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Antigenic Properties of *Borrelia burgdorferi* Isolated from *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan

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Spirochete strains HP3 and HO14, isolated from *Ixodes persulcatus* and *I. ovatus* in Hokkaido in 1989, were the first isolates of *Borrelia burgdorferi*, the etiological agent of Lyme disease, to be recognized in Japan. Antigenic properties of the Japanese strains were compared with those of the strains isolated in the United States (B31 and 297) and Europe (IRS, P/Gau, P/Bi, 2/B45, and 3/B56) by Western blotting (immunoblotting), by using monoclonal antibodies (MAbs) against strains B31 and P/Bi. The Japanese strains reacted with MAb U40 against the 41-kDa antigen. MAb E34a31 against Osp A reacted with all the strains tested except for strain HP3. Furthermore, MAb U31b against Osp A reacted with all the American and European strains but did not react with the Japanese strains. When MAbs against Osp B were used, MAb E34b reacted only with European strains and MAb U34b reacted only with the American strains. However, neither showed reactivity to two Japanese strains. MAb E60 against 60-kDa antigen reacted with all the U.S. and European strains and strain HP3 but did not react with Japanese strain HO14. These results indicate that the antigenicity of the Japanese strains isolated from two species of ixodid ticks is different from that of the strains isolated in the United States and Europe. It is suggested that the Japanese strains are much more suitable than the U.S. or European strains as the antigen source for the serodiagnosis of Lyme disease in Japan.

The etiologic agent of Lyme disease, *Borrelia burgdorferi* (9), has been isolated or detected from various species of ixodid ticks, *Ixodes dammini* (5), *I. ricinus* (2), *I. pacificus* (7), and others (6, 17).

Lyme borreliosis was first reported in Japan in a patient in the Nagano Prefecture in 1987 (10). Spirochetes were isolated from two species of ixodid ticks, *I. persulcatus* and *I. ovatus*, in Hokkaido, Japan, and were determined to be *B. burgdorferi* by genus-specific monoclonal antibody (MAb) H9724 (3) against flagella and MAb H5332 (4) against outer surface protein A (Osp A) (16). Although *I. persulcatus* is considered to be a vector of Lyme borreliosis in European and Asian USSR, China, and Japan (6), isolation of *B. burgdorferi* from *I. ovatus* was a novel observation and is of interest because of the ecological features of this tick species. A comparative study of the antigenic properties of *B. burgdorferi* among Japanese, American, and European strains is important for the establishment of a confident serodiagnosis and is necessary for the development of a vaccine for Lyme borreliosis in Japan.

In this study, the antigenic characteristics of Japanese strains were compared with those of the strains isolated in the United States and Europe by Western blotting (immunoblotting) by using MAbs against strain B31 and P/Bi.

MATERIALS AND METHODS

Strains and cultivation. The borrelial strains used in this study are shown in Table 1. Strains B31, 297, IRS, P/Bi, P/Gau, and 2/B45, and 3/B56 were kindly provided by R. C. Johnson, the University of Minnesota, Minneapolis, and by A. Schönberg, the Institute for Veterinary Medicine, Berlin, Federal Republic of Germany. The Japanese strains, HO14 and HP3, were isolated from the midgut of *I. ovatus* and *I. persulcatus* in Hokkaido, Japan, in 1989 by Sato et al. (16). Borrelia strains were cultivated at 33°C in BSK II medium (1).

Rabbit antiserum. Polyclonal antisera to *B. burgdorferi* 297, B31, and P/Bi were prepared from rabbits. Rabbits were injected intravenously four times at 7-day intervals with lyophilized cells (100 μg) in saline.

Development of MAbs. Hybridomas secreting MAbs were prepared by the method of Köhler and Milstein (11). Briefly, BALB/c mice (Japan SLC Co., Hamamatsu, Japan) were injected intraperitoneally with 10^8 lyophilized cells of strain B31 or P/Bi four times at 7-day intervals. The spleen cells of immunized BALB/c mice were fused with P3-X63-Ag8.653 mouse myeloma cells at a ratio range of 5:1 to 10:1 by using 50% polyethylene glycol 4000 (E. Merck AG, Darmstadt, Federal Republic of Germany) by the method described previously (13). Fused cells were cultured in hypoxanthine-

