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Evaluation of Genetic Divergence of Borrelial Isolates from Lyme Disease Patients in Hokkaido, Japan, by rRNA Gene Probes

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Eight spirochetal isolates (JEM1 to JEM8) were obtained from cutaneous lesions of patients with Lyme disease in Hokkaido, Japan, and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reactivities with monoclonal and polyclonal antibodies, and Southern blot hybridization. The protein profiles of these borrelial isolates were variable and differed markedly from that of *Borrelia burgdorferi* B31. The 41-kDa flagellin protein was present in all isolates, but the outer surface protein A that reacted with monoclonal antibody H5332 was absent from four clinical isolates (JEM1, JEM5, JEM7, and JEM8). Genomic hybridization with rRNA gene probes demonstrated the genetic divergences among those isolates. These findings indicate that the borrelial isolates from patients in Japan are quite characteristically unique.

The etiological agent of Lyme disease, *Borrelia burgdorferi*, was discovered and described about 10 years ago (8, 16). Since then, additional isolates of *B. burgdorferi* have been obtained from patients, rodents, and various species of ixodid ticks in all parts of the world (3, 8, 10, 28). *B. burgdorferi* sensu lato was first thought to be a homogeneous species. However, on the basis of DNA homology, specific rRNA gene restriction patterns, and the differential reactivities of monoclonal antibodies, three genospecies were recently delineated (2). Two of them are named *B. burgdorferi* sensu stricto and *B. garinii*, and the third genospecies is referred to as "group VS461."

We have studied the organization of the genes coding for the rRNAs of *B. burgdorferi* and found a unique organization of rRNA genes in this organism (11, 13, 15, 27). Two sets of 23S/5S rRNA genes (*rrl/rrf*) have 3.2-kb tandem repeats in that genome (13). A recent typing method based on developments in DNA analysis is the use of rRNA gene probes to study the restriction fragment length polymorphisms (RFLPs) of rRNA genes (30). This procedure is most successful for characterizing bacterial species and is also useful for identifying geographic isolates and for epidemiological studies (2, 27). On the basis of these findings, we performed an RFLP analysis of *Borrelia* strains isolated from Europe, the United States, and Asia (14). Our results indicated that the European and U.S. isolates could be classified into three distinct RFLP groups. By this method, we were able to associate certain RFLP groups with three genospecies exclusively. However, some isolates from Japanese ticks (*Ixodes persulcatus*) clustered into different RFLP groups which did not belong to those of any of the genospecies (14).

We report here the genetic divergences of clinical isolates obtained from patients with Lyme disease in Japan. One clinical isolate (JEM4) was classified as group II (corresponding to genospecies *B. garinii*), while the other isolates were dissimilar to all representatives of the three genospecies. Six clinical isolates clustered into a fourth group, and another isolate (JEM3) did not belong to any of the groups.

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MATERIALS AND METHODS

Bacterial isolates and culture. Eight borrelial isolates from humans, designated JEM1 to JEM8, were obtained by culturing cutaneous tissues of erythema migrans lesions in BSKII medium at 31°C for 4 weeks as described previously (25). These biopsy specimens from the patients are described in Table 1. In all cases, we could not identify the species of ticks because the patients were admitted to the hospitals after tick removal.

SDS-PAGE and Western blot analysis. The borrelial isolates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The procedures for SDS-PAGE and Western blot (immunoblot) analysis were as described previously (25, 26). Murine monoclonal antibodies H5332 and H9724 were obtained as hybridoma supernatants (4–6). Polyclonal antiserum to outer surface protein A (OspA) of *B. burgdorferi* B31 was prepared from BALB/c mice.

Southern hybridization and probe. Cells at the late exponential phase of growth in 100 ml of BSKII medium were collected and washed by centrifugation. Genomic DNAs were extracted by a previously described method (12, 13) and were digested with the enzymes indicated in the legend to Fig. 3. After separation of DNA digests by electrophoresis, DNA fragments in an agarose gel were stained with ethidium bromide, photographed, and transferred to a nylon membrane as described previously (12).

Southern hybridization was performed by using rRNA gene probes as described previously (13, 14). We used two types of probes. The DNA fragment generated by *Nhe*I and *Pst*I digestion of the *rrl* sequence from *B. burgdorferi* B31 (5′ part of the gene; probe NP) and the fragment including the 3′ part of the gene from the same organism generated by *Sty*I digestion (probe Sty) were used. DNA fragments were amplified by polymerase chain reaction as described previ-

TABLE 1. Origin of human-derived spirochetes and results of OspA Western blotting and RFLP ribotyping

