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Association of Treatment-Resistant Chronic Lyme Arthritis with HLA-DR4 and Antibody Reactivity to OspA and OspB of *Borrelia burgdorferi*

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Chronic Lyme arthritis that is unresponsive to antibiotic therapy is associated with an increased frequency of the HLA-DR4 specificity. To determine whether the immune response to a particular polypeptide of *Borrelia burgdorferi* may be associated with treatment-resistant chronic Lyme arthritis, we correlated the clinical courses and HLA-DR specificities of 128 patients with Lyme disease with their antibody responses to spirochetal polypeptides. Antibody reactivity was determined by Western blotting (immunoblotting) with sonicated whole *B. burgdorferi* and recombinant forms of its outer surface proteins, OspA and OspB, as the antigen preparations. Of 15 patients monitored for 4 to 12 years, 11 (73%) developed strong immunoglobulin G responses to both OspA and OspB near the beginning of prolonged episodes of arthritis, from 5 months to 7 years after disease onset. When single serum samples from 80 patients with Lyme arthritis, were tested, 57 (71%) showed antibody reactivity to recombinant Osp proteins; in contrast, none of 43 patients who had erythema migrans or Lyme meningitis ($P < 0.00001$) and 1 of 5 patients who had chronic neuroborreliosis but who never had arthritis ($P = 0.03$) showed antibody reactivity to these proteins. Among the 60 antibiotic-treated patients with Lyme arthritis, those with the HLA-DR4 specificity and Osp reactivity had arthritis for a significantly longer time after treatment than those who lacked Osp reactivity (median duration, 9.5 versus 4 months; $P = 0.009$); a similar trend was found for the HLA-DR2 specificity. For other HLA-DR specificities, arthritis resolved within a median duration of 2 months in both Osp-reactive and nonreactive patients. We conclude that the combination of the HLA-DR4 specificity and OspA or OspB reactivity is associated with chronic arthritis and the lack of a response to antibiotic therapy.

Lyme disease, which is caused by the tick-borne spirochete *Borrelia burgdorferi* (21), usually begins with a localized infection of the skin, erythema migrans, followed days to weeks later by dissemination of the spirochete to many sites, including joints (22). Months later, brief attacks of arthritis, lasting weeks, may occur in a few large joints and sometimes may be followed by prolonged episodes of arthritis, lasting months (26). Particularly during the second or third years of illness, approximately 10% of affected patients develop continuous joint inflammation for 1 year or longer; we have termed this condition chronic Lyme arthritis (25, 26). The histology of the synovial lesions in these patients is similar to that seen in other forms of chronic inflammatory arthritis, including rheumatoid arthritis (10, 23). However, even in untreated patients, chronic Lyme arthritis usually resolves within several years (26).

In a previous immunogenetic study of 130 patients with various manifestations of Lyme disease, 57% of those with chronic Lyme arthritis had the class II major histocompatibility complex (MHC) molecule HLA-DR4 and 43% had HLA-DR2; particularly those with HLA-DR4 often failed to respond to antibiotic therapy (24). These observations suggested that *B. burgdorferi* may trigger an untoward immune response, perhaps with autoreactive features, in patients with particular MHC genes, leading to chronic Lyme arthritis.

The cellular and humoral immune responses to *B. burgdorferi* develop gradually in Lyme disease (1, 5, 20, 28). In patients with erythema migrans, the cellular responses to *B. burgdorferi* are often minimal (20), whereas in patients with prolonged arthritis, marked reactivity to multiple spirochetal antigens is often found (28). Using serial serum samples from patients seen prior to the use of antibiotic therapy for this illness, many of whom participated in the immunogenetic study (24), we showed that the humoral immune response to *B. burgdorferi* expands over a period of months to years to an increasing array of 10 or more spirochetal polypeptides (1, 5). In the current study, our goal was to use this unique set of serum samples to determine whether the humoral response to a particular polypeptide of *B. burgdorferi* was associated with chronic Lyme arthritis.