TABLE 1. Type and source of borrelial strains

Organism	Source of organism	Origin of source
B. burgdorferi		
B 31	*I. dammini*	New York
297	Human CSF	Connecticut
IRS	*I. ricinus*	Switzerland
P/Bi	Human CSF	FRG[a]
P/Gau	Human skin	FRG
2/B45	*I. ricinus*	FRG
3/B56	*I. ricinus*	FRG
HP3	*I. persulcatus*	Hokkaido, Japan
HO14	*I. ovatus*	Hokkaido, Japan
B. coriaceae	*Ornithodoros coriaceus*[b]	California
B. parkeri	*Ornithodoros parkeri*[c]	Western United States

[a] FRG, Federal Republic of Germany.
[b] The presence of *B. coriaceae* may be associated with epizootic bovine abortion.
[c] The presence of *B. parkeri* has been associated with New World tick-borne relapsing fever.

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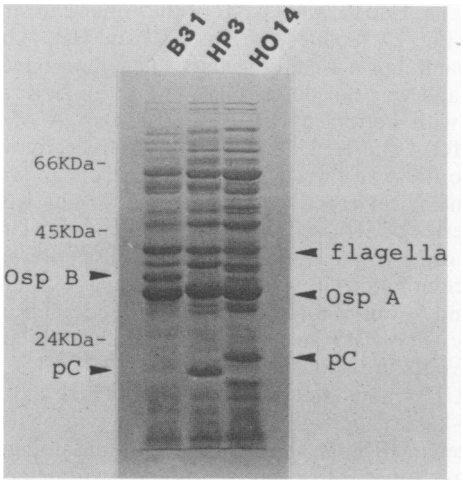

FIG. 1. SDS-PAGE profiles. Numbers on the left and arrowheads indicate the molecular masses of the standard proteins used and positions of flagellum Osp A, Osp B, and pC proteins, respectively.

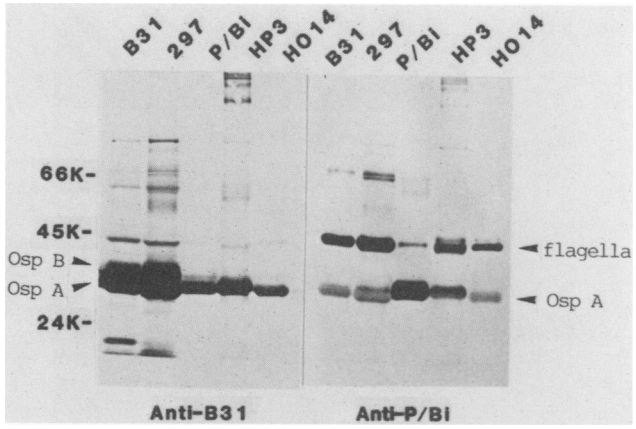

FIG. 2. Antigenic characteristics of Japanese strains by Western blotting with rabbit polyclonal sera. Numbers on the left and arrowheads indicate the molecular masses (daltons) of the standard proteins used and positions of flagellum, Osp A, and Osp B proteins, respectively.

aminopterin-thymidine medium. Two weeks later, the culture supernatants were tested for the formation of specific antibody against homologous strains by immunoblotting.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (12), with a vertical slab gel of 10 or 15% polyacrylamide and a stacking gel of 5% polyacrylamide. The gels were stained with Coomassie brilliant blue. Antigens from another gel strip were electrophoretically transferred onto nitrocellulose membranes (Toyo-Roshi Co., Tokyo, Japan) by the method of Towbin et al. (19).

The immunologically reactive bands were stained with MAbs as previously described (14). The nitrocellulose membranes were incubated with MAbs at 37°C for 2 h. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG)-IgM-IgA (Organon Teknika, Malvern, Pa.) as the second antibody was added and incubated at 37°C for 1 h. Substrate solution prepared by the method of Hawkes et al. (8) was added, and specific antigen bands were visualized. MAbs H9724 against flagella (3), H5332 against Osp A (4), and H68 against Osp B were used for a comparison of reactivities. The two MAbs were kindly provided by Alan G. Barbour, University of Texas, San Antonio.

