with *Ehrlichia* infection. CD25 was also reduced with coinfection (Fig. 7B). Fifty-six percent of the CD4+ T cells from *B. burgdorferi*-infected mice expressed CD25, while HGE-infected and coinfected mice showed 36 and 13% expression, respectively (Fig. 7B). Three different experiments showed that the average reduction in CD25 in mixed infection was 65% ± 13% compared with *B. burgdorferi* infection and 35% ± 30% compared with *Ehrlichia* infection. We also evaluated the activation status of the neutrophils and macrophages by examining levels of the IFN-γ receptor, MHC class II, and CD11b (Mac-1). Macrophages from the coinfected mice demonstrated reduced expression of IFN-γ receptor compared to those from the singly infected mice (Fig. 7D). From three experiments, the average reduction in IFN-γ receptor in mixed infection was 72% ± 32% compared with *B. burgdorferi* infection and 63% ± 42% compared with *Ehrlichia* infection. Expression of MHC class II was not affected (Fig. 7C). Neutrophils from coinfected mice also showed levels of IFN-γ receptor (Fig. 7E) and CD11b (Fig. 7F) similar to those of neutrophils from either *B. burgdorferi* - or HGE-infected mice.

DISCUSSION

Infection with more than one pathogen, including *P. chabaudi* and *S. mansoni* (37), *S. mansoni* and *T. gondii* (53), and *Candida albicans* and *E. coli* (2), can result in altered host responses and disease. We now show that coinfection with *B. burgdorferi* and the HGE agent, two organisms that can be transmitted by the same *I. scapularis* ticks, results in elevated bacterial burden, modified immune responses, increased Lyme...
arthritis, and enhanced pathogen transmission from the host back to the vector. Dual infection may therefore assist in the persistence of these microbes, which are ecologically linked and influence the clinical outcome of human infection.

Our results revealed that coinfection with *B. burgdorferi* and the HGE agent enhances *B. burgdorferi* pathogenesis. We observed that in coinfection the spirochete burden was markedly elevated at the peak phase of disease (2 weeks) and was accompanied by an increase in the severity of acute murine Lyme arthritis. Paradoxically, this was associated with reduced levels of IL-12, TNF-α, and IFN-γ, Th1-type cytokines that are generally associated with the development of more severe disease, during coinfection. However, IL-6, a cytokine shown to direct Th2 responses (60) and to help decrease Lyme arthritis in C57BL/6 (B6) mice (5), was increased in the sera during dual infection. The relative influence of *B. burgdorferi* numbers and

![Image of diagrams showing decreased activation of CD4+ T cells and macrophages during coinfection.](image-url)
specific cytokine responses may contribute to the disease outcome, depending on the situation (4, 14, 15, 55, 74). For example, bacterial numbers can directly affect disease, because resistance to Lyme arthritis in BALB/c mice can be overcome by infecting the animals with higher numbers of spirochetes (51) and the lack of pathogenesis of long-term-passage cN40 spirochetes (N40-75) is directly correlated with reduced bacterial burden in the joint tissues (6). On the other hand, inhibition of IL-12 in immunocompetent mice results in decreased joint inflammation, even when B. burgdorferi levels are elevated (4). During dual infection, the increased bacterial burden and elevated IL-6 levels in sera could have been more important factors than the reduction in the levels of IFN-γ, IL-12, and TNF-α in the sera.

Antibodies have been shown to influence B. burgdorferi and HGE bacterial clearance and the course of Lyme arthritis (12, 24, 25, 29, 32, 46, 69). Our data show that CD4+ T cells from coinfected mice had reduced levels of expression of CD40 ligand, an important costimulatory signal for B-cell activation, suggesting that antibody responses may be diminished. However, consistent with previous observations that protective antibodies to B. burgdorferi can arise in the absence of T-cell help (25, 26), antibody titers to these pathogens were not affected by coinfection, except for the slight decrease in IgG3 antibodies to HGE-44 during coinfection. Therefore, humoral responses are not likely to play a dominant role in affecting the course of disease during coinfection.

Dual infection also resulted in the decreased activation of macrophages, cells that are important in innate immune responses and bacterial clearance. In particular, expression of the IFN-γ receptor was lower on macrophages. Since antigen presentation and antimicrobial activity can be induced by IFN-γ, the reduction in expression of the IFN-γ receptor suggests that activation of these phagocytic cells may be impaired during coinfection, resulting in increased numbers of each pathogen.

Our results also demonstrated that coinfection influences levels of the HGE agent. Recent reports demonstrate that IFN-γ may be involved in the control of HGE infection, because mice begin to clear Ehrlichia after IFN-γ levels become readily detectable (3, 54). Moreover, HGE infection of IFN-γ-deficient mice resulted in elevated levels of Ehrlichia (3). The reduced levels of IFN-γ and the IFN-γ receptor in coinfection may create a favorable environment for survival of the HGE agent, thereby resulting in an increased percentage of neutrophils with morulae during coinfection.

Enhanced larval acquisition of both pathogens was also observed. At 1 week of infection, ticks that fed on coinfectred mice acquired larger numbers of spirochetes than ticks that engorged on B. burgdorferi-infected mice. Surprisingly, at 1 week of infection, the HGE agent was more readily detected in larvae that fed on coinfected mice and not in larvae that engorged on mice infected with HGE alone. Levin and Fish have recently shown that nymphal ticks infected with either Ehrlichia or B. burgdorferi can acquire the second pathogen from infected mice (50). Nymphal procurement of a second pathogen is a different phenomenon from larval acquisition of both pathogens from coinfected mice and suggests that the interplay between vector-host and pathogen may be dependent upon the infection status of the host, developmental stage of the tick, and other multifactorial influences. Indeed, the HGE agent must be able to complete the transition from mouse to tick in the absence of B. burgdorferi, because in the natural environment both mice and ticks can harbor HGE bacteria without concomitant B. burgdorferi infection. The present studies demonstrate, however, that the natural transmission of each pathogen in the vector-host-vector life cycle is facilitated by coinfection.

We have demonstrated that coinfection can influence the B. burgdorferi and Ehrlichia pathogen burden, the pathogenesis of Lyme arthritis, and transmission of the HGE agent and B. burgdorferi from the murine host to the tick vector. Coinfection influences the host in several ways, and a single response is not directly responsible for enhancing disease. These findings may have implications for human Lyme disease, which can range from acute cutaneous disease with erythema migrans to persistent infection with cardiac, musculoskeletal, and neurologic involvement, and for HGE, which may be self-limited or severe. In particular, the increase in joint inflammation in the murine studies suggests that coinfection may influence the severity of human Lyme arthritis. Epidemiological studies have previously suggested that concurrent Lyme disease and babesiosis (a disease caused by a protozoan that is transmitted by I. scapularis) result in increased Lyme disease severity, providing one clinically relevant example of this phenomenon (46a). Our present murine studies now suggest that B. burgdorferi and the agent of HGE transmission and pathogenicity increase during coinfection, and this may be one explanation for the similar natural reservoirs, vectors, and geographic distributions of these pathogens and for differences in the severity of disease.

ACKNOWLEDGMENTS

We thank Deborah Beck for technical assistance. This work was supported by grants from the National Institutes of Health, the Arthritis Foundation, and the American Heart Association and by a gift from SmithKline Beecham Biologicals. Erol Fikrig is a recipient of a Burroughs Wellcome Clinical Scientist Award in Translational Research.

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Editor: D. L. Burns