MATERIALS AND METHODS

Patients. For the 130 patients with Lyme disease who participated in the previous immunogenetic study (24), single serum samples were still available from 123 patients: 80 had arthritis, 17 had meningitis, and 26 had erythema migrans. Of the 80 patients with arthritis, 60 had been given antibiotics sometime during the course of the arthritis; the remaining 20, who had been cared for in the 1970s, before the role of antibiotic therapy in this illness was known, had not received antibiotic treatment. For 15 of the arthritis patients, 10 or more serial serum samples were available from disease onset through 4 to 12 years of illness. At the time of examination, joint swelling was rated as severe (score of 4), marked (score of 3), moderate (score of 2), or mild (score of 1). The HLA-DR specificities of each patient were identified in the previous study (24) by standard lymphocyte microcytotox-

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icity testing (16) with a panel of local and exchanged alloantisera (15). In addition to the serum samples from these 123 patients, single serum samples from five patients who had chronic neuroborreliosis but who never had arthritis were also tested. For comparison, serum samples from 36 normal control subjects were tested.

Western blotting (immunoblotting). Serum samples were first tested by Western blotting with sonicated spirochetal lysates as the antigen preparations. Fifty micrograms of sonicated, filtered *B. burgdorferi* G39/40 was loaded on a preparative gel (7.5 by 5 by 0.08 cm) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the Protean II minigel system (Bio-Rad, Richmond, Calif.) as previously described (6). Proteins were transferred to Immobilon filters (polyvinylidene difluoride [PVDF]; Millipore, Bedford, Mass.) and blocked in blocking buffer (Tris, 0.04 M; NaCl, 1 M; thimerisal, 0.01%; Tween 20, 0.1%; nonfat dry milk, 5%; pH 7.5) overnight at 4°C. The filters were cut into 2-mm strips, and each strip was incubated with patient serum (diluted 1:250 in blocking buffer) and then with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (diluted 1:3,000) (Tago Inc., Burlingame, Calif.), each at room temperature for 1 h. Bound antibody was detected by incubation of the strips with 175 μg of 5-bromo-4-chloro-3-indolyl phosphate and 350 μg of nitroblue tetrazolium per ml in 0.1 M $NaHCO_3$–0.5 mM $MgCl_2$ (pH 9.8). Sequential serum samples from the same patient were tested on strips cut from the same filter.

Recombinant Osp proteins. Because initial studies indicated that the immune response to outer surface protein A (OspA) and OspB of *B. burgdorferi* may be associated with chronic Lyme arthritis, we constructed and purified hybrid proteins containing OspA and OspB. The *ospA* and *ospB* genes in the plasmid vectors pTRH44 and pTRH46 were kindly provided by Alan Barbour (8). Restriction fragments containing segments of the *ospA* and *ospB* genes were inserted at the 3′ end of the *malE* gene of *Escherichia coli*, which encodes maltose binding protein (MBP). Plasmid vectors pCG807 and pIH770 carry the *malE* gene expressed under the control of the inducible promoters P_{lac} and P_{tac}, respectively (14). The OspA fusion protein, designated MBP-OspA61, lacked the first 60 of the 273 amino acids of native OspA. The OspB fusion protein, designated MBP-OspB16, lacked the first 15 of the 297 amino acids of native OspB. The fusion proteins were purified on cross-linked amylose columns as previously described (11).

Serial or single serum samples from 128 patients with Lyme disease and from 36 normal control subjects were tested with these purified MBP-Osp fusion proteins as the antigen preparations in Western blots. The amounts of protein loaded on preparative gels were 10 μg of MBP-OspA61, 0.75 μg of MBP-OspB16, and 5 μg of MBP. In the few patients whose serum contained antibodies to MBP, Western blotting was repeated after adsorption of the serum with MBP. Twenty micrograms of MBP in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 8.0) was spotted onto nitrocellulose filters (3 by 5 mm). The filters were dried at room temperature for at least 90 min. The filters were then incubated overnight at 4°C in 10 μl of serum diluted 1:1 in phosphate-buffered saline–0.5% bovine serum albumin. The adsorbed serum was then used in Western blotting.

