Heterogeneity in Immunoblot Patterns Obtained by Using Four Strains of *Borrelia burgdorferi* and Sera from Naturally Exposed Dogs

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Received 25 April 1988/Accepted 5 July 1988

This study evaluated the differences in immunoblot patterns when serum specimens from seropositive dogs were reacted against four strains of *Borrelia burgdorferi*. Intense bands were consistently detected for the 83-, 66-, 61- to 60-, 41-, and 31-kilodalton regions for all four strains. Most variations were observed in the regions of 45 to 34 and 26 to 15 kilodaltons. Adsorption studies suggested that one reason for the observed variability was a difference in proteins among the organisms, rather than a difference in migration of proteins. Therefore, knowledge and consistency of the test antigen are essential when evaluating and comparing canine immunoblot patterns to *B. burgdorferi*, but for diagnostic purposes all of the serum samples would have been considered positive regardless of the strain used.

Immunoblots have been used diagnostically in human Lyme disease to evaluate the specificity of immunoglobulins (2, 6, 7, 10). The immunoblot patterns obtained by using sera from infected human patients have been found to correlate with the stage of the disease (6, 7). In addition, under certain circumstances, immunoblots have been found to be the best method to detect serum antibodies in human Lyme disease patients (10).

In a previous study with canines, immunoblot patterns were evaluated by using sera from naturally and experimentally exposed seropositive dogs (9). Sera from the naturally exposed dogs had antibodies that normally reacted with at least 15 protein bands. Differences in immunoblot patterns existed among dogs, and these differences were observed in both clinically ill and asymptomatic seropositive dogs. Sera from experimentally exposed dogs reacted differently than did those from the naturally exposed dogs.

Reasons for the differences in immunoblot patterns may have been due to in vivo factors, such as spirochete strain differences, temporal differences during infection, or differences in the immune responses of the dogs. It is difficult to determine which of these factors play predominant roles in the pattern variability of the immunoblots because the antibodies produced under experimental conditions do not appear to be comparable to those produced in naturally exposed dogs. In addition, the absence of clinical signs soon after natural infection delays diagnosis and makes collection of sequential serum samples from recently infected dogs difficult.

Differences in laboratory methodologies may also lead to variability in immunoblot patterns. In particular, use of different *Borrelia burgdorferi* strains as antigens in immunoblots may be a source of interlaboratory pattern differences. The purpose of this study was to evaluate the degree of heterogeneity that occurred in immunoblot patterns when the same sera were reacted with different strains of *B. burgdorferi*. This information is important, since different laboratories may perform immunoblots with different isolates as antigens.

This report represents a portion of a thesis submitted by R.T.G. as partial fulfillment of the requirements for the Ph.D. degree.

MATERIALS AND METHODS

**Bacterial strains.** Four previously described isolates of *B. burgdorferi* were used as antigens for the immunoblots. *B. burgdorferi* B31 (ATCC 35210; American Type Culture Collection, Rockville, Md.; hereafter referred to as B31) and *B. burgdorferi* IRS (ATCC 35211; hereafter referred to as IRS) had been subjected to limiting dilutions such that cultures were derived from single spirochetes (3, 5). The number of in vitro passages these strains have undergone is unknown. Massachusetts isolate (MI) was isolated from the midgut of an *Ixodes dammini* nymph from Ipswich, Mass. (8). This organism had been passaged in vitro six times. Wisconsin isolate (WI) was isolated from the blood of an adult white-footed mouse (*Peromyscus leucopus*) from Fort McCoy, Wis. (1), and had been passaged 13 times in medium. The last two strains have not been subjected to limiting dilutions. All isolates reacted with genus (H9724) and species (H5332)-specific monoclonal antibodies (graciously provided by Alan G. Barbour, Department of Microbiology, University of Texas Health Science Center, San Antonio).

**Sera.** Serum samples were obtained from 12 clinically ill dogs from Wisconsin. These dogs were lame and often febrile or depressed. All dogs had high anti-*B. burgdorferi* antibody titers (immunofluorescence assay titers were greater than 1:512, and enzyme-linked immunosorbent assay values [8] were greater than 50).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Preparation of whole-cell suspensions has been described previously (8, 9). Briefly, 2-week-old cultures were centrifuged at 10,000 × g for 30 min at 4°C and washed three times in sterile phosphate-buffered saline, pH 7.38. The pellet was resuspended in enough sterile phosphate-buffered saline that 100 μl of a 1:4 dilution of the solution had an *A*₄₁₀ of 0.165 in

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where the silver stain identifies molecular weights. Lanes: 1, B31; 2, IRS; 3, MI; 4, WI. Numbers on the left indicate molecular weights of the protein standard (in thousands).