Isolate	Patient characteristic			Size (kDa) of OspA[a]	RFLP pattern[b]
	Sex	Age (yr)	Biopsy site[c]		
JEM1	Female	62	Right upper arm		IV-2
JEM2	Female	49	Right chest	31.0	IV-4
JEM3	Female	52	Abdomen	30.0	V
JEM4	Male	57	Left popliteal fossa	31.0	II-8
JEM5	Male	65	Right shoulder		IV-2
JEM6	Male	29	Right shoulder	31.5	IV-3
JEM7	Male	40	Dorsal center		IV-1
JEM8	Female	67	Right earlobe		IV-2

[a] Molecular sizes indicate the proteins that were reactive with monoclonal antibody H5332.
[b] RFLP groups were designated as described previously (14).
[c] Human clinical isolates were obtained by culturing the cutaneous tissues (about 5 to 10 mm^3) of erythema migrans lesions.

ously (14) and were radiolabeled by using [α-^{32}P]dCTP (222 TBq mmol; Amersham Japan Ltd., Tokyo, Japan) and a random primer labeling kit (Takara Shuzo Co. Ltd., Kyoto, Japan).

RESULTS

SDS-PAGE and Western blot analysis. Eight clinical isolates were examined by SDS-PAGE, and the resulting profiles are shown in Fig. 1. The protein profiles of those isolates were different from one another, and the major proteins of the isolates were found to differ from those of the North American type strain *B. burgdorferi* B31. The molecular sizes of proteins from borrelial isolates from patients with Lyme disease in Hokkaido varied for proteins with lower molecular sizes (20 to 35 kDa) but were similar to each other for proteins of 36 kDa or larger. The reactivities of monoclonal antibodies H9724 and H5332 and polyclonal antibody against the clinical isolates are shown in Fig. 2. For comparison with human isolates, 32 isolates from *Ixodes ovatus* ticks were subjected to SDS-PAGE and immunoblotting. The protein profiles and reactivities of monoclonal and polyclonal antibodies of all isolates obtained from *I. ovatus* ticks were almost identical. Therefore, the results for one isolate (HT103) are shown in Fig. 1 and 2 together with those for the isolates from humans. The epitope for H9724 was present in the 41-kDa flagellin proteins of all isolates. However, the reactivities of the proteins with H5332 were quite different. Four clinical isolates (JEM2, JEM3, JEM4, and JEM6) and the isolate from *I. ovatus* possessed the proteins that reacted with H5332, but their molecular sizes were heterogeneous and ranged from 30 to 31.5 kDa. The other four isolates (JEM1, JEM5, JEM7, and JEM8) failed to react with H5332, whereas these isolates bore major OspA-like protein bands. Mouse polyclonal antibodies directed against

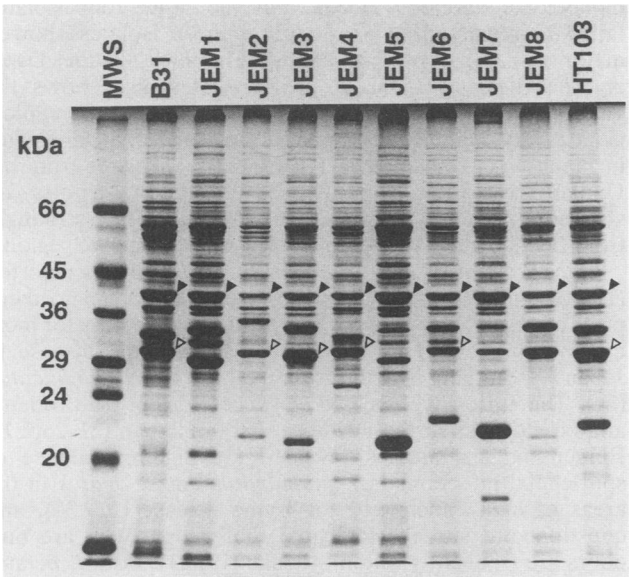

FIG. 1. Coomassie brilliant blue-stained proteins in whole-cell lysates of tick isolates. Lane B31, *B. burgdorferi* B31; isolates JEM1, JEM2, JEM3, JEM4, JEM5, JEM6, JEM7, and JEM8, spirochetal isolates from Lyme disease patients in Hokkaido, Japan; isolate HT103, spirochete from an *I. ovatus* tick. Molecular mass standards (MWS) are bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20-kDa). The arrowheads indicate Western blots of isolates that reacted with monoclonal antibodies (H9724 and H5332). The solid and open arrowheads indicate the H9724- and H5332-reacting proteins, respectively.