Statistical analysis. The identity of groups was compared with the Fisher exact test, and the distribution of values among groups was compared with the Wilcoxon rank sum test. All *P* values were two tailed.

RESULTS

Development of the antibody response in Lyme disease. In an effort to determine the natural history of the antibody response in Lyme disease, we first tested serial serum samples from 15 patients who were monitored in the 1970s and 1980s from disease onset through 4 to 12 years of illness. The courses of these patients were representative of the range of severity of joint involvement in Lyme arthritis. Of the 15 patients, 5 had the HLA-DR4 specificity, 4 had the HLA-DR2 specificity, and 6 had other HLA-DR specificities. For seven of the patients, antibiotic therapy had been given in the 1980s, late in the course of Lyme arthritis, and for eight patients, whose illness had resolved by that time, antibiotics had not been given.

In these 15 patients, the immunoglobulin G antibody response to *B. burgdorferi* expanded gradually to an increasing array of spirochetal polypeptides over a period of months to years, as determined by Western blotting with sonicated spirochetal lysates as the antigen preparations (the clinical course and serial blots for one representative patient are shown in Fig. 1). Common early responses prior to the onset of arthritis were to the 41-kDa flagellar protein of the spirochete, the 21-kDa OspC protein, and the 58- and 66-kDa heat shock proteins. During the initial brief attacks of arthritis, the patients continued to show expansion of the immune response to 10 or more spirochetal polypeptides, including those at 28 and 30 kDa, but the first attack of arthritis did not appear to be associated with the development of reactivity to a particular spirochetal polypeptide. In all 15 patients, the final point in the expansion of the antibody response was the recognition of polypeptides with apparent molecular masses of 31 and 34 kDa, and this event seemed to occur near the beginning of prolonged periods of arthritis. However, since it was difficult to differentiate the responses to the 30- and 31-kDa polypeptides, it was unclear, with this methodology, whether the 31-kDa polypeptide response was associated with prolonged periods of arthritis.

Thirty-one and 34 kDa are the molecular masses of the two major outer surface proteins of the spirochete, OspA and OspB, respectively. To test directly whether the 31- and 34-kDa polypeptides were indeed OspA and OspB, we produced and purified recombinant MBP-Osp fusion proteins (see Materials and Methods) and used them in Western blots. When the blots with each antigen preparation were compared, the onset of strong reactivity against the recombinant OspA and OspB proteins correlated precisely with the appearance of the 31- and 34-kDa bands on blots with sonicated spirochetes (Fig. 1). Sera from 2 of the 15 patients reacted weakly with MBP; this reactivity, but not the reactivity with the MBP-Osp fusion proteins, was abolished by adsorption of the sera with MBP. We concluded that the MBP-Osp fusion proteins could be used to analyze the Osp antibody response in Lyme disease.

In Western blots with the MBP-Osp fusion proteins, 11 of the 15 patients (73%), including all 9 with the HLA-DR4 or HLA-DR2 specificity, developed strong IgG responses to both OspA and OspB near the beginning of their most prolonged episodes of arthritis (Fig. 2). This response occurred from 5 months to 7 years (median duration, 18 months) after disease onset. When the recombinant proteins were used, it also became apparent that 5 of the 11 patients had previously developed weak responses to either OspA or OspB before or near the beginning of early, brief episodes of arthritis, a median duration of 6 months (range, 3 to 12

