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Prevalence of Antibodies against *Borrelia* Species in Patients with Unclassified Uveitis in Regions in Which Lyme Disease Is Endemic and Nonendemic

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We studied 93 patients with unclassified uveitis from two regions in Japan (Hokkaido and Kanagawa) to assess the contribution of *Borrelia* species to this condition. The seroprevalence of antibody to *Borrelia* species was higher in patients from Hokkaido than in those from Kanagawa. The unclassified uveitis of seropositive patients was probably a complication of Lyme borreliosis since (i) the antibody titers were as high as those in clinically diagnosed Lyme disease patients, (ii) healthy controls from Hokkaido showed low seroprevalence in contrast with unclassified uveitis patients from Hokkaido, and (iii) the reaction pattern of antibodies in sera from patients with unclassified uveitis was the same as that in patients with Lyme disease. This is the first report to reveal the high risk of Lyme borreliosis in patients with unclassified uveitis in regions endemic for Lyme disease. In cases of unclassified uveitis as well as in cases of inflammatory disease of unknown origin, Lyme disease should be taken into consideration, especially in regions in which Lyme disease is endemic, even if it is reported only in animals.

Lyme disease is an acute, persistent zoonotic infection caused by the spirochete *Borrelia burgdorferi*, which is transmitted by ixodid ticks. Lyme disease typically begins with the symptoms of flu, which are associated with a characteristic skin rash, erythema chronicum migrans (ECM), after tick bite (2–4). However, in many cases of Lyme disease, ECM is not manifested, and only such nonspecific symptoms and signs as arthritis, carditis, complete heart block, and encephalitis are observed in the patients. Ocular complication is one of the symptoms of Lyme disease (1, 7). Since ocular Lyme disease sometimes results in loss of sight, differential diagnosis in the early stage is essential to exclude other inflammatory ocular diseases. We have recently reported that some cases of unclassified uveitis were probably complications of Lyme disease (7). In the present study, we examined the seroprevalence of antibody to *Borrelia* species in patients with unclassified uveitis from two regions in Japan where seropositivity rates in animals are different.

MATERIALS AND METHODS

Bacterial cells. *Borrelia japonica* (*B. burgdorferi* HO14) and *Borrelia garinii* (*B. burgdorferi* HP3) (15), isolated from the midgut of *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan, were used for this study. *Borrelia* species were cultivated in BSK II medium at 32°C for 5 to 7 days by the method described by Burgdorfer and associates (3). The bacterial cells grown in the medium were harvested by centrifugation at 10,000 × *g* for 20 min and washed three times in 20 ml of phosphate-buffered saline (PBS; pH 7.2). The final pellet was suspended in carbonate buffer (0.05 M, pH 9.6) and then sonicated three times on ice for 30 s each time. The resultant extract was centrifuged, and the protein concentration of the supernatant was then adjusted to 10 μg/ml. This solution was used as the bacterial antigen for an enzyme-linked immunosorbent assay (ELISA).

Patients. Serum specimens were obtained from 53 patients with unclassified uveitis in Hokkaido and from 40 patients with unclassified uveitis in Kanagawa (Fig. 1). The clinical symptoms of the unclassified uveitis patients were iridocyclitis, uveoretinitis, and panuveitis. To establish criteria, we obtained sera from one patient with clinically diagnosed neuro-Lyme disease (with progressive neurologic manifestations after tick bite and subsequent localized ECM), seven patients with clinically diagnosed Lyme disease with mild to moderate flu-like symptoms (with high levels of immunoglobulin G [IgG] and IgM antibodies to *B. burgdorferi* in serum and/or ECM after tick bite), one patient with tick (*I. persulcatus*) bite without clinical signs, and 84 healthy controls, all of these patients being in Hokkaido.

ELISA. ELISA was performed by a previously described method (9). Briefly, microplates were coated with the bacterial antigens overnight at 4°C. After repeated washings, 100-μl aliquots of the serum samples, diluted in PBS (1:130 and 1:120 for the assay of IgG and IgM against *B. japonica* and 1:120 and 1:130 for IgG and IgM against *B. garinii*, respectively, were added, and the plates were then incubated for 1 h at 37°C. Peroxidase-conjugated antihuman IgG (100 μl, goat IgG fraction; Cappel Co., Ltd., Malvern, Pa.) or IgM (100 μl, goat IgG fraction; Cappel), diluted to 1:500 in PBS containing 0.05% Tween 20 and 10% Block Ace (Yukijirushi Co., Ltd., Tokyo, Japan) was added to each well, and then the plates were incubated at 37°C for 1 h. One hundred microliters of *o*-phenylenediamine (4 mg/ml) in 0.1 M citrate-phosphate buffer (pH 5.0), containing 0.02% hydrogen peroxide, was then added to each well. The colorimetric value was determined by A_{492}. The degree of serum dilution was determined with a reference positive control, so that the results agreed with those in our previous study (7). Since the cutoff value used in the previous study was 0.6, we employed the same cutoff value in this study.