RESULTS

Comparison of protein antigen bands by SDS-PAGE. The main antigens of the Japanese strains were characterized by SDS-PAGE. The two Japanese strains, HP3 and HO14, had the Osp A (molecular mass, 29 to 30 kDa), flagellum (41-kDa), and 60-kDa antigens. These electrophoretic profiles were very similar to that of strain B31 (Fig. 1). Strains HO14

TABLE 2. Reactivities of MAbs to *B. burgdorferi* strains isolated in Japan compared with reactivities to non-Japanese *Borrelia* strains

Organism	Origin[a]	Molecular mass (kDa) of reactive band with the following MAb (class and subclass):										
		U17 (IgM)	U31b (IgG3)	E31 (IgG3)	E34a31 (IgG1)	U34b (IgM)	E34b (IgG1)	U40 (IgG2a)	E60 (IgM)	H5332[b] (IgG1)	H68[b] (NR[c])	H9724[b] (IgG2a)
B. burgdorferi												
B31	USA	11–16	29	ND[d]	29	32	ND	41	60	29	32	41
297	USA	11–14.3	29	ND	29	32	ND	41	60	29	32	41
IRS	EUR	12	29	ND	29	32	ND	41	59	29	32	41
P/Bi	EUR	ND	30	30	30	ND	31	ND	61	30	ND	41
2/B45	EUR	ND	30	30	30	ND	31	ND	61	30	ND	41
3/B56	EUR	ND	30	30	30	ND	31	ND	61	30	ND	41
P/Gau	EUR	ND	29	ND	29	ND	ND	41	58	29	ND	41
HO14	JPN	ND	ND	ND	30	ND	ND	41.5	ND	30	ND	41
HP3	JPN	ND	ND	ND	ND	ND	ND	41	61	30	ND	41
B. coriaceae co53	USA	ND	ND	ND	ND	ND	ND	ND	60	ND	ND	39
B. parkeri	USA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	41

[a] USA, United States; EUR, Europe; JPN, Japan.
[b] These MAbs were kindly provided by A. G. Barbour.
[c] NR, class and subclass of MAb H68 were not reported.
[d] ND, specific reactive bands were not detected.

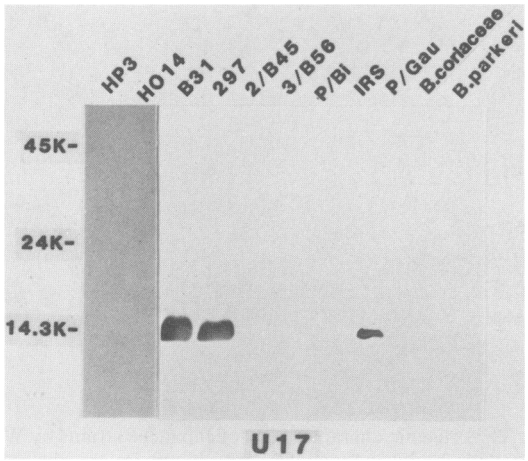

FIG. 3. Immunoblotting of borrelial strains by using MAb U17. Numbers on the left indicate the molecular masses (daltons) of the standard proteins used.

and B31 showed the Osp B band (31 to 32 kDa), which was absent in strain HP3. Protein C (pC), the major protein in the 20- to 22-kDa range, was observed in the two Japanese strains.

Immunological reactivity of Japanese strains with polyclonal anti-rabbit sera. Anti-strain B31 antiserum reacted with flagella, Osp A, and Osp B of the American strains, B31 and 297 (Fig. 2). Japanese strains HP3 and HP14 showed the Osp A band and a weakly reactive flagellum band, but the Osp B band was not detectable. Anti-strain P/Bi antiserum reacted with periplasmic flagella and Osp A of Japanese strains HO14 and HP3. These reactivity patterns were very similar to those of the American and European strains.

Immunological reactivity of borrelial strains with MAbs. The U series of MAbs, U17, U31b, U34b, and U40, and the E series of MAbs, E31, E34a31, E34b, E60, were prepared from mice immunized with strain B31 isolated in the United States and strain P/Bi isolated in the Federal Republic of Germany. The class and subclass of each MAb and immunoblot results are shown in Table 2.

MAb U17 reacted to antigens of strains B31 and 297 in the 11- to 16-kDa range, and a weak reaction was observed with the antigen of IRS in a similar molecular mass range (Fig. 3). In contrast, other European strains and two Japanese strains were nonreactive with this MAb. MAb U31b reacted to Osp A of strains B31, 297, 2/B45, 3/B56, P/Bi, and IRS (Fig. 4A); it had a weak reaction to Osp A of strain P/Gau (arrow). MAb E31 prepared from mice immunized with strain P/Bi reacted with Osp A of European strains 2/B45, 3/B56, and P/Bi, but it did not react with other strains tested (Fig. 4B). Another MAb against Osp A, MAb E34a31, showed potent reactivity against Osp A of European strains 2/B45, 3/B56, and P/Bi. Weakly reactive bands were observed with Japanese strain HO14, North American strains B31 and 297, and

