

Decreased Interleukin-4 and Increased Gamma Interferon Production by Peripheral Blood Mononuclear Cells of Patients with Lyme Borreliosis

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Spontaneous and *Borrelia burgdorferi*-stimulated proliferation of peripheral blood mononuclear cells (PBMCs) and their interleukin-4 (IL-4), gamma interferon (IFN- γ), and NO production were measured in 36 patients with second- or third-stage Lyme borreliosis (LB) and 11 control subjects. Spontaneous proliferation of PBMCs was significantly higher ($P = 0.0003$) in the LB patients than in the control subjects. Spontaneous production of IL-4 was significantly lower in patients than in control subjects ($P = 0.0007$), but spontaneous production of IFN- γ was slightly higher in patients. The proliferative response of PBMCs to stimulation with *B. burgdorferi* was significantly higher ($P = 0.01$) in patients. The *B. burgdorferi*-induced production of IFN- γ ($P = 0.002$) was also significantly higher in patients. The spontaneous and *B. burgdorferi*-induced production of NO showed no significant difference between patients and control subjects. These findings indicate that the activation of PBMCs in patients with late LB is enhanced in vivo. Furthermore, the production of IL-4 is effectively suppressed spontaneously, whereas the production of IFN- γ by PBMCs is slightly increased spontaneously and significantly enhanced during stimulation with *B. burgdorferi* in vitro. The "spontaneous" or disease-induced alterations in cytokine levels of patients, in this case suppression of a Th2-type cytokine production and activation of a Th1-type cytokine production, may contribute to the pathogenesis of LB.

Lyme borreliosis (LB) is a multisystem disease with a strong tendency to chronicity. The severity of its symptoms has been associated with spirochete burdens in experimentally infected animals (31). However, it is likely that indirect mechanisms are partly responsible for the symptoms (11). The inciting pathogen, *Borrelia burgdorferi*, persisting in tissues, may cause continuous activation of lymphocytes and production of cytokines (14). The effects of tissue and circulating cytokines may explain many of the symptoms and inflammatory reactions of LB (14, 15, 25). Previous studies have shown that Lyme disease patients have elevated concentrations of interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha in the blood (3, 7, 20).

T-cell clones derived from patients with chronic LB and T cells from animals experimentally infected with *B. burgdorferi* have been found to exhibit the Th1 phenotype exclusively; i.e., they produce gamma interferon (IFN- γ) (32). Interestingly, the development of clinical arthritis seems to be associated with the production of Th1-type cytokines (IFN- γ) whereas resistance to the development of overt arthritis seems to be associated with the production of Th2-type cytokines (IL-4) in experimentally infected mice (18). CD4⁺ Th2 cells elicited by immunization also seem to protect mice from experimental borrelia infection (24). One study found striking differences in mouse spleen cell production of cytokines in vitro between disease-susceptible and -resistant mice, indicating the importance of IL-4 in early control of spirochete growth. In vivo studies on these experimentally infected mice further confirmed the above finding. Furthermore, the results indicated

that IFN- γ may inhibit the early control of spirochete growth in susceptible mice (13).

IFN- γ can also be linked to tissue damage via NO. IFN- γ induces intensive NO production by macrophages incubated with minute amounts of outer surface protein A (OspA) of *B. burgdorferi* (29). Although NO has potential antimicrobial properties, it may also be detrimental to the host (17).

We investigated spontaneous and *B. burgdorferi*-stimulated lymphocyte proliferation and production of IL-4, IFN- γ , and NO by peripheral blood mononuclear cells (PBMCs) in 36 patients with second- or third-stage LB and in 11 control subjects.

MATERIALS AND METHODS

Patients and healthy controls. The mean age of the patients, 18 women and 18 men, was 44.2 years (range, 4 to 77 years). The mean age of the control subjects, seven women and four men, was 32.5 years (range, 25 to 39 years). All patients were suffering from second- or third-stage LB, diagnosed according to Centers for Disease Control and Prevention criteria (23). Of the 36 patients, 30 had musculoskeletal manifestations of LB (18 had arthritis, 1 had myositis, and 11 had arthralgia); 19 had neurologic manifestations (1 had encephalitis, 3 had leukoencephalitis, 3 had facial palsies, 3 had meningitis, 1 had radiculoneuritis, 1 had epilepsy and cerebral vasculitis, 5 had severe dizziness, 1 had optic neuritis, 3 had neuritis or neuropathy, 2 had encephalopathy, 1 had transient hemiparesis, 1 had diplopia, and 1 had tinnitus); 20 had multisystem disease (two or more organ systems involved); 33 had serum antibodies against *B. burgdorferi* as measured by enzyme-linked immunosorbent assay (ELISA) with whole sonicated bacteria as the antigen; and 24 had DNA of *B. burgdorferi* in their plasma, cerebrospinal fluid, or synovial fluid as detected by PCR with primers for a chromosomal gene encoding the flagellin protein (22). Immunoglobulin M and G antibodies against sonicated *B. burgdorferi* were measured by an in-house ELISA (27). Seropositivity was determined by comparing antibody results for test serum samples with those for samples from 110 healthy controls. The cutoff value for weakly positive results was the mean plus 2 standard deviations of results from the controls. The three seronegative patients were all PCR positive.

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TABLE 1. Lymphocyte proliferation and production of IL-4, IFN- γ , and NO by PBMCs of patients with late LB and control persons

Subjects and conditions ^a	Lymphocyte proliferation (cpm)	Amt of:			
		IL-4 (pg/ml)	IFN- γ (pg/ml)	NO (μ M)	NO (μ M) (2 days)
Spontaneous					
Patients (<i>n</i> = 36)					
\bar{x} (SEM)	1,624 (367)	74 (18)	501 (301)	2.8 (0.3)	
Md (Q1, Q3)	851 (474, 1,213)	54 (0, 87)	24 (0, 112)	2.6 (1.0, 4.5)	
Controls (<i>n</i> = 11)					
\bar{x} (SEM)	354 (150)	374 (53)	71 (13)	3.6 (0.3)	
Md (Q1, Q3)	131 (86, 414)	432 (412, 469)	92 (9, 101)	3.8 (3.7, 4.2)	
<i>P</i>	0.0003	0.0007	0.3	0.5	
<i>B. burgdorferi</i> stimulated ^b					
Patients (<i>n</i> = 36)					
\bar{x} (SEM)	2,563 (487)	8 (7)	753 (232)	0.06 (0.09)	0.37 (0.18)
Md (Q1, Q3)	1,362 (438, 3,184)	0 (-5, 20)	482 (58, 850)	-0.05 (-0.29, 0.29)	0.29 (-0.05, 0.64)
Controls (<i>n</i> = 11)					
\bar{x} (SEM)	672 (148)	5 (51)	51 (27)	-0.26 (0.13)	-1.27 (1.42)
Md (Q1, Q3)	439 (294, 995)	-19 (-5, 20)	18 (-25, 89)	-0.19 (-0.63, 0.0)	-0.15 (-0.42, 0.55)
<i>P</i>	0.01	0.05	0.005	0.1	0.2
PHA stimulated					
Patients (<i>n</i> = 36)					
\bar{x} (SEM)	72,779 (6,350)				
Md (Q1, Q3)	63,011 (44,489, 91,001)				
Controls (<i>n</i> = 11)					
\bar{x} (SEM)	42,305 (7,143)				
Md (Q1, Q3)	46,852 (13,781, 55,539)				
<i>P</i>	0.01				

^a The mean (\bar{x}) values and in parentheses the standard errors of mean (SEM) are shown on the first row for different variables. The corresponding median (Md) and in parentheses lower (Q1) and upper (Q3) quartiles are given on the second row. P values were obtained by the Mann-Whitney U test.

^b Net production of IL-4, IFN- γ , and NO by stimulated cell cultures.

The control subjects were recruited among laboratory workers (n = 6) and hospital personnel (n = 5).

Mononuclear cell cultures. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation, washed three times, and adjusted to a final concentration of 10^6 cells per ml in heparin-RPMI (1 ml of heparin [5,000 IU/ml] in 100 ml of RPMI) supplemented with 10% AB serum and 0.1 mM 2-mercaptoethanol. For lymphocyte proliferation assays, 10^5 cells per well were added to microtiter wells (96 wells per plate) and stimulated with heat-killed *B. burgdorferi* cells (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The original bacterial suspension containing 0.5% (wt/vol) wet-packed cells in dextran solution was diluted 1:30, 1:60, 1:120, and 1:240 for stimulation. Cell cultures were incubated in a humidified atmosphere of 5% carbon dioxide at 37°C. Radioactive thymidine was added to the wells 18 h before the cells were harvested at 7 days. Cells were collected with a cell harvester onto fiberglass filters, and the radioactivity of the cells was determined with a scintillation counter. For measurement of mitogen-induced lymphocyte proliferation, PBMCs were stimulated with phytohemagglutinin (PHA; at 62.5 μ g/ml, previously found to be the optimal concentration) in wells containing 10^6 cells each. Radioactive thymidine was added to the wells 24 h before the cells were harvested at 4 days. Harvesting of cells and detection of radioactivity were carried out as described above.

Cytokine production and measurement. For cytokine and NO production assays, 3×10^5 PBMCs per ml were stimulated with sonicated *B. burgdorferi* cells (a high-passage strain, ATCC 35210) at a final concentration of 50 μ g of bacterial protein per ml. The incubation volume was either 800 or 1,600 μ l. The control tubes contained medium only. Cell cultures were incubated in a humidified atmosphere of 5% carbon dioxide at 37°C.

Culture supernatants were collected after 2 days for measurement of IL-4 and after 4 to 5 days for measurement of IFN- γ . These days were chosen on the basis of results of previous studies (12). The measurements were carried out with both unstimulated and *B. burgdorferi*-stimulated cell cultures. The collected supernatants were stored frozen at -70°C before measurement. IL-4 and IFN- γ were both measured by a sandwich ELISA as described previously (6). The sensitivity of the assay was 10 pg/ml for IL-4 and 20 pg/ml for IFN- γ .

NO $_2^-$ titration. Culture supernatants of both unstimulated and *B. burgdorferi*-stimulated cell cultures were collected after 2 and 4 to 5 days, and the NO concentrations were assayed by the Griess reaction as described previously (10). Briefly, 100 μ l of the supernatants to be tested and 100 μ l of a reactive solution composed of 1% sulfanilamide in 30% CH₃COOH plus 0.1% N-1-naphthyl ethylenediamine dihydrochloride in 60% CH₃COOH were added to wells of 96-well

microplates. The standard curve was prepared with NaNO₂ diluted in Iscove medium. Optical densities were measured at 540 nm with an autoreader (Dynatech Laboratories, Alexandria, Va.).

Statistical analysis. The data are summarized by using the means and the respective standard errors. Furthermore, we have also used medians and quartiles Q1 and Q3, which divide the ordered data into four sets such that the number of observations within each set is equal to 25% of the total.

The statistical analyses were carried out by the nonparametric Mann-Whitney U test and the Kolmogorov-Smirnov test. The former test searches for a difference between the study groups in the locations of the two distributions of measurements based on the medians. The latter test analyzes the difference between the groups in the shape and the location in general. This means that in certain cases the results will disagree. In our study, the results usually agreed; accordingly, we report results based on the Mann-Whitney U test. In cases when there was disagreement between the two tests, the results were analyzed in greater detail as described in Results.

RESULTS

Table 1 shows the proliferation (as expressed in counts per minute) of lymphocytes and the concentrations of IL-4, IFN- γ , and NO in culture supernatants.

Spontaneous proliferation of PBMCs was significantly higher ($P = 0.0003$) in the LB patients than in the controls. Statistical analysis of proliferative responses of PBMCs to stimulation with *B. burgdorferi* or PHA showed a marked difference ($P = 0.01$ for both) in the Mann-Whitney U test but no difference in the Kolmogorov-Smirnov test. The reason for this can be seen in the frequency distributions. The shapes of the distributions in both cases are analogous, but the medians differ. It can be concluded that the proliferative responses of PBMCs to *B. burgdorferi* stimulation were significantly higher ($P = 0.01$) in LB patients. Maximum proliferation was detected with a 1:120 dilution of the original bacterial suspension.

PHA-stimulated proliferation of PBMCs was significantly higher ($P = 0.01$) in LB patients than in controls.

The spontaneous production of IFN- γ was slightly higher (not statistically significant) in the patients than in the control subjects. In contrast, the spontaneous production of IL-4 was significantly suppressed ($P = 0.0007$) in the LB patients. The production of IFN- γ during a 4-day cultivation with *B. burgdorferi* was significantly higher ($P = 0.005$) in the patients. The levels of IL-4 in culture supernatants during the cultivation of PBMCs in the presence of spirochetal antigens remained unchanged (median net production, 0.0 pg/ml) in the culture derived from LB patients, whereas IL-4 levels in most cultures derived from control subjects decreased (median net production, -19 pg/ml). This difference between patients and controls was statistically significant ($P = 0.05$) by the Mann-Whitney U test but insignificant by the Kolmogorov-Smirnov test.

The spontaneous and *B. burgdorferi*-induced production of NO did not show significant differences between patients and control subjects. The only statistically significant P value ($P < 0.05$) for differences in NO production was obtained in the Kolmogorov-Smirnov test when spontaneous production of NO on day 2 was compared between groups. Spontaneous production of NO on day 4 and all *B. burgdorferi*-stimulated levels of NO production showed no significant differences between the groups. Therefore, it was concluded that there was no difference in NO production between the patients and control subjects.