Western blotting (immunoblotting). The cultured cells were harvested, washed three times in PBS, and then suspended in sterilized water. The suspensions were sonicated and then heated for 5 min at 100°C in the presence of 2-mercaptoethanol and sodium dodecyl sulfate (SDS). After centrifugation, the supernatants were subjected to SDS–12% polyacrylamide gel electrophoresis as described by Laemmli (11). The protein bands were transferred to a nitrocellulose membrane. The membrane was then cut into strips, and each strip

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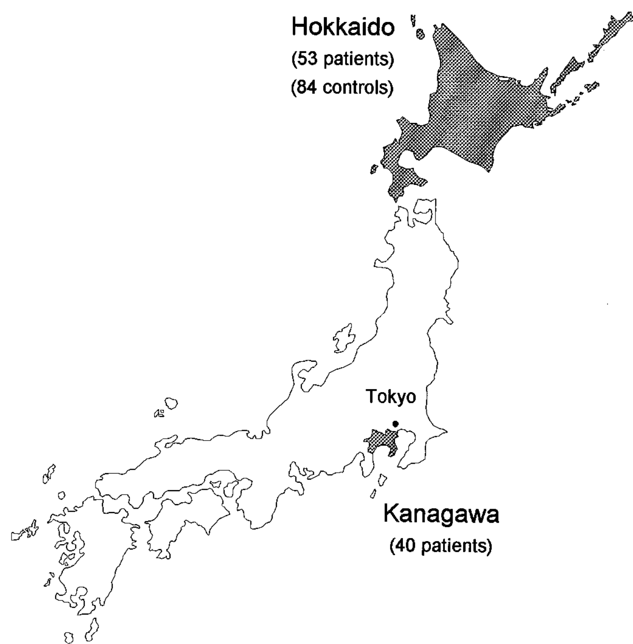

FIG. 1. Map of Japan indicating regions of origin of patients surveyed in this report. Hokkaido is endemic for Lyme disease in animals.

was placed in an incubation tray containing 2 ml of 10% skim milk in PBS and incubated for 1 h at room temperature, with gentle stirring. The membrane strips were washed with 20 mM Tris buffer (pH 7.5) containing 0.05% Tween 20 and 0.5 M NaCl (T-TBS), and the serum samples, diluted to 1:100 in T-TBS, were then reacted for 1 h at room temperature. After repeated washes with T-TBS, 2 ml of alkaline phosphatase–anti-human IgG conjugate (Promega Corp., Madison, Wis.), diluted 1:7,500 with T-TBS was reacted for 30 min at room temperature. The membrane strips were then washed three times, and alkaline phosphatase activity was detected with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 2 to 10 min. Monoclonal antibodies, i.e., H9724 against flagella, H5332 against OspA, and L22-1F8 against pC, were used to confirm the identity of the proteins. The monoclonal antibodies H9724 and H5332 were kindly provided by Alan G. Barbour, and the L22-1F8 monoclonal antibody was kindly supplied by B. Wilske. (L22-1F8 reacted with pC of *B. japonica* but did not react with the pC of *B. garinii*.)

RESULTS

IgG antibody to *B. japonica* (*B. burgdorferi* HO14) was positive in 19 of the 53 unclassified uveitis patients (35.8%) in Hokkaido, in one of the 40 patients in Kanagawa (2.5%), and in one of the 84 healthy controls (1.2%). IgM antibody to *B. japonica* was detected in nine patients (17.0%) in Hokkaido and in three patients (7.5%) in Kanagawa (Table 1; Fig. 2). In the patients with unclassified uveitis, there were significant differences between the two regions ($P < 0.01$ and $P < 0.05$, respectively) in the IgG- and IgM-positive rates. In Hokkaido, significant differences were observed between the patients and the healthy controls in the seropositivity rates ($P < 0.01$). Similar results were obtained with the ELISA using *B. garinii* (*B. burgdorferi* HP3). No IgM antibodies to *Borrelia* species were detected in the 84 healthy controls (Table 1; Fig. 2).

Western blot analysis was carried out in an attempt to determine the relationship between the clinical symptoms of Lyme disease and the reaction patterns of antibodies in the patient sera. A protein with a molecular weight of 41,000, which could be flagellin protein (M_r, 41,000) of the *Borrelia* species, was the major antigen detected by antibodies in sera. A 30.5- to 32-kDa protein and a 19-kDa protein, which could

TABLE 1. Antibodies to *Borrelia* species in patients with unclassified uveitis

Borrelia species	Region	No. and type of patients	No. (%) of ELISA-positive patients[a]	
			IgG	IgM
B. japonica	Kanagawa	40 (unclassified uveitis)	1 (2.5)	3 (7.5)
	Hokkaido	53 (unclassified uveitis)	19 (35.8)*	9 (17.0)**
	Hokkaido	84 (healthy controls)	1 (1.2)	0 (0.0)
B. garinii	Kanagawa	40 (unclassified uveitis)	1 (2.5)	2 (5.0)
	Hokkaido	53 (unclassified uveitis)	20 (37.7)*	13 (24.5)*
	Hokkaido	84 (healthy controls)	1 (1.2)	0 (0.0)