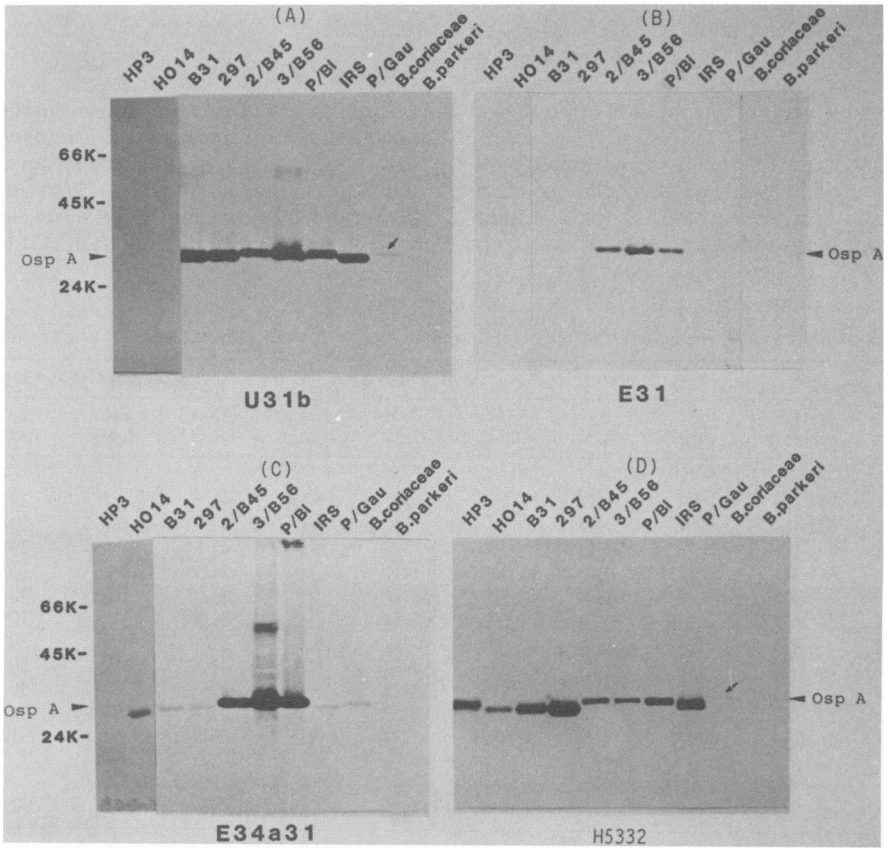

FIG. 4. Immunoblotting of borrelial strains by using MAbs against Osp A. (A) U31b; (B) E31; (C) E34a31; (D) MAb H5332. Numbers on the left and arrowheads indicate the molecular masses (daltons) of the standard proteins used and the position of the Osp A band, respectively. The arrows in panels A + D indicate the presence of the faintly reactive bands.

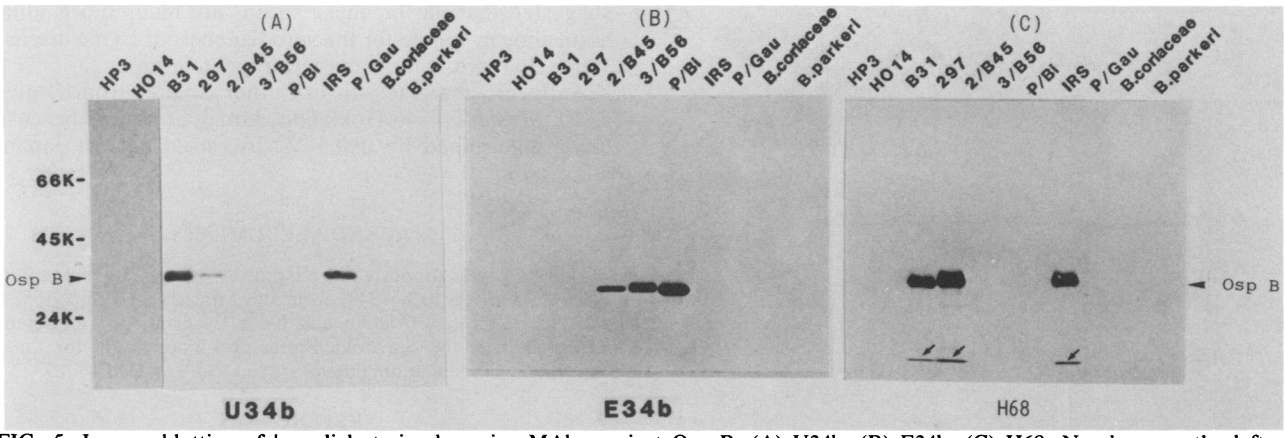

FIG. 5. Immunoblotting of borrelial strains by using MAbs against Osp B. (A) U34b; (B) E34b; (C) H68. Numbers on the left and arrowheads indicate the molecular masses (daltons) of the standard proteins used and the position of the Osp B band, respectively. The arrows in panel C indicate low-molecular-mass bands.

European strains IRS and P/Gau, but this MAb did not react with Japanese strain HP3 (Fig. 4C). Positively reacting strains with MAbs U31b and E34a31 showed weakly and/or potently cross-reactive bands of 58 to 62 and 18 to 20 kDa (Fig. 4A and C). Furthermore, MAb H5332 reacted with all the *B. burgdorferi* strains tested, while the reactivity with strain P/Gau was very weak (Fig. 4D, arrow). These results suggested that MAbs U31b, E31, E34a31, and H5332 recognized Osp A as antigen, but antigenic determinants recognized by each MAb were different from each other.

MAb U34b, prepared from mice immunized with strain B31, reacted with Osp B of strains B31, 297, and IRS, but it did not react with other strains tested including two Japanese strains (Fig. 5A). Another MAb against Osp B, E34b, prepared from mice immunized with strain P/Bi, reacted with Osp B of European strains 2/B45, 3/B56, and P/Bi (Fig. 5B). MAb H68 reacted with Osp B and low-molecular-mass antigens (arrows) of strains B31, 297, and IRS (Fig. 5C). The reactivity of MAb U34b was very similar to that of MAb H68, but MAb U34b did not show cross-reaction with the low-molecular-mass antigen. MAb U40 reacted with the 41- to 41.5-kDa antigen of strains HP3, HO14, B31, 297, IRS, and P/Gau, while the reactivity of U40 with strains HP3 and HO14 was weaker than that of other strains tested (Fig. 6A). MAb H9724 reacted with a 39- to 41-kDa antigen, flagellin, of the tested strains (Fig. 6B). As shown in Fig. 4C, the molecular mass of reactive bands with MAb U40 was slightly smaller than that with H9724. Furthermore, MAb U40 did not react to periplasmic flagella by using the immunoelectron-microscopic method. By two-dimensional electrophoresis, it was revealed that MAb U40 reacted to a 41-kDa protein antigen which was more acidic than the flagellum protein (data not shown). MAb E60 recognized the 60-kDa antigen and reacted with 9 of 11 strains tested but did not react with Japanese strain HO14 or *B. parkeri* (Fig. 7).

DISCUSSION

This study demonstrates that blot patterns of the main antigens (flagellum, Osp A, and the 60-kDa protein) of Japanese strains (*B. burgdorferi* HO14 and HP3) are quite similar to those of American and European strains in West-