DISCUSSION

The most striking difference between LB patients and control subjects was the suppression of IL-4 production in PBMC culture supernatants from the former. This suppression may be either characteristic of the subjects developing chronic forms of LB, or it may be a result of the ability of the spirochete to suppress or turn off the activation of Th2 cells. The tendency toward decreasing levels of IL-4 produced by PBMCs derived from controls when cultured with *B. burgdorferi* favors the latter possibility. The down-regulation of Th2 cells may lead to impaired humoral immune responses against the spirochete and worsening of the disease, since protection against LB is considered to be antibody mediated (2, 8, 18).

Our findings indicate that the PBMCs of patients with second- or third-stage LB are activated in vivo. This was evidenced by the increased spontaneous proliferation of the cells. It is possible that the activated cells are of the Th1 type, since the spontaneous production of IFN- γ was slightly increased whereas IL-4 production was significantly depressed. In vitro stimulation of PBMCs with *B. burgdorferi* further enhanced the production of IFN- γ in the LB patients but not in the control subjects. From these observations, it can be suggested that *B. burgdorferi* is able to stimulate the T cells of the Th1 phenotype both in vivo and in vitro. The enhanced production of IFN- γ in our patients further supports the previous findings of cytokine patterns in LB obtained in studies on experimentally infected animals and studies on limited numbers of LB patients (9, 13, 18, 32). In a recent study, two patients with neuroborreliosis eliciting Th1-type responses had simultaneous suppression of Th2 cell responses (9). Our findings with a larger group of patients are in agreement with this result.

IFN- γ is instrumental in activation of macrophages (1), stimulating them to release monokines, including the proinflammatory cytokines IL-1, IL-6, and tumor necrosis factor alpha (30). Thus, the activation of Th1 cells and production of Th1-type cytokines may contribute to the pathology of LB. Recent investigations indicate that IFN- γ may be involved in the de-

velopment of vasculitis and arteritis (30). Vasculitis is one of the mechanisms that can be an underlying cause of organ damage in LB. *B. burgdorferi* infection may trigger autoimmune reactions, which may also be attributable to Th1-type cytokines, known to be involved in the induction of autoimmune diseases (16, 26, 28).

Besides autoimmune reactions, chronic infection is another possible explanation for the persistent symptoms of LB (4, 14). Very small numbers of bacteria invading a site may cause intense inflammation and may also explain local inflammatory reactions (29). One candidate for the cause of the inflammation is NO (19). In studies on experimental animals, OspA and OspB of *B. burgdorferi* stimulated NO production by activating the cytokine-inducible NO synthase gene of macrophages. IFN- γ further enhanced NO production by activating macrophages (17). In certain tissues frequently affected by LB, such as the synovium, macrophages may account for half of the mononuclear cells (17). Invading *B. burgdorferi* organisms have ample opportunity to cause NO-mediated damage to the synovium. The damage may be mediated by vasculitis, which is a typical histologic finding in Lyme arthritis (17, 21).

We found no increased production of NO by PBMCs in our LB patients. Our study probably underestimated the production of NO during stimulation with *B. burgdorferi* by PBMCs, since the samples contained only a few macrophages: the isolation method had not been developed for obtaining macrophages. Further studies on adherent cells isolated from the peripheral blood are needed to obtain valid information about the true magnitude of NO production stimulated by *B. burgdorferi*.

A large number of plasma samples obtained from LB patients contained DNA of *B. burgdorferi* as detected by PCR. The increased production of IFN- γ by PBMCs obtained from the patients and even the presence of *B. burgdorferi* antigens in cell cultures without the addition of spirochetes to the media may explain the "spontaneous" proliferation of lymphocytes in LB patients.

The finding that PHA-stimulated proliferation of PBMCs was significantly higher in LB patients than in controls is in contrast to the results of one previous study, i.e., that antigens of *B. burgdorferi* may inhibit mitogen-induced lymphocyte proliferation (5). At present, we have no explanation to offer for this discrepancy.

Our findings indicate that PBMCs of patients with second- or third-stage LB are activated in vivo. The production of IFN- γ , a Th1-type cytokine, was increased spontaneously and further enhanced during stimulation with *B. burgdorferi* in vitro. The production of IL-4, a Th2-type cytokine, was significantly suppressed spontaneously. The "spontaneous" or disease-induced alterations in cytokine levels in patients, in this case suppression of a Th2-type cytokine and activation of a Th1-type cytokine, may contribute to the pathogenesis of LB either by activating macrophages to produce tissue-active substances or by inducing autoimmune reactions.

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