[a] * and **, $P < 0.01$ and $P < 0.05$, respectively, compared with value for patients from Kanagawa with unclassified uveitis and with value for healthy controls.

be OspA (outer surface protein A; 30.5 to 32 kDa) and pC (protein C; 20 to 22 kDa), respectively (14), were also detected. However, the specificity of the reaction pattern was dependent mainly on the intensity of the band, and no different specific

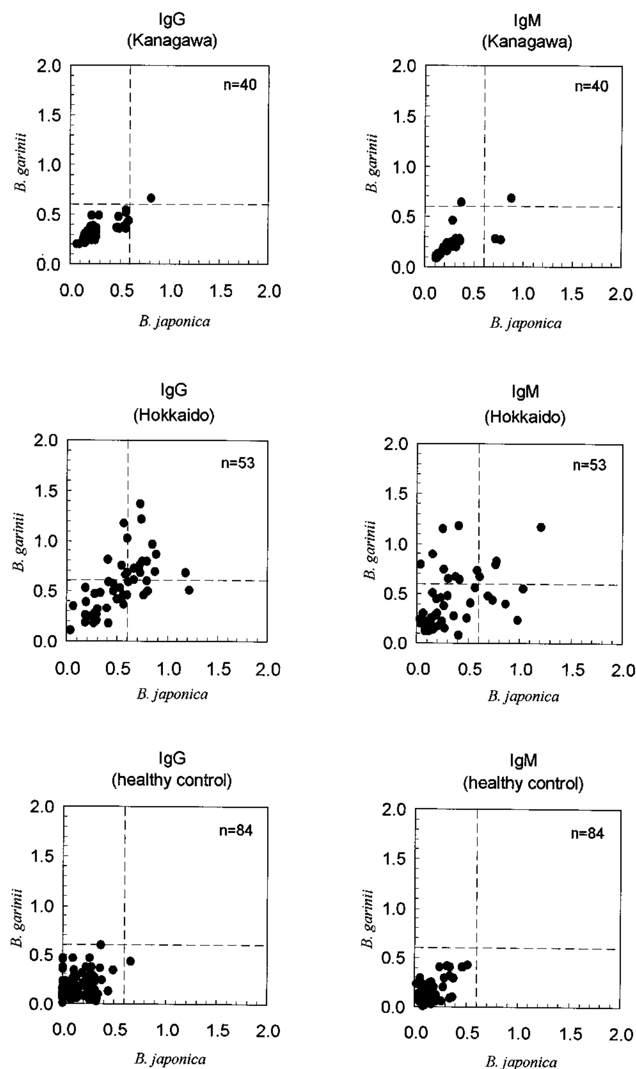

FIG. 2. Levels of antibodies to *Borrelia* species in unclassified uveitis patients and healthy controls. The antibody titers are expressed as optical density values at 492 nm in an ELISA. The cutoff value is indicated by dotted lines. *n*, sample number.

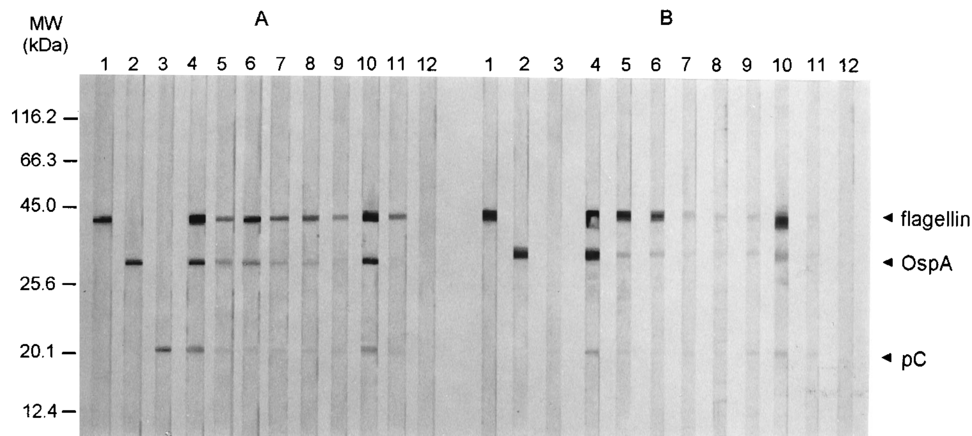

FIG. 3. Western blot analysis of *Borrelia* species extracts with serum samples obtained from patients. (A) *B. japonica* (*B. burgdorferi* HO14); (B) *B. garinii* (*B. burgdorferi* HP3). Lanes: 1, monoclonal antibody H9724 against flagella; 2, monoclonal antibody H5332 against OspA; 3, monoclonal antibody L22-1F8 against pC; 4 and 5, neuro-Lyme disease; 6 to 9, unclassified uveitis; 10, Lyme disease with ECM; 11, tick bite with no clinical signs; 12, healthy control. MW, molecular size.

patterns were observed in the types of Lyme disease, including unclassified uveitis (Fig. 3).

DISCUSSION

Lyme disease is a multