Outer Surface Lipoproteins of *Borrelia burgdorferi* Stimulate Nitric Oxide Production by the Cytokine-Inducible Pathway

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The outer surface lipoproteins of *Borrelia burgdorferi*, OspA and OspB, stimulate the production of nitric oxide (NO) by murine bone marrow-derived macrophages from BALB/c, C3H/HeN, and C3H/HeJ mice. Gamma interferon (IFN- γ) caused a three- to fivefold enhancement of this production of NO, and the L-arginine analog *N*-guanidino-monomethyl L-arginine inhibited it. Activation of transcription of the inducible NO synthase gene in stimulated macrophages was demonstrated by reverse transcriptase rapid PCR. Although IFN- γ increased the amount of NO produced in macrophage cultures, it did not cause transcription of the inducible NO synthase gene greater than that seen with the *Borrelia* proteins. OspA and OspB also induced the production of high levels (40 to 150 ng/ml) of IFN- γ in cultures of macrophages incubated with interleukin-2 (IL-2)-elicited cells from normal (T and NK cells) and *scid* (NK cells) mice but not in macrophages or IL-2-elicited cells cultured individually. This suggests that OspA stimulated macrophage production of cytokines, which, in turn, stimulated the production of IFN- γ by NK and T cells. Reverse transcriptase rapid PCR demonstrated that OspA and sonicated *B. burgdorferi* stimulated production of several inflammatory cytokines in macrophage cultures, including IL-1, IL-6, IL-12, IFN- β , and tumor necrosis factor alpha. As tumor necrosis factor alpha, IFN- β , and IL-12 are potent activators of IFN- γ production by T and NK cells, their presence in these cocultures could be responsible for the IFN- γ production. Lymphocytes from infected C3H mice also produced IFN- γ when stimulated with *B. burgdorferi*; thus, immune cells may also modulate NO responses. The generation of NO during infection with *B. burgdorferi* may be important, as NO has potent antimicrobial properties. NO can also be involved in pathological inflammatory processes in which its generation is detrimental to the host. Thus, the colocalization of *B. burgdorferi* lipoproteins, NO-producing cells, and regulatory cytokines may determine the outcome of infection.

Lyme disease is caused by infection with the tick-borne spirochete *Borrelia burgdorferi* (11, 30). The spirochete is capable of invading many tissues (6, 61) and has recently been shown to persist in synovial fluid from patients with chronic arthritis (46). Studies with a murine model of Lyme disease also suggest that spirochete persistence is correlated with severe pathology and may be responsible for localized inflammation (6, 61). The association of tissue invasion with localized inflammation may be explained by the observation that the bacterium possesses potent B-cell mitogen and cytokine stimulatory properties in its outer surface lipoproteins (9, 25, 33, 36, 49, 55, 59). In the mouse model (5), inbred mice experiencing severe arthritis have intense inflammatory and immune responses, including highly elevated levels of interleukin-6 (IL-6) in plasma (60). Lyme disease patients also have elevated concentrations of IL-6, tumor necrosis factor alpha (TNF- α), and IL-1 in plasma and/or synovial fluid (7, 16, 42), and IL-6 has been found in synovial fluids from individuals with rheumatoid arthritis (45). Moreover, transgenic mice that constitutively produce TNF- α spontaneously develop severe arthritis (32). Taken together, these data support a role for inflammatory cytokines in the development of Lyme arthritis.

In humans, there is great variation in the severity of symptoms experienced by infected patients (31, 51, 53). The severity of arthritis also varies in strains of inbred mice, suggesting that

genetic factors regulating the host inflammatory response may control the pathological outcome of infection (5). We previously demonstrated that B lymphocytes and macrophages from severely and mildly arthritic strains of mice respond intensely to the stimulatory properties of the outer surface lipoproteins OspA and OspB in vitro (36). In vivo, the severely arthritic mice have more intense systemic inflammatory responses, indicated by circulating IL-6 and elevation in immunoglobulin G (60). This difference could be explained by the recent finding that severely arthritic C3H mice harbor larger numbers of persisting spirochetes throughout infection than do mildly arthritic BALB/c mice (61). This would result in a greater presence of the stimulatory lipoproteins OspA and OspB in tissues of the arthritic C3H mouse.

Many host factors potentially contribute to the ability of the host to clear tissue spirochetes and could be responsible for the difference between BALB/c and C3H mice. One of these is the bioactive molecule nitric oxide (NO). NO is a crucial component of macrophage killing of intracellular parasites such as *Leishmania major* (24), *Chlamydia trachomatis* (39), and *Toxoplasma gondii* (1) and has been implicated in neutrophil defenses against extracellular pathogens such as *Staphylococcus aureus* (37) and the larva stage of *Schistosoma mansoni* (29). NO can also be involved in pathological inflammatory events such as streptococcal cell wall arthritis (40) and autoimmune disorders (57); therefore, its production could also contribute to arthritis development. In this study, we wished to determine if *B. burgdorferi* and its outer surface lipoproteins could stimulate NO production. We further assessed the effect

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of the cytokines produced by OspA- and *B. burgdorferi*-stimulated innate and immune cells on the expression of NO.

MATERIALS AND METHODS

Bacterial strains. The N40 strain of *B. burgdorferi* was provided by Stephen Barthold, Yale University (5), and a clone expressing only the full-length OspB was obtained by plating on solid agar. The OspB-deficient variant of HB-19 was provided by Alan Barbour, University of Texas (50). Stocks were stored frozen at -70°C and cultured in BSK-H medium (Sigma) (3).

Purification of OspA and OspB. OspA was purified from the OspB-negative variant of the HB-19 strain, and OspB was purified from the N40 strain of *B. burgdorferi*, as described before (36). Briefly, spirochetes were collected from 600-ml cultures and sonicated, and the membrane-enriched fraction was collected by centrifugation at $100,000 \times g$ for 90 min. The pellet was suspended in 10 ml of phosphate-buffered saline (PBS) and extracted with 8 ml of *n*-butanol by rocking for 1 h at 4°C (23). The aqueous phase was recovered by centrifugation at $27,000 \times g$ for 90 min at 4°C and dialyzed against affinity chromatography starting buffer (10 mM Tris [pH 7.4], 5 mM NaCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin). The starting material was brought to 30 mM with *n*-octyl- β -glucoside and applied sequentially to 3-ml Sepharose affinity columns coupled with bovine serum albumin, an irrelevant murine immunoglobulin G2a monoclonal antibody (HB-5), and monoclonal antibodies to OspA, clone H5332 (4), or OspB, clone H614 (10), provided by Alan Barbour. Material bound to the anti-OspA and anti-OspB affinity columns was eluted with 0.2 M acetic acid (pH 2.5) and dialyzed against starting buffer. This removed the detergent and resulted in highly purified preparations of OspA and OspB, as determined by silver stain analysis (36). Purified lipoproteins were stored at 4°C and retained solubility in the low-salt buffer.

Endotoxin analysis. Extreme care was taken to prevent contamination of OspA and OspB with endotoxin during their purification. Glassware was baked, equipment that could not be baked was washed with E-Toxa-Clean (Sigma), and water was filtered through a Barnstead Nanopure II system equipped with the ULTRAFILTER accessory kit for the removal of pyrogens. The level of endotoxin contamination in the purified OspA and OspB was less than 0.03 endotoxin units (EU) per 500-ng sample, using a *Limulus* amebocyte lysate assay (Associates of Cape Cod, Inc.). A slight presence of endotoxin (2 EU/ml, or approximately 0.4 ng/ml) was found in the lymphocyte medium containing 1% Nutridoma, antibiotics, glutamine, and 2-mercaptoethanol.

Mice. C3H/HeJ, C3H/HeN, and BALB/c mice were obtained from Charles River Laboratories or Jackson Laboratories. C3H *scid* mice were obtained from Jackson Laboratories. Mice were housed in the Animal Resource Center of the University of Utah Medical Center.

Infection of mice. C3H/HeJ mice, 5 to 6 weeks old, were infected with 2×10^5 *B. burgdorferi* N40 organisms by intradermal injection in the (shaven) back (60). Controls received an equal volume of sterile medium.

Bone marrow-derived macrophages. Bone marrow-derived macrophages were obtained from mouse femurs and suspended in RPMI containing 30% L929 conditioned medium, 20% horse serum (Hyclone), 0.5 mM 2-mercaptoethanol, 2 mM L-glutamine, and 0.01 mg of gentamicin sulfate per ml (41). Bone marrow cells were cultured for 5 to 6 days at 37°C under a humidified atmosphere of 5% CO_2 in petri dishes

(Falcon). The plates were rinsed with media, and macrophages to be studied for cytokine or nitrite production were transferred to 24-well dishes. Macrophages were recovered from the petri dishes by incubation with 10 ml of cold PBS for 20 min. Cells were replated in 24-well dishes at a density of 3×10^5 per well in lymphocyte medium (LM). After overnight culture at 37°C , nonadherent cells were removed, and the indicated stimuli were added. Macrophages analyzed for mRNA induction were maintained in the petri dishes, rinsed with LM, and incubated for 24 h with 8 ml of the indicated stimuli. Medium was removed, and RNA was directly prepared from adherent cells that were released by incubation with cold PBS.

IL-2-elicited cells. Splenocytes from normal C3H mice were incubated for 3 days in RPMI containing 10% fetal bovine serum, 30% IL-2-conditioned medium (supernatant from the human IL-2-expressing transfectant cell line, 653-IL-2), 1% nonessential amino acids, 0.2 μg of gentamicin per ml, 1% L-glutamine, 1 mM sodium pyruvate, and 5×10^{-5} M 2-mercaptoethanol. The final concentration of IL-2 was 300 U/ml as determined by antibody capture enzyme-linked immunosorbent assay (ELISA) (2).

To obtain pure populations of NK cells, single cell suspensions were prepared from spleens and bone marrow of C3H *scid* mice and cultured for 3 days in the IL-2-containing medium. Cultures from the two types of tissues were pooled to achieve sufficient numbers of cells for these experiments.

NO assay. Nitrite levels in supernatants from macrophage cultures were assayed with the Greiss reagent, made by mixing equal volumes of 1% sulfanilamide in 30% acetic acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 60% acetic acid (18). The color change was immediate, and samples were read with an ELISA reader at 570 nm.

RT rapid PCR (RPCR) detection of mRNA. Total cellular RNA was prepared from macrophages, as described by Chirgwin et al. (12). Reverse transcription (RT) was performed on 5 μg of RNA in $1 \times$ RT buffer (GIBCO-BRL), 10 mM dithiothreitol, 0.125 mM (each) four deoxyribonucleotide triphosphates, 0.5 μg of random primers (New England Biolabs), and 400 U of Moloney murine leukemia virus RT (GIBCO-BRL) in a 50- μl total volume (56). Samples were incubated for 60 min at 37°C , and 2 μg of DNase-free RNase was added for 5 min at 37°C . Samples were extracted with phenol-chloroform and chloroform and precipitated with ethanol.

RPCR was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences. Oligonucleotide sequences were derived from sequences in GenBank: actin (GTA ACA ATG CCA TGT TCA AT and CTC CAT CGT GGG CCG CTC TAG), IL-1 (CAT GAG ACT TGC ACA GAT CAG and GGG TTG GAT GGT CTC TTC CAG), TNF- α (CTC AGA TCA TCT TCT CA and CAC CAC TAG TTG GTT GT), IL-12 p35 (ACC AGC ACA TTG AAG ACC TG and GAT TGC ATC AGC TCA TCG AT), IL-12 p40 (AGA TGA CAT CAC CTG GAC CT and GCC ATG AGC ACG TGA ACC GT), and inducible NO synthase (iNOS; GCA TGG ACC AGT ATA AGG CAA GCA and GCT TCT GGT CGA TGT CAT GAG CAA). Oligonucleotide pairs were designed to span an intervening sequence so that products from cDNA templates could be distinguished from products of genomic DNA. RPCR was performed on 120 ng of cDNA in a 10- μl final volume containing reaction buffer (50 mM Tris [pH 8.3], 3 mM MgCl_2 , 20 mM KCl, 0.5 mg of bovine serum albumin), 70 pmol of each oligonucleotide pair, 0.8 mM (each) deoxyribonucleotide triphosphates (Boehringer Mannheim), 2.5 μCi of ^{32}P -dCTP (New England Nuclear Research Products), and 0.72 U of *Taq* DNA polymerase (5,000 U/ml;

BRL-GIBCO) (56). Conditions used for amplification of actin cDNA were as follows: denaturing at 94°C for 1 s; annealing at 59°C for 1 s; elongation at 72°C for 6 s; 14 cycles. Conditions for IL-1 and TNF- α were as follows: denaturing at 94°C for 1 s; annealing at 62°C for 1 s; elongation at 72°C for 6 s; 24 cycles. Conditions for IL-12 (p35 and p40) and gamma interferon (IFN- γ) were as follows: denaturing at 94°C for 1 s; annealing at 59°C for 1 s; elongation at 72°C for 6 s; 24 cycles. Conditions for iNOS were the following: denaturing at 94°C for 1 s; annealing at 65°C for 1 s; elongation at 72°C for 6 s; 24 cycles. Amplification was carried out in the 1605 Air Thermocycler (Idaho Technologies), which has an extremely rapid cycle time, approximately 24 s (56). Fine-tune adjustments for DNA concentration were made on the basis of the quantity of product obtained with the β -actin primers.

Bacterial cytostasis assay. Log-phase cultures of *Escherichia coli* (XL1-Blue) or *B. burgdorferi* were cultured at 2×10^7 /ml in microtiter plates (200 μ l per well) containing BSK-H and 2.5 μ Ci of 3 H-adenine per ml. Equal volumes of freshly made solutions of 0.4 M glutathione and 0.4 M NaNO₂ were mixed to form 0.2 M S-nitrosoglutathione, a reaction that generates NO gas. Tris (2 M) was added to the S-nitrosoglutathione to bring the pH to 7.6, and the indicated dilutions were made directly into BSK-H and mixed with bacterial cultures in the microtiter wells. Cultures were incubated at 37°C under a humidified atmosphere of 5% CO₂. 3 H-adenine incorporation was determined from samples collected with a PHD cell harvester.

IFN- γ ELISA. R4GA2 (Pharmingen) was used as the capture antibody, and biotin-conjugated XMG1.2 (Pharmingen) was used for the developing antibody. Recombinant IFN- γ was used for the standard curve (Genzyme). Samples and standards were diluted in PBS containing 10% fetal bovine serum and incubated overnight at 4°C in the antibody-coated wells. Plates were developed by incubation with 0.4 mg of o-phenylenediamine per ml–0.01% H₂O₂ and read at an optical density of 492 nm with a $V_{\max}$ 96-well microtest plate spectrophotometer (Molecular Devices).

TNF- α ELISA. MP6-XT3 (Pharmingen) was used as the capture antibody, and biotin-conjugated MP6-XT22 (Pharmingen) was used for the developing antibody. Recombinant TNF- α was used for the standard curve (Pharmingen). Samples and standards were prepared and assayed as described for the IFN- γ ELISA.

IFN- γ was obtained from both Genzyme and Genentech. The L-arginine analog N-guanidino-monomethyl L-arginine (NMA) was obtained from Chem Biochem Inc., Salt Lake City, Utah. The anti-CD3 monoclonal antibody was from clone 145-2C11 (48).

RESULTS

Sonicated *B. burgdorferi* and purified OspA and OspB were tested for the ability to induce NO production by bone marrow-derived macrophages (Fig. 1). NO is rapidly converted to nitrite and nitrate; thus, nitrite levels in culture supernatants measured with the Greiss reagent are an indication of the production of NO (18). Sonicated *B. burgdorferi* and purified OspA and OspB had potent stimulatory effects on macrophage NO production. OspA and OspB were approximately as active as sonicated bacteria and commercially prepared endotoxin. OspA and OspB were free of bacterial endotoxin, as determined by the *Limulus* amoebocyte lysate assay (36). Furthermore, the stimulatory properties were not dependent on the presence of small amounts of endotoxin in the medium, as polymyxin B (44) had a minimal effect on responses to OspA

FIG. 1. IFN- γ -enhanced stimulation of nitrite production by OspA and OspB. Bone marrow-derived macrophages (3×10^5) from BALB/c (A), C3H/HeN (B), and C3H/HeJ (C) mice were incubated for 48 h with the indicated stimuli. Supernatants were removed and assayed for the presence of nitrite. LM, samples receiving LM alone; LPS, samples with 100 ng of bacterial endotoxin per ml; I-MAB, samples with the eluate of an irrelevant monoclonal antibody column; OspA and OspB, samples that received the purified protein preparations at the indicated concentrations. Closed bars indicate samples incubated without polymyxin B, open bars are samples incubated with 10 μ g of polymyxin B per ml, and hatched bars are samples that received both polymyxin B and 1 U of IFN- γ per ml.

and OspB while completely blocking the response to lipopolysaccharide (LPS) of C3H/HeN and BALB/c mice (Fig. 1). Macrophages from the endotoxin-resistant C3H/HeJ mice did not produce NO when incubated with a commercial source of LPS but produced at least as much NO when incubated with OspA and OspB as did the endotoxin-sensitive strains BALB/c and C3H/HeN.

The addition of IFN- γ to cultures of macrophages and other cells does stimulate the production of iNOS (19, 24, 39). The addition of 1 U of IFN- γ per ml to the macrophage cultures in Fig. 1 did not induce the production of NO when added as a single stimulus but did enhance NO production in macrophages which received a second stimulus with either sonicated *B. burgdorferi*, OspA, or OspB. Optimal costimulation was found with 1 U of IFN- γ per ml from two commercial sources (data not shown). Although a potent costimulator, IFN- γ was

FIG. 2. Antibody to TNF- α inhibits nitrite production by macrophages incubated with OspA. Bone marrow-derived macrophages from C3H/HeJ mice were incubated with LM, with sonicated *B. burgdorferi* (5 μ g/ml), or with the indicated concentration of purified OspA for 48 h. Supernatants were assayed for nitrite. Solid bars indicate samples incubated with the stimuli alone; open bars indicate samples incubated with anti-TNF α .

not absolutely required for induction of NO production by OspA and OspB, best indicated in Fig. 1B and C and Fig. 6B. In other experiments, very little NO was produced without IFN- γ costimulation (Fig. 1A and Fig. 5B). This could reflect variability in the presence of other cytokines stimulated by the lipoproteins that could be acting in an autocrine fashion to coincide iNOS expression. TNF- α is a macrophage product previously reported to be stimulated by OspA and known to activate iNOS production by macrophages (19). Therefore, we determined whether neutralizing antibody to TNF- α could block the appearance of NO (Fig. 2). Partial, but not complete, blockage of NO production was obtained by adding anti-TNF- α to the culture media, yielding an average of 44.5% inhibition of induction by the three OspA concentrations ($P < 0.05$ by Student's t test). This treatment prevented the appearance of detectable TNF- α in the media, as measured by antibody capture ELISA (data not shown). This suggests that TNF- α production contributes to the level of NO produced and supports involvement of iNOS in this NO response.

Cytokine-stimulated production of NO arises from metabolism of L-arginine by iNOS (19). The production of NO by this enzyme is inhibited by NMA (26, 27). NO production stimulated by OspA and sonicated *B. burgdorferi* was blocked by NMA, indicating that L-arginine is the macrophage source of NO (Fig. 3). IFN- γ -enhanced production of NO was also inhibited by NMA, suggesting that the inducible enzyme was responsible for *B. burgdorferi*-stimulated NO production. The high level of NO production is also consistent with the activity of the inducible enzyme, as the constitutive enzyme produces NO at much lower levels and is not affected by IFN- γ (27). To confirm the involvement of iNOS in the macrophage response to OspA and OspB, the induction of mRNA transcription in macrophages incubated with sonicated *B. burgdorferi* or OspA was assessed. RT-PCR indicated that transcription of the iNOS gene was directly stimulated by both sonicated *B. burgdorferi* and purified OspA. Transcription of iNOS did not require IFN- γ , nor was it enhanced by IFN- γ (Fig. 4). This is interesting in light of the enhanced nitrite presence in culture supernatants of IFN- γ -containing cultures (Fig. 1).

Macrophages produce many cytokines with potent inflammatory activities, including the potential to activate IFN- γ production by other cell types. Our previous study demonstrated that OspA and OspB were potent stimulators of the

FIG. 3. The L-arginine analog NMA blocks the production of nitrite by OspA- and OspB-stimulated macrophages. Bone marrow-derived macrophages from C3H/HeJ mice were incubated with LM, with sonicated *B. burgdorferi* (5 μ g/ml), or with the indicated concentrations of purified OspA. After 48 h, the supernatants were assayed for nitrite. Closed bars represent samples incubated with the indicated stimuli alone, hatched bars show samples that received 0.8 nM NMA, open bars indicate samples that received 1 U of IFN- γ per ml, and stippled bars represent samples that received 0.8 mM NMA and 1 U of IFN- γ per ml.

production of IL-6 and TNF by macrophages (36). Investigations of the full complement of cytokines that can be induced by *B. burgdorferi* lipoproteins was hindered by the lack of availability of antibody reagents required for ELISAs. Direct

FIG. 4. Induction of mRNA-encoding inflammatory mediators by *B. burgdorferi* and OspA. RT-PCR was performed on mRNA obtained from bone marrow-derived macrophages incubated with the indicated stimuli. LM, samples incubated with medium alone; sonicate, samples incubated with 5 μ g of *B. burgdorferi* per ml; OspA, samples that received 500 ng of purified OspA per ml. Indicated samples received 1 U of IFN- γ per ml. The amplification products were analyzed by electrophoresis on a 6% acrylamide sequencing gel and identified by autoradiography. The sizes of the amplification products were 222 bases for the iNOS gene, 162 bases for IFN- β , 148 bases for TNF- α , 210 bases for IL-1, 83 bases for IL-6, 272 bases for the p35 chain of IL-12, 260 bases for the p40 chain of IL-12, and 135 bases for β -actin.

detection of mRNA for cytokines circumvents the need for ELISAs and provides a very sensitive technique for assessing transcriptional activation in response to OspA stimulation. Thus, the ability of sonicated *B. burgdorferi* and OspA to induce several other cytokines was determined by RT-PCR. OspA and *B. burgdorferi* stimulated the production of a variety of cytokine mRNAs, including strong activation of IL-1, IFN- β , IL-12 p35 and p40 (Fig. 4). NO production peaked at 48 h (data not shown) but was strongly induced by 24 h and not present at 6 h. Therefore, a single time point was chosen for these experiments to detect messages for many different inflammatory proteins. This may explain why mRNA for TNF- α and IL-6 was less abundant, as these messages peak at earlier time points and are turned over rapidly. Our previous ELISA results provided clear indication of the accumulation of both TNF- α and IL-6 in cultures of OspA-stimulated macrophages (36). mRNA for IFN- α was not detected in *B. burgdorferi*-stimulated macrophage cultures (data not shown).

Because the macrophage products TNF- α , IFN- β , and IL-12 are inducers of IFN- γ production by NK and T cells (15, 21, 58), we sought to determine if coculture of OspA-stimulated macrophages with NK and T cells would lead to the generation of IFN- γ . This hypothesis was tested by using OspA to prime bone marrow-derived macrophages alone, IL-2-elicited splenocytes alone, or cocultures of macrophages and IL-2-elicited cells (Fig. 5). T and NK cells are the major cell populations derived from splenocytes which have been cultured with IL-2 for 3 days. Neither the macrophages nor the splenocytes, when cultured individually with OspA, produced significant amounts of IFN- γ . Coculture of these cells in the presence of OspA led to the production of large amounts of IFN- γ , up to 40 ng/ml. These cocultures also produced higher levels of NO than did macrophages or IL-2-elicited cells individually. This suggests that the enhancing effects of IFN- γ on NO production demonstrated in Fig. 1 could have relevance to those in vivo situations in which macrophages and lymphocytes are colocalized.

To determine if T cells were absolutely required for IFN- γ production in macrophage-IL-2-elicited cell cocultures, IL-2-elicited cells were prepared from C3H *scid* mice (Fig. 6). IL-2-dependent cells from bone marrow and spleens of *scid* mice lack T lymphocytes and are highly enriched for NK cells. NK cells from *scid* mice produced very high levels of IFN- γ when cultured with macrophages and OspA, 80 to 150 ng/ml. This was even higher than the 25 to 40 ng of IFN- γ produced per ml with IL-2-elicited cells from immunocompetent mice. Thus, cocultures of cells strictly of the innate immune response are capable of generating high levels of IFN- γ in response to OspA. It is unlikely that this observation could be explained by T-cell contamination due to leakiness of the *scid* mutation as the levels of IFN- γ were actually higher than seen with T-cell-containing populations and similar results were obtained in several experiments. Interestingly, the presence of NK cells did not cause increased production of NO in these cocultures, even though very high levels of IFN- γ were present (Fig. 6B). This could be explained by a detrimental effect of extremely high levels of IFN- γ in the NK cell cocultures. Alternatively, NK cells may make substances that are inhibitory for iNOS induction or activity, or T cells may make products other than IFN- γ that are important in iNOS activation.

We sought to determine if there was an increase in IFN- γ production during infection with *B. burgdorferi* by culturing splenocytes and lymph node cells from infected or control C3H/HeJ mice in the absence or presence of sonicated *B. burgdorferi*. High levels of IFN- γ were produced in *B. burgdorferi*-

FIG. 5. OspA or OspB stimulates the production of IFN- γ by cocultures of macrophages and IL-2-elicited cells. Bone marrow-derived macrophages (3×10^5) and IL-2-elicited splenocytes (2×10^5) from C3H/HeJ mice were cultured individually or together for 48 h in the presence of medium alone (LM), 5 μ g of commercially prepared LPS per ml, 5 μ g of sonicated *B. burgdorferi* per ml, or 250 ng of purified OspA or OspB per ml. Supernatants were assayed for IFN- γ (A) and nitrite (B) as described in Materials and Methods. Closed bars indicate macrophages alone, open bars indicate IL-2-elicited cells alone, and hatched bars indicate cocultures of these cells.

feri-stimulated cultures of spleen and lymph node cells from infected mice, in the range of that stimulated by the T-cell mitogen anti-CD3, which stimulates the production of high levels of IFN- γ (Fig. 7). Lymph node cells from control mice produced no IFN- γ when incubated with sonicated *B. burgdorferi*; however, splenocytes did produce low levels of IFN- γ . The low level of IFN- γ produced by control splenocytes may reflect the presence of 10% NK cells in the spleen acting as in the cocultures of Fig. 5. NK cells are absent from the lymph nodes. Previous analysis of splenocyte and lymph node populations during infection with *B. burgdorferi* revealed no increase in the proportions of cells that were NK cells (i.e., non-B, non-T [60]). Thus, the increased production of antigen-stimulated IFN- γ by lymph node cells from infected mice suggests the induction of antigen-specific T cells (Fig. 7).

To determine if *B. burgdorferi* was susceptible to NO-mediated cytostasis, cultures were incubated in the presence of S-nitrosoglutathione and the effect on DNA synthesis was determined. *E. coli* was used as a positive control for susceptibility to killing in the complex BSK-H medium. The production of S-nitrosoglutathione from glutathione and sodium nitrite results in the release of NO gas into the growth medium, which effectively prevented the incorporation of 3 H-adenine by

FIG. 6. IFN- γ production by cultures of NK cells and macrophages incubated with OspA or OspB. Bone marrow-derived macrophages (3×10^5) from C3H/HeJ mice and IL-2-elicited splenocytes from C3H/HeJ *scid* mice (2×10^5), NK cells, were cultured individually or together for 48 h in the presence of medium alone (LM), 5 μ g of commercially prepared LPS per ml, 5 μ g of sonicated *B. burgdorferi* per ml, or 250 ng of purified OspA or OspB per ml. Supernatants were assayed for IFN- γ (A) and nitrite (B) as described in the text. Bars represent samples as described for Fig. 5.

both bacterial cultures (Table 1). The addition of equivalent concentrations of either glutathione or sodium nitrite alone did not block DNA synthesis, indicating that the NO product was responsible for inhibition of DNA synthesis (data not shown).

DISCUSSION

The outer surface lipoproteins of *B. burgdorferi* are extremely potent stimulators of cytokine production (9, 36, 49, 55) and B-cell activation (17, 36, 55, 59), properties which could be involved in the inflammation (7, 16, 25, 42, 51) and B-cell hyperactivation associated with Lyme disease (52, 55, 60, 64). In this report, we have documented that *B. burgdorferi* and its outer surface lipoproteins are also potent stimulators of NO production by bone marrow-derived macrophages (Fig. 1). To our knowledge, this is the first demonstration of bacterial lipoproteins directly activating NO production. This macrophage activation was stimulated by the addition of IFN- γ to cultures but did not require it. *B. burgdorferi* and OspA directly activated transcription of the cytokine-inducible NO synthase gene, iNOS. The activation of iNOS gene transcription (Fig. 4) was probably enhanced by the presence of other cytokines in OspA-stimulated macrophage cultures, as demonstrated by the partial inhibition of NO production by neutralizing anti-TNF- α

FIG. 7. Antigen-specific lymphocytes from *B. burgdorferi*-infected mice make IFN- γ . Splenocytes or pooled peripheral lymph node cells (axillary, inguinal, and popliteal) from control C3H/HeJ mice (closed bars) or mice infected 3 weeks previously with *B. burgdorferi* (open bars) were incubated with medium (LM), with a mitogenic concentration of anti-CD3, or with 5 μ g of sonicated *B. burgdorferi* per ml for 24 h. Supernatants were assayed for IFN- γ as described in Materials and Methods.

(Fig. 2). Partial inhibition of NO production by anti-TNF- α suggests that other *B. burgdorferi*-stimulated macrophage products known to enhance NO production, such as IL-1 or IFN- β (39, 63), were also contributing to the induction of iNOS. The expression of mRNA encoding the iNOS-inducing cytokines IFN- β , IL-1, and TNF- α was stimulated by *B. burgdorferi* and OspA (Fig. 4); thus, many factors may contribute to the ultimate level of NO production.

Other cell types could also contribute to stimulation of iNOS activity. This was demonstrated by coculturing macrophages and IL-2-elicited cells in the presence of *B. burgdorferi* and OspA (Fig. 5). In this case, OspA and *B. burgdorferi* could not stimulate IFN- γ production in cultures of these cell types alone; however, the interaction of the cell types led to the production of large amounts of IFN- γ . Taken with the induction of mRNA synthesis for IL-12, TNF- α , IFN- β , and IL-1 in OspA and *B. burgdorferi*-stimulated macrophages (Fig. 4), these findings suggest that inflammatory cytokines made by macrophages stimulate the production of IFN- γ by IL-2-

TABLE 1. Inhibition of DNA synthesis by *S*-nitrosoglutathione^a

Organism	³ H-adenine incorporation (cpm)			
	Medium alone	Medium with <i>S</i> -nitrosoglutathione at:		
		5 mM	7.5 mM	10 mM
<i>E. coli</i>	31,788	1,152	ND	925
<i>B. burgdorferi</i>	54,556	6,024	1,853	1,290

^a *E. coli* or *B. burgdorferi* was cultured at 2×10^7 /ml in BSK-H medium containing the indicated concentrations of *S*-nitrosoglutathione. The incorporation of ³H-adenine was determined after 4 h for *E. coli* and 24 h for *B. burgdorferi*. ND, not determined.

FIG. 8. Potential sites for the involvement of the stimulatory properties of *B. burgdorferi* in the resolution of infection and in the development of arthritis. *B. burgdorferi* induces inflammatory cytokine and NO production in invaded tissues. These cytokines cause T and NK cells to produce IFN- γ , which upregulates macrophage responses to the spirochete. Inflammatory cytokines and NO may function to eliminate the spirochetes or may participate in the pathophysiology of localized infection.

elicited cells. The IFN- γ can, in turn, enhance the production of inflammatory cytokines and NO by the macrophages, as suggested in the model in Fig. 8. IL-2-elicited cells from immunocompetent mice contain both T and NK cells. To focus on the involvement of NK cells in the production of IFN- γ , we also tested IL-2-elicited cells from *scid* mice (Fig. 6). These NK cells also responded to *B. burgdorferi*- and OspA-stimulated macrophages by producing high levels of IFN- γ , even higher than when the IL-2-elicited cells were from immunocompetent mice. Surprisingly, in these IFN- γ -containing cocultures of *scid* NK cells and macrophages, the production of NO was not enhanced. This suggests the presence of an inhibitory element in these cultures, as IFN- γ alone could stimulate NO production by macrophages (Fig. 1). These results suggest that cell types such as NK and T cells could function early in infection to upregulate the production of NO through IFN- γ or to modulate the effects of IFN- γ .

In infected mice, antigen-specific cells may also participate in the control of NO production. Antigen-specific stimulation of IFN- γ production was observed with splenocytes and lymph node cells from mice 3 weeks after infection when cultured with *B. burgdorferi* antigens (Fig. 7). This provides a mechanism for stimulating NO activity during the host response to infection. We would predict that the IFN- γ -producing cells in these cultures from infected mice would be antigen-specific T cells, which may be an important source of this cytokine later in infection.

Our finding that *B. burgdorferi* and OspA stimulate activation of the iNOS gene and the production of NO is important as NO plays a crucial antimicrobial role against many pathogens (1, 24, 29, 37, 39). The conversion of L-arginine to NO is inducible by IFN- γ and is the main mechanism by which murine macrophages eliminate intracellular pathogens. Although the importance of this pathway to human macrophages has not been demonstrated in vivo, NO production has been documented under some conditions by human hepatocytes and monocytes and in patients undergoing IL-2 therapy (28, 47, 62). Our findings indicate that *B. burgdorferi* can stimulate macrophage iNOS production; however, in vivo, the actual responding cell is not known. *B. burgdorferi* has been demonstrated within endothelial cells, macrophages, and fibroblasts (14, 22, 35, 43) in vitro and can transcytose endothelial cell monolayers (13, 54); however, whether intracellular localiza-

tion is important for survival in the host has not been established. NO is also part of the antimicrobial defenses of human neutrophils, where it may function in killing extracellular bacteria, such as *S. aureus*, that have been trapped within phagocytic vacuoles (37). NO released by cytokine-activated macrophages kills adjacent tumor cells and schistosomula, acting as an extracellular defensive mechanism (27, 29). Thus, the induction of NO by *B. burgdorferi* and its products could play an important role in limiting growth or persistence of the organism, whether it be in a protective intracellular environment or in an extracellular niche. The bacteriostatic and bactericidal effects of NO are thought to be mediated through the formation of complexes with Fe-thiol-containing enzymes such as ribonucleotide reductase and through the production of peroxynitrite and subsequent generation of hydroxyl free radical (8, 34). Interestingly, we previously demonstrated a 5- to 10-fold-greater presence of persistent *B. burgdorferi* in tissues of infected C3H mice than in BALB/c mice (61). The findings of Fig. 1 and 4 indicate that there is no genetically determined deficiency in the activation of iNOS by cells of C3H mice that could explain their reduced ability to clear spirochetes in vivo. This suggests that understanding the regulation of iNOS and its regulatory cytokines in the infected animal may be required to explain the observed strain differences.

Other effects of NO have also been demonstrated, particularly its involvement in immune-mediated injury. Two examples are the MRL mouse, which spontaneously develops lupus, and the streptococcal cell wall arthritis model (40, 57). Although it has been difficult to culture *B. burgdorferi* from synovial fluid of patients, Lyme arthritis has been thought to be infection based. Recent DNA-based detection of *B. burgdorferi* in synovial fluid or joint tissues from humans and mice supports this (6, 46, 61). Synovium contains approximately 50% macrophages and is infiltrated by neutrophils. Since both of these cell types are capable of NO production, invading *B. burgdorferi* organisms have ample opportunity to cause NO-mediated damage to inflamed joints. Although we have demonstrated macrophage production of NO in response to lipoproteins, the response of neutrophils to OspA or *B. burgdorferi* remains to be determined.

In the infected animal, it is possible that the presence of cytokines that regulate the activity of iNOS could play a role in the interplay of bacterial killing and pathological development. Because NO is very nonspecific in its actions, it is possible that NO stimulated by *B. burgdorferi* could be detrimental to both the bacterium and the host. We have shown that *B. burgdorferi* can be killed by NO; however, we do not know if this is the means by which the infected host eliminates the spirochete. The model presented in Fig. 8 depicts our understanding of the possible involvement of NO and inflammatory cytokines during infection with *B. burgdorferi*. The stimulatory effect of IFN- γ on the NO response suggests that the presence of IFN- γ or other cytokines in the local environment of inflamed tissue could strongly affect the ultimate level of NO produced in vivo. Many differences in cytokines produced in response to various infectious agents by C3H and BALB/c mice have been documented (38). In the case of murine leishmaniasis, the induction of IFN- γ and activation of iNOS have been shown to result in control of the infection, whereas the absence of NO production results in overwhelming infection (20, 21). Further studies designed to understand the regulation of iNOS during *B. burgdorferi* infection in severely and mildly arthritic strains of mice are needed to understand its role in the prevention or development of pathology.

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