The lysates were electrophoresed in a 4% acrylamide stacking gel and a 10% acrylamide resolving gel. Electrophoresis was performed at a constant current of 35 mA for approximately 4 h. Coomassie brilliant blue G250 (Sigma Chemical Co., St. Louis, Mo.) and silver stains (Bio-Rad Laboratories, Richmond, Calif.) were used to visualize the proteins in the gels.

**Immunoblot analysis.** The procedure used for electrophoretic transfer of *Borrelia* proteins to a nitrocellulose membrane has been described previously (9). Transfer in a transblot cell (Bio-Rad Laboratories) was performed overnight at 0.1 A. After transfer was complete, the nitrocellulose was blocked for 1 h at room temperature with 26 mM Tris-buffered saline (TBS; pH 7.5) with 3% (wt/vol) gelatin, using constant rocking. The membranes were washed three times for 5 min per wash with 0.05% Tween-TBS. The membranes were incubated for 1 h with constant rocking at 25°C with 2 ml of a 1:50 dilution of the test serum in 1% gelatin-TBS (antibody buffer). The wash cycle was repeated. The nitrocellulose membranes were incubated for 1 h at room temperature with a 1:500 dilution of horseradish peroxidase-conjugated goat anti-dog immunoglobulin G (heavy chain specific) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) in antibody buffer. Strips were washed twice with Tween-TBS and then once with TBS. Nitrocellulose strips were developed by the addition of a freshly prepared solution of 0.05% (wt/vol) 4-chloro-1-naphthol (Bio-Rad Laboratories), 0.015% hydrogen peroxide, and 20% methanol in TBS. The reaction was stopped by washing the strips with cold distilled water.

**Adsorptions.** Suspensions of spirochetes were prepared such that 100 μl of a 1:4 dilution of the suspensions had an A410 of 0.222. For the whole-cell adsorption, 500 μl of the bacterial suspension to be used for adsorption was centrifuged at 8,200 × g for 10 min at 25°C. The supernatant was discarded, and the pellet was suspended in 0.5 ml of a 1:100 dilution of test serum in antibody buffer. This suspension was incubated for 30 min at 37°C and then centrifuged at 8,200 × g for 10 min. The supernatant was used to resuspend a new pellet of the same organism. A total of three adsorptions with each organism were performed. After the adsorption steps, the final 0.5 ml of supernatant was added to 1.5 ml of antibody buffer for use in the immunoblotting. For the adsorption with sonicated organisms, 1.5 ml of whole cells was sonicated (Branson Sonifier Cell Disruptor 200; Branson Sonic Power, Danbury, Conn.) for 4 min with alternating pulse and rest periods of 15 s in an ice bath, as previously described (8). Test serum (5 μl) was then added to the sonicated antigen, and this suspension was incubated for 1.5 h at 37°C. After the adsorption, the suspension was centrifuged at 8,200 × g for 10 min and the supernatant was added to 1.5 ml of antibody buffer. This was then used for immunoblotting. For controls, test sera were processed by these steps, with the adsorption antigen excluded.

**RESULTS**

The four strains of *B. burgdorferi* had similar but not identical electrophoretic patterns when stained with the Coomassie blue and silver stains (Fig. 1 and 2). With the Coomassie blue stain, major differences were noted in the 34- and 20-kilodalton (kDa) regions (Fig. 1). In the 34-kDa region, both B31 and WI had dark bands, while bands for IRS and MI were only faintly visible (Fig. 1). Faint bands...

**FIG. 1.** Coomassie blue stain of whole-cell lysates of four strains of *B. burgdorferi*. Two arrowheads identify the 34- and 20-kDa areas, where heterogeneity among the strains can be seen. The arrow identifies the 22-kDa region, where a difference is noted with the silver stain. Lanes: 1, B31; 2, IRS; 3, MI; 4, WI. Numbers on the left indicate molecular weights of the protein standard (in thousands).

**FIG. 2.** Silver stain of whole-cell lysates of four strains of *B. burgdorferi*. Two arrowheads identify the 34- and 20-kDa areas, where heterogeneity with the Coomassie blue stain can be seen. The arrow identifies the 22-kDa region, where IRS has a dark protein band not seen in the other three isolates. Lanes: 1, B31; 2, IRS; 3, MI; 4, WI. Numbers on the left indicate the molecular weights of the protein standards (in thousands).
which were not present in B31 or WI were detected in IRS and MI just above the 34-kDa region. The 20-kDa region varied with the Coomassie stain: B31 and WI lacked bands, while IRS and MI had distinct bands (Fig. 1). The silver stain revealed that all four isolates had protein bands in the 34- and 20-kDa regions (Fig. 2). In addition, the silver stain revealed a definite 22-kDa band in IRS which was not seen in the other three strains (Fig. 2). Overall, B31 most resembled WI, while IRS, with the exception of its 22-kDa band, was similar to MI.

For all 12 serum specimens, immunoblots for the 83-, 66-, 61- to 60-, 41-, and 31-kDa regions were similar for all four strains (Table 1), showing major bands that reacted identically. Among the serum samples there were occasional differences in the intensity of the reactions in these regions (Fig. 3). The immunoblots obtained with three representative serum specimens are shown in Fig. 3. These correspond to dogs 10 through 12 in Table 1.

Two general areas in the immunoblot patterns showed variations among the four strains. These were in the regions of 45 to 34 kDa and 26 to 15 kDa (Fig. 3, Table 1). For example, at the 45-kDa region, seven serum specimens had the same reaction with all four strains (a band was either present or absent with all four strains), but for five serum specimens there was a difference in reactions among the strains. Differences were characterized by a band being present in the immunoblot with one or more strains, but not in the others. There was no apparent consistency as to the strain for which the bands were present or absent. Another difference occurred just above the 41-kDa band. In 8 of the 12 immunoblots, a dark band was present when MI was used as the antigen but was not present in the reactions with the other strains (Fig. 3, where 3 of the 12 serum specimens are shown). Heterogeneity in the 34-kDa region was seen in two cases. There was an intense reaction with B31 and IRS as antigens but not with MI or WI (Table 1). In the other 10 cases, the reactions were the same among all of the strains (often a very weak reaction or none at all).

The most dramatic fluctuations in the immunoblots were in the 26- to 15-kDa region (Fig. 3, Table 1). Bands at 26, 24, 22, 20, 19, 17, and 15 kDa were evaluated in this area. The pattern variations included differences in intensity of reactions or presence or absence of bands. In the 19-kDa region there appeared to be a consistent difference accounted for by a particular strain. With all 12 serum specimens, immunoblots with WI as the antigen showed a band in the 19-kDa region. With 11 of the 12 serum samples, the band was dark. This band was absent or barely visible in the immunoblots with the other strains as antigens (Fig. 3, Table 1).

In the cross-adsorption experiment with B31 and WI, reactions to fewer bands were attributed to the lower concentration of serum used (Fig. 4). The homologous systems were more efficient at adsorbing the antibodies (Fig. 4). Approximately six faint bands remained when the serum that was reacted against B31 was first adsorbed with WI (heterologous system). The adsorbed bands were less intense than the nonadsorbed bands. This decrease in intensity of bands was different when WI was used as the immunoblot antigen. Two bands (19 and 17 kDa) were as intense as the nonadsorbed immunoblots when the serum was adsorbed with the heterologous system (B31). These bands were faint or eliminated when the serum was adsorbed with WI (the homologous reaction). Adsorptions with the sonicated antigens (not shown) were similar to those with whole cells.
Differences cause the numbers continued in vitro in variations developed types three (6, 7, 10). Extrapolation of the progression disease strain (A, B31, ATCC 35210; C, MI; D, WI. Dark bands are shown in boldface type.

**DISCUSSION**

Immunoblots have been evaluated in a sequential manner during the course of infection with *B. burgdorferi* in humans (6, 7, 10). Investigators have found that with time and progression of the disease, the number of antibodies in the sera of patients increases. The lack of an early marker for the disease in dogs has made it difficult to determine progression of the immune response to *B. burgdorferi* in canine infections. Extrapolation from the human immunoblot data would suggest that the sera used in this study were from dogs with chronic Lyme disease, or at least with chronic exposure to the organism. Regardless of the strain used as the antigen, the sera from these dogs consistently reacted against the 83-, 66-, 61/- to 60-, 41-, and 31-kDa regions. Perhaps these antigens would be cross-protective among different strains of *B. burgdorferi* if used in a canine vaccine.

This study evaluated heterogeneity in the immunoblot patterns with four different strains of the organism. The results indicate that there are differences due to the particular strain used as an antigen, but in all cases the serum samples appeared reactive regardless of the strain used. Three types of pattern variations were observed. One type was in the intensity of reaction against certain proteins. In particular, intense reactions were occasionally seen with one or more strains, and the reactions with the others were only weak. A second type was the difference in height of the band that developed in certain regions. The final pattern variation observed was that a protein band developed in the reaction with one strain but not with one of the others.