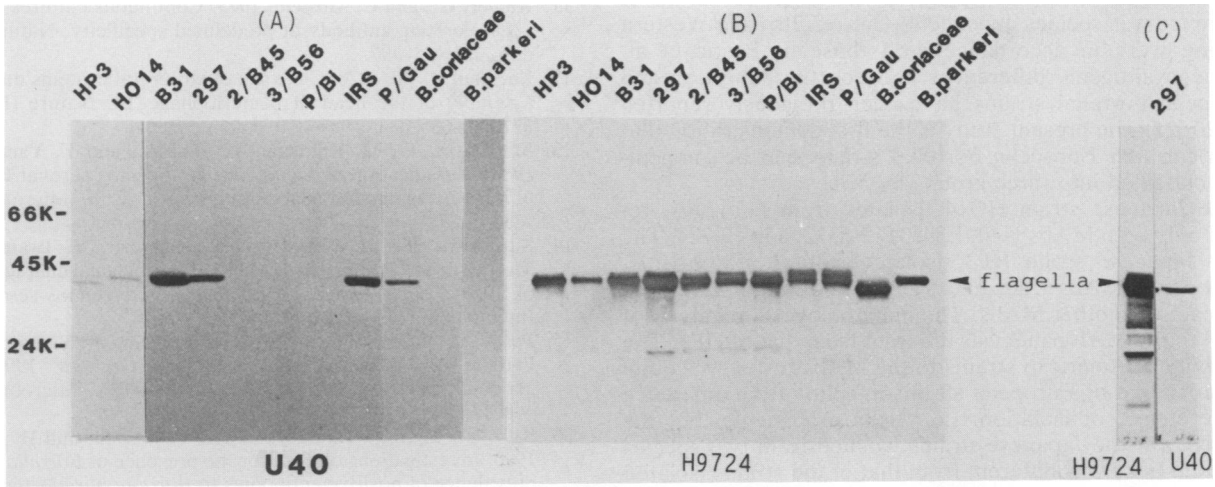

FIG. 6. Immunoblotting of borrelial strains by using MAbs against 40- to 41-kDa antigens. (A) U40; (B) H9724; (C) strain 297 with MAbs U40 or H9724 on the same membrane. Numbers on the left and arrowheads indicate the molecular masses (daltons) of the standard proteins used and the position of the flagellum band, respectively.

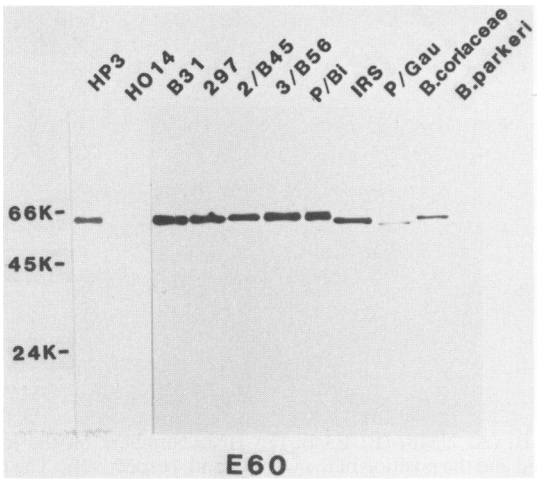

FIG. 7. Immunoblotting of borrelial strains by using MAb E60. Numbers on the left indicate the molecular masses (daltons) of the standard proteins used.

ern blotting with polyclonal antisera against American and European strains. Furthermore, SDS-PAGE profiles of the Japanese strains are very similar to that of American strain B31. The two Japanese strains reacted with MAb H5332 directed against Osp A of *B. burgdorferi* and with H9724 directed against the *Borrelia* flagellum antigen. These results suggested that the two Japanese strains were strains of *B. burgdorferi*.

In addition, antigenic properties of the Japanese strains were compared with those of American and European strains by using a variety of MAbs (Table 2). U.S. strains B31, isolated from *I. dammini*, and 297, isolated from human cerebrospinal fluid (CSF), and Swiss strain IRS, isolated from *I. ricinus*, showed similar blot profiles with these MAbs. Strains 2/B45 and 3/B56, isolated from *I. ricinus* in the Federal Republic of Germany, and P/Bi, isolated from human CSF in the Federal Republic of Germany, also showed similar profiles that differed from those of B31, 297, and IRS. Postic et al. (15) reported that two borrelial genomic DNA hybridization groups could be differentiated by rRNA gene restriction patterns and suggested that this difference was species dependent. Our results from Western blotting were in accordance with those of Postic et al. Although antigenic differences between the American and European borrelial strains have been previously reported (18, 20, 21), the present study is the first demonstration that American and European borrelial strains can be antigenically classified into three groups by MAbs.

The Japanese strain HO14, isolated from *I. ovatus*, reacted only with MAbs U40, E34a31, H5332, and H9724. The other Japanese strain HP3, isolated from *I. persulcatus*, reacted with MAbs U40, E60, H5332, and H9724, but it did not react with other MAbs. The immunological reactivity of HO14 to the MAb panel was different from that of HP3. The reactivity of American strains to the MAbs tested was quite similar to that of European strains in spite of the difference of the source of isolation, i.e., ticks or patients, but the reactivity of the Japanese strains isolated from two species of ixodid ticks was different from that of the strains isolated in the United States and Europe.

Antigenic differences among Japanese, American, and European strains in this study are clearly shown, and it is suggested that the Japanese strains are much more suitable as an antigen source for the serodiagnosis of Lyme disease in Japan than are American or European strains.

Antigenic characteristics of other isolates from *I. ovatus* and *I. persulcatus* in Hokkaido, Japan, are in the process of being determined by using MAbs raised against Japanese strains.

ACKNOWLEDGMENTS

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