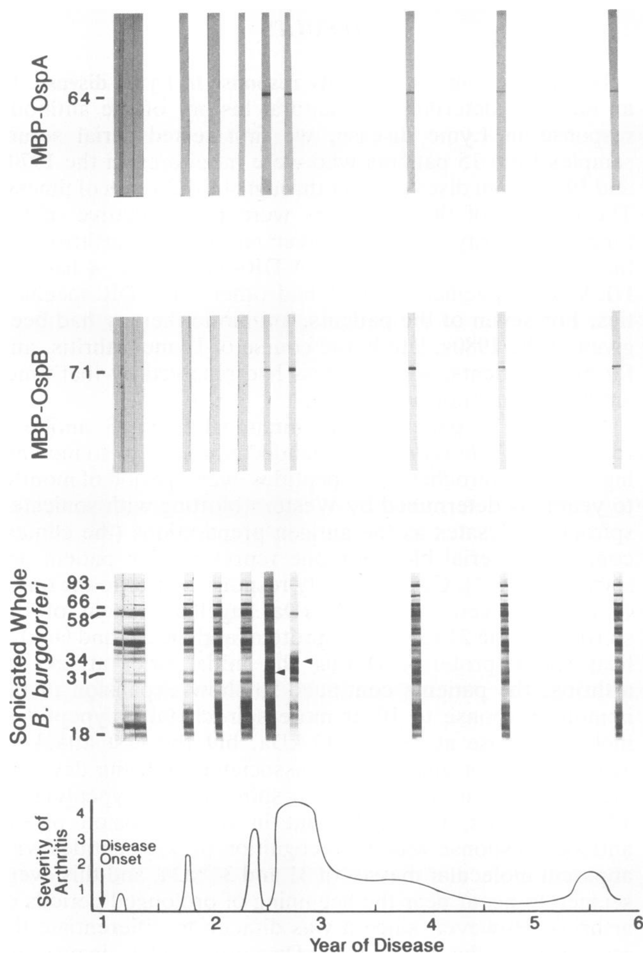

FIG. 1. Western blots of serial serum samples from a representative patient with Lyme disease. This patient had erythema migrans followed by brief attacks of arthritis and then chronic arthritis that lasted for 4 years. The duration and severity of arthritis, rated on a scale of 1 (mild) to 4 (severe) on the basis of the clinical joint examination, were correlated with the immunoglobulin G antibody response to *B. burgdorferi* when sonicated spirochetes (third panel) or recombinant MBP-Osp proteins (first and second panels) were used as the antigen preparations. The onset of the antibody response to the 31- and 34-kDa proteins (arrowheads) correlated precisely with the onset of reactivity with the OspA and OspB fusion proteins. This immune response occurred near the beginning of the most severe and prolonged period of arthritis.

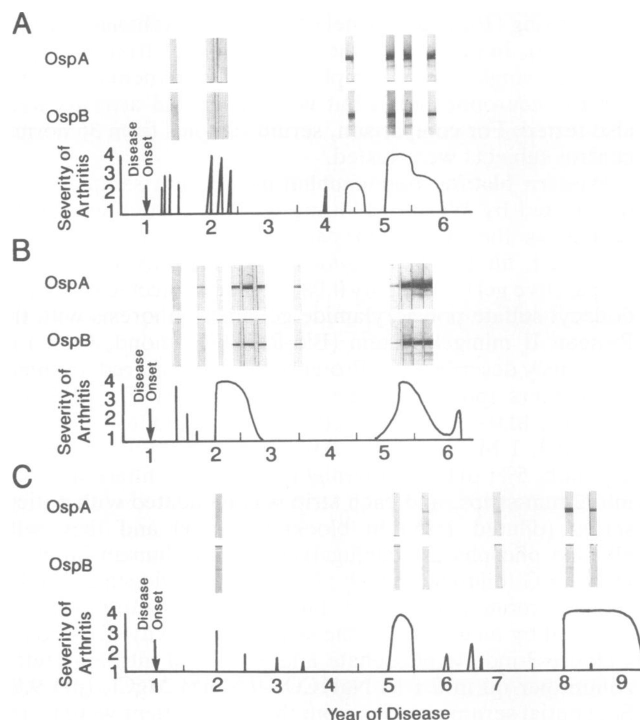

FIG. 2. Western blots of serial serum samples from three patients with chronic Lyme arthritis, demonstrating the range of patterns of reactivity with the MBP-Osp fusion proteins. The duration and severity of arthritis were correlated with the IgG antibody response to *B. burgdorferi* when MBP-Osp fusion proteins were used as the antigen preparations. After several brief attacks of arthritis, patient A, who had the HLA-DR4 specificity, generated a strong response to both Osp proteins near the beginning of prolonged periods of joint involvement. Patient B, who also had the HLA-DR4 specificity, developed faint reactivity with the OspA protein after 3 months of illness along with a first brief attack of arthritis. During the second year of illness, as the episodes of arthritis became longer, strong reactivity to both Osp proteins developed. During the fifth and sixth years of illness, the patient again had a prolonged period of arthritis accompanied by the most intense reactivity with the Osp proteins. Patient C, who had the HLA-DR2 specificity, developed faint responses first to the OspB protein and then to the OspA protein during brief attacks of arthritis. Not until the eighth year of illness did the patient develop strong reactivity to the OspA protein and a prolonged period of arthritis.

months) after disease onset. The remaining four patients, all of whom had other HLA-DR specificities, had only brief episodes of arthritis. They developed responses to either OspA or OspB, or in one instance to both, before or during the period of joint involvement, a median duration of 7 months (range, 3 to 41 months) after disease onset.

As the arthritis resolved, reactivity to OspA and OspB diminished, as demonstrated by the patient whose course is shown in Fig. 1. At 6 or 7 years after disease onset, 2 of the 15 patients developed chronic neurologic abnormalities of the disorder (12). For these two patients, Western blots with sonicated spirochetes as the antigen preparations showed no further expansion of the immune response after the period of arthritis (data not shown).

OspA and OspB antibody responses and manifestations of disease. We next looked for reactivity with the OspA and OspB fusion proteins in single serum samples from 128 patients with various manifestations of Lyme disease and from 36 normal control subjects (Table 1). None of the 43 patients with early manifestations of Lyme disease (erythema migrans or meningitis) and none of the 36 normal control subjects showed reactivity with the Osp proteins. Compared with these individuals, 57 of the 80 patients (71%) with Lyme arthritis showed reactivity to OspA or OspB ($P < 0.00001$). Of the 57 patients, 49 showed reactivity with both OspA and OspB, 7 showed reactivity with only OspA, and 1 showed reactivity with only OspB. Compared with the 80 patients with arthritis, only 1 of the 5 patients who had chronic neuroborreliosis and who never had arthritis showed weak reactivity with the Osp proteins ($P = 0.03$).

Osp reactivity and treatment response in Lyme arthritis. In an effort to determine whether reactivity to OspA and OspB was associated with treatment response, we compared clinical and laboratory findings for the 41 Osp-reactive and the

TABLE 1. Osp reactivity in 128 patients with various manifestations of Lyme disease and in normal control subjects

Individuals tested and disease manifestation (n)	No. (%) of patients with a response to:	
	OspA	OspB
Patients with Lyme disease		
Erythema migrans (26)	0	0
Meningitis (17)	0	0
Arthritis (80)	56 (70)[a]	50 (63)[a]
Chronic neuroborreliosis without arthritis (5)	1	1
Normal control subjects (36)	0	0

[a] $P < 0.00001$ for patients with arthritis versus those with erythema migrans or meningitis or normal control subjects and $P = 0.03$ for patients with arthritis versus those with chronic neuroborreliosis without arthritis.

19 non-Osp-reactive patients who had Lyme arthritis and who were treated with antibiotic therapy (Table 2). Of the 15 patients from whom serial serum samples were available throughout the course of the illness, the 7 who were treated with antibiotics were included in this analysis. The age and sex distributions of the patients and the antibiotic regimens used were similar for both Osp-reactive and non-Osp-reactive patients; each HLA-DR group had a similar frequency of Osp-reactive and non-Osp-reactive patients; and the median duration of disease prior to treatment did not differ significantly among the groups. In the pretreatment period, patients who had the HLA-DR4 specificity and showed OspA or OspB reactivity had significantly longer cumulative periods of active arthritis than those who lacked Osp reactivity (median duration, 11.5 versus 3.5 months; $P = 0.024$). Likewise, in the posttreatment period, the Osp-reactive patients had arthritis significantly longer than the non-Osp-reactive patients (median duration, 9.5 versus 4 months; $P = 0.009$). A similar trend in the posttreatment response was seen for patients with HLA-DR2 (median duration, 4 versus 1 month; $P = 0.073$). For other HLA-DR specificities, the arthritis resolved after treatment within a median duration of 2 months in both Osp-reactive and non-Osp-reactive patients.

Finally, we attempted to correlate the pre- and posttreatment durations of arthritis with the presence or absence of reactivity to other spirochetal polypeptides by using blots with sonicated *B. burgdorferi* as the antigen preparations (data not shown). All or almost all of the patients had reactivity to most of the other spirochetal polypeptides; for these antigens, statistical comparisons were meaningless since there were so few nonreactive patients. Twenty-four of the 80 patients with arthritis lacked reactivity with the 28-kDa polypeptide and 51 lacked reactivity with the 21-kDa OspC protein, but the durations of arthritis did not differ significantly between patients who did or did not show reactivity with these antigens. A more thorough analysis of this sort must await the development of a large library of recombinant borrelial proteins.

DISCUSSION

The purpose of the current study was to correlate the development of the humoral immune response to *B. burgdorferi* with the occurrence of chronic Lyme arthritis. For this purpose, we used a unique set of sera that included samples from previously studied patients who were monitored prior to the use of antibiotic therapy for this illness. All 15 patients from whom serial samples were available developed antibody reactivity to the 58- and 66-kDa borrelial heat shock proteins early in the illness (13). These heat shock proteins show homology to the GroEL protein of *E. coli* (4) and the 65-kDa heat shock protein of *Mycobacterium tuberculosis* (14), which is thought to trigger adjuvant arthritis in rats by an autoimmune mechanism (7). However, the responses to these borrelial heat shock proteins were not associated with the development of Lyme arthritis.

Instead, the development of prolonged episodes of arthritis occurred months to years later, near the onset of strong immunoglobulin G reactivity to the 31- and 34-kDa polypeptides of the spirochete; 31 and 34 kDa are the molecular masses of the OspA and OspB proteins (9). OspA and OspB, two of the major outer surface proteins of the spirochete, are closely related lipoproteins (3). They are encoded on the same linear plasmid (8) and share 53% amino acid homology (2). Because it was difficult to distinguish the responses to the 31- and 34-kDa polypeptides from those directed against other borrelial proteins of similar molecular masses (Fig. 1), we constructed MBP-Osp fusion proteins for use in Western blots. Although potential N-terminal epitopes as well as the lipid moiety were lacking in the fusion proteins, none seemed to be critical, since the appearance of the 31- and 34-kDa bands correlated precisely with the onset of strong reactivity to the OspA and OspB fusion proteins. Shanafelt et al. found that in a small group of patients with Lyme arthritis, antibody to OspA recognized only the carboxy-terminal portion of this protein (19). Sears et al. also found that sera from six patients with late Lyme disease bound

TABLE 2. Duration of arthritis before and after antibiotic treatment in 60 patients according to HLA-DR specificity and OspA or OspB antibody responses

HLA specificity	Total no. (%) of patients who were[a]		Median duration (range) of arthritis in the indicated patients			
			Before treatment		After treatment	
	Osp^+	Osp^-	Osp^+	Osp^-	Osp^+	Osp^-
DR4[b]	14 (64)	8 (36)	11.5 (1–28)[c]	3.5 (0–11)	9.5 (4–46)[d]	4 (0–9)
DR2	12 (71)	5 (29)	4.5 (3–28)	5 (1–9)	4 (1–48)[e]	1 (0.75–8)
Other DR	15 (71)	6 (29)	9 (1–42)	9 (2–20)	2 (0–9)	2 (0.25–12)

[a] Osp^+, Osp reactive; Osp^-, non-Osp reactive.
[b] The four patients who had both HLA-DR4 and HLA-DR2 specificities were included only in the HLA-DR4 group.
[c] Duration of arthritis before treatment, HLA-DR4, Osp^+ versus Osp^-, $P = 0.024$.
[d] Duration of arthritis after treatment, HLA-DR4, Osp^+ versus Osp^-, $P = 0.009$; duration of arthritis after treatment, Osp^+, HLA-DR4 versus other HLA-DR, $P < 0.001$.
[e] Duration of arthritis after treatment, HLA-DR2, Osp^+ versus Osp^-, $P = 0.073$.

primarily to the region of OspA containing amino acids 133 to 273, the C-terminal portion of the protein (18).

When single serum samples from 128 patients with different manifestations of Lyme disease were tested, 71% of the patients with arthritis but none of those with earlier manifestations of the illness showed strong IgG reactivity with the OspA or OspB fusion proteins and, in most instances, with both. Is it possible that the methods used here failed to identify a response to these proteins in patients with earlier manifestations of the illness? Our preparations of Osp proteins may not have had an epitope recognized early in the illness, antibodies generated to such an epitope may not bind in Western blots, or our methods may not have been sensitive enough to detect a minimal response. However, the important point is that the appearance of the IgG response to one or more epitopes present on the OspA and OspB fusion proteins occurred near the beginning of prolonged episodes of arthritis.

In a further effort to determine whether this association is only a result of the duration of infection, we attempted to develop a control group consisting of patients who had chronic neuroborreliosis but who never had arthritis. However, in our experience, this expression of the disease is rare. Of the five such patients that we have seen, only one showed weak reactivity with the Osp proteins. Because the onset of the strong antibody response to OspA and OspB occurred near the beginning of prolonged episodes of arthritis and because these events occurred together from 5 months to 7 years after disease onset, we doubt that this association is simply the chance occurrence of two events late in the illness.

In the previous immunogenetic study of 130 patients with Lyme disease, which was done primarily by serologic typing methods, chronic Lyme arthritis and a lack of response to antibiotic therapy were associated with an increased frequency of the HLA-DR4 specificity and, secondarily, the HLA-DR2 specificity (24). Subsequently, the HLA-DR4 association was confirmed for several representative patients by use of nucleotide sequence typing (24) and oligonucleotide probes (17). However, of the four HLA-DR2-positive patients whose HLA-DR alleles were determined by the latter method, two had DR2 alleles and two had the serologically cross-reactive DRw13 allele (17). We believe that the weak association between chronic Lyme arthritis and HLA-DR2, noted again in the current study (Table 2), suggests that only a subgroup of patients with this serologic specificity are susceptible to chronic Lyme arthritis; the actual marker is not yet clear.

The current study shows that the combination of the HLA-DR4 specificity and OspA or OspB reactivity is associated with chronic Lyme arthritis and a lack of response to antibiotic therapy. Is the interpretation of this association confounded by the fact that HLA-DR4-positive, Osp-reactive patients had a longer pretreatment duration of arthritis than HLA-DR4-positive, non-Osp-reactive patients? We think not, since the pretreatment duration of disease, as opposed to the cumulative duration of attacks of arthritis, was not significantly different between these two groups. The important point is that the HLA-DR4-positive patients with Osp reactivity had continuous arthritis for months or years after antibiotic treatment. This treatment-resistant course may not be different from the natural history of the illness, since chronic Lyme arthritis usually resolves within several years, even without antibiotic treatment (26). In contrast, the resolution of arthritis within 1 or 2 months after treatment, without a recurrence, which was the course in most of the other patients, suggests a treatment response.

Chronic Lyme arthritis is one of the few forms of chronic inflammatory arthritis in which the causative agent is known with certainty. In rheumatoid arthritis, disease susceptibility has been mapped to the third hypervariable region of the β1 chain of the HLA-DR4 molecule (27), but the foreign or endogenous peptides that may be presented by this molecule to initiate the disease-associated immune response remain a mystery. The work reported here suggests that certain class II MHC genes in combination with the immune response to OspA and OspB of *B. burgdorferi* may play a role in persistent Lyme arthritis that is unresponsive to antibiotic therapy. We believe that these clinical and epidemiologic observations will help guide studies aimed at defining the pathogenetic mechanisms of chronic Lyme arthritis. The elucidation of these mechanisms may serve as a model for helping to understand other forms of chronic inflammatory arthritis for which the cause is not yet known.

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