Proposed reasons for the varied reactivity patterns include the presence of different proteins within the strains or variations in the electrophoretic migration of those proteins. In vitro passage of these strains may have contributed to strain differences because the strains had been passaged different numbers of times. Antigenic changes associated with continued in vitro cultivation have been documented to cause differences in immunoblot patterns (12). Protein migration for the four strains was similar on the Coomassie blue and silver stains, but it was apparent that several immunogenic bands were not visualized with either stain. For example, the bands in the 19- to 15-kDa region consistently had immunoblot reactions but were not visualized when the two protein strains were used. It is possible that these bands do not represent proteins or that the immunoblots are more sensitive in detecting proteins.

The silver stain is more sensitive than Coomassie blue at staining proteins (11). Therefore, the finding that some proteins were better visualized with the silver stain was expected. The 22-kDa band of IRS, which stained more darkly than did the 22-kDa bands of the other strains, was not associated with a consistent reactive band in this area. It is possible that this protein is poorly immunogenic, that it is not presented to the immune system during natural infections, or that the strains that infected these dogs did not have this protein. Since this strain is an isolate from Europe, any of several explanations may be possible. Importantly, the electrophoretic differences detected with the protein stains did not correlate with consistent differences in the immunoblot patterns.

Some of these naturally exposed dogs may have been exposed to several different strains of *B. burgdorferi* such that antibodies developed to numerous proteins. It is difficult to determine which strain(s) elicited the immune response, since isolation is rarely successful in naturally exposed dogs. We used an isolate from Wisconsin as one antigen source and sera from Wisconsin dogs in an effort to simulate the conditions of natural exposures. This approach may be more theoretical than practical, because several isolates of *B. burgdorferi* have been obtained in Wisconsin (1).

The consistent reaction to a 19-kDa band for the immunoblots with WI indicated a difference in protein migration or a different protein in this strain than in the others. In the adsorption studies with WI as the antigen in the immunoblot, two dark bands (19 and 17 kDa) remained when B31 was used as the adsorption antigen. These bands were markedly decreased in intensity or absent when WI was used as an
adsorption antigen. These findings suggest that differences in electrophoretic migration of the same protein were not the cause and that the antibodies in the sera recognized epitopes present in WI that were not in B31. Similarly, the persistence of approximately six faint bands when B31 was used as the reacting antigen and WI was used for adsorption suggests that these proteins are slightly different in WI or are present in insufficient quantities in the adsorption suspension to completely eliminate the bands. The similarity between the adsorptions with whole cells and sonicated antigens implies that the differences were not due to the internal structural location of proteins. It is unlikely that the unadsorbed bands represent nonspecific reactions, since seronegative samples do not react on immunoblots (R. Greene, unpublished data).

It is interesting that all four strains had the 34-kDa protein, a major outer surface protein (Osp B), yet the sera rarely reacted in this region with any of the strains. This has been observed in previous human and canine immunoblots (2, 6, 7, 9). It has been proposed that this protein is somehow masked by an outer layer (4, 6, 7). The other major outer surface protein (Osp A) migrates to the 31-kDa region. None of the sera tested from naturally exposed dogs reacted against this protein (9). Instead, the reaction in the 31-kDa region was to a protein that migrated just ahead of the Osp A. It is unclear why all dogs had a response to the flagellar (41-kDa) protein, since in spirochetes the flagella are found beneath the outer membrane. Humans also react to this 41-kDa protein before they react to the 31-kDa protein (6, 7). Possibly, this finding relates to the pathogenesis of the organism or to the antigen-processing capabilities of the host. The lack of an immune response to these outer proteins may be partly responsible for the persistence of the organism and the chronic nature of the disease syndromes.

In summary, major protein bands were consistently detected for all 12 serum samples at the 83-, 66-, 61- to 60-, 41-, and 31-kDa regions with four different strains of B. burgdorferi. The major heterogeneity among the canine immunoblot patterns when these four strains were used was in two broad regions (45 to 34 kDa and 26 to 15 kDa). Adsorption studies suggested that one mechanism for the pattern variation was the presence or absence of certain proteins or epitopes in some strains. When canine immunoblot patterns to B. burgdorferi are evaluated, it is essential that differences due to the strain used in the immunoblots be considered. The differences were not drastic enough to affect the results when this technique was used as a supplementary diagnostic procedure.

ACKNOWLEDGMENTS

We thank William L. Nicholson for his technical assistance. Resources to support this research were provided by the State of North Carolina.

LITERATURE CITED