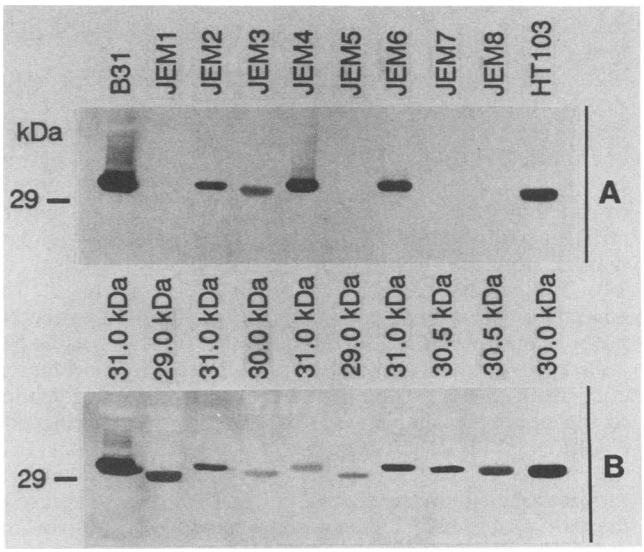

FIG. 2. Antigenic characteristics of clinical and tick isolates determined by Western blotting against the OspA protein. All spirochetal isolates were the same as those in Fig. 1. (A) Monoclonal antibody H5332; (B) mouse polyclonal sera against purified OspA protein from *B. burgdorferi* B31.

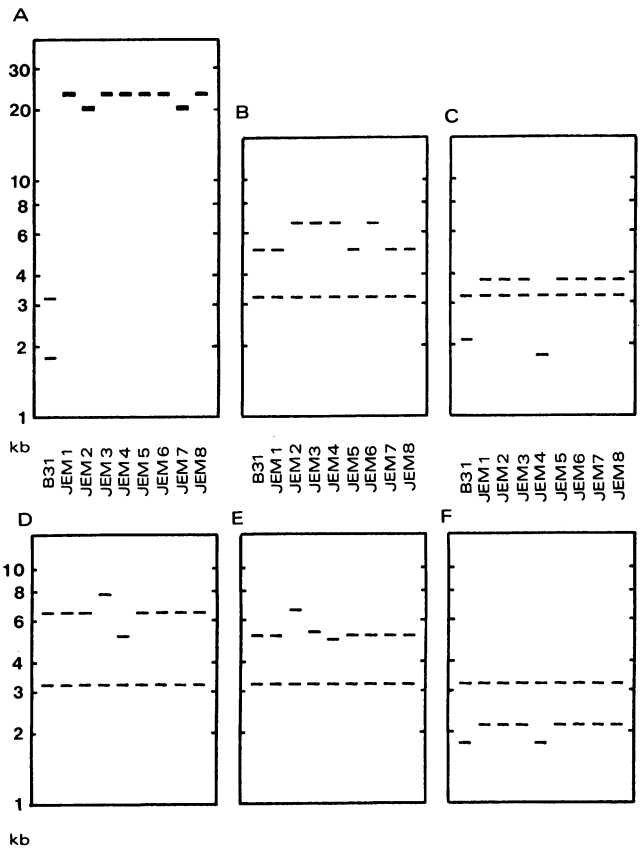

FIG. 3. Schematic representation of the hybridization patterns obtained with the probe. Genomic DNAs were digested with *Pst*I (A), *Hpa*I (B and C), *Eco*RV (D), and *Hin*cII (E and F), electrophoresed in agarose gels, blotted, and probed with radiolabeled rRNA gene fragments. Probe NP (A, B, D, and E) or Sty (C and F) were used as hybridization probes. All other experimental conditions are described in the text.

the OspA protein of strain B31 reacted with all of these OspA-like proteins (Fig. 2B).

Genomic Southern hybridization and schematic representation of RFLP patterns. The genomic DNA of each clinical isolate was extracted and digested with *Pst*I, *Eco*RV, *Hpa*I, or *Hin*cII. DNA digests were separated, blotted, and hybridized with ^{32}P-labeled probes (NP or Sty). The patterns obtained by genomic Southern hybridization showed that the strains usually shared a common 3.2-kb fragment which was derived from the rRNA gene repetition in this organism (13, 14). The results are shown schematically in Fig. 3. The genomic Southern hybridization of *Pst*I digests with the NP probe is shown in Fig. 3A. None of the clinical isolates had a *Pst*I site in their *rrl* sequences and presented profiles in contrast to that for strain B31. In contrast, the hybridization of the other digests revealed two bands. The sharing of a common 3.2-kb fragment suggests that a fragment begins at the enzyme cleavage site in the first *rrl* sequence and ends at the cleavage site in the second copy of the gene. The use of the NP probe revealed a second band consisting of the remainder of the first *rrl* sequence and the upstream region to the next enzyme cleavage site (B, D, and E). A second band which comprised the remainder of the second *rrl* gene and the downstream region to the next restriction site (C and F) was detected by using the Sty probe. By probing of *Eco*RV digests with the NP probe, six clinical isolates and strain B31 gave a second band of 6.5 kb which contained the 5′ half of the first *rrl* sequence and the upstream region to the next *Eco*RV restriction site (D). JEM3 yielded a second band of 7.8 kb, and JEM4 gave a distinct second band of 5.2 kb. With *Hpa*I digestion, four isolates (JEM2, JEM3, JEM4, and JEM6) showed different upstream *Hpa*I sites, but only JEM4 had a distinct restriction site downstream of the genes (B and C). JEM2 and JEM3 showed a different upstream *Hin*cII site, whereas JEM4 revealed different *Hin*cII sites both upstream and downstream of the rRNA genes (E and F). The groupings obtained on the basis of RFLP analysis are also presented in Table 1.

DISCUSSION

Lyme disease is frequently diagnosed on the basis of the presence of the characteristic skin lesion, erythema chronicum migrans (29). The first patient with Lyme disease in Japan was diagnosed by serological detection of antibodies to antigens of *B. burgdorferi* and was reported in 1987 (17). Subsequently, spirochetes were actually detected in a culture of patient specimens (18, 25). The present study was undertaken to analyze and compare the antigenic and genetic characteristics of *Borrelia* isolates in Japan. Eight clinical isolates were obtained from erythema migrans lesions of patients admitted to the Asahikawa Medical College Hospital and related clinics during the last 3 years (from 1990 through 1992). Those clinical isolates were examined by SDS-PAGE and genomic Southern hybridization by using rRNA gene probes. The isolates exhibited a variety of protein profiles and considerable antigenic heterogeneity. Four clinical isolates possessed proteins that reacted with monoclonal antibody H5332, but the other four isolates failed to react with H5332. All of these isolates showed major OspA-like protein bands. The lack of the OspA epitopes against H5332 in these isolates also shows the variations in the antigenic properties of the Japanese clinical isolates. By using RFLP analysis, almost all clinical isolates in Japan were dissimilar to representative isolates from the United States and Europe. One of our previous reports (14) showed that some Japanese tick isolates differ strikingly from tick isolates recovered from other geographic regions.

Since the first case of Lyme disease in Japan was reported (17), the number of cases has increased gradually in northern parts of Japan (1, 18, 24). However, the tick vectors for most cases were not identified. The agent of Lyme disease in Japan was initially isolated from the hard tick *I. persulcatus* (21). Thereafter, we isolated spirochetes from wild rodents and ixodid ticks in the northern parts of Japan (21–26, 31). Epidemiological studies revealed the high prevalence of spirochetal infections in *I. persulcatus* and *I. ovatus* in the areas of high endemicity for Lyme disease (22, 31), and questionnaire surveys indicated that people who are outdoors are frequently bitten by ticks (19, 23). Both *I. persulcatus* and *I. ovatus* are the most common tick species in Hokkaido. We demonstrated that the isolates from *I. ovatus* ticks collected at various locations in Japan were homogeneous and distinguishable from the heterogeneous isolates from *I. persulcatus* ticks with respect to their protein compositions (Fig. 1) (26). The data obtained in the present study and from our previous work (14) indicated that Japanese borrelial isolates obtained from patients and the isolates from *I. persulcatus* ticks are clustered into the same RFLP groups. Our RFLP analysis of isolates from ticks also indicated that the borrelial isolates obtained from *I. ovatus*

were quite distinct from members of the groups associated with Lyme disease (data not shown). All of these results, therefore, suggested that *I. persulcatus* is the most important vector of Lyme disease in Japan.

Lyme disease is a severe human illness primarily initiated by infection with the pathogenic spirochete *B. burgdorferi* (7, 9). Following the skin rash, severe arthritis and neurologic and cardiac manifestations may occur (8, 16, 28). A number of *Borrelia* isolates have been obtained from clinical specimens, rodents, and tick vectors. Those isolates were examined by biochemical, immunological, and genetic procedures, and now, borrelial isolates associated with Lyme disease in the United States and Europe are thought to consist of three genospecies (2, 20, 32, 33). In contrast, symptoms of Lyme disease in Japan associated with cardiac and nervous system manifestations have not been well documented.

We found that clinical isolates obtained in the Hokkaido area can share phenotypic and genetic heterogeneity. The characteristics of Japanese isolates are dissimilar to those of strains from Europe and the United States. This dissimilarity is thought to be related to the species of tick vector. Ticks belonging to the *Ixodes ricinus* species complex, *Ixodes scapuralis* and *Ixodes pacificus* in North America and *I. ricinus* and *I. persulcatus* in Eurasia, are important vectors to humans. *I. ricinus* is distributed in European countries, and the habitat of *I. persulcatus* ranges continuously from eastern Europe to the Far East. The wide variation in antigenic and genetic properties of Japanese isolates may suggest an evolution of the borreliae in the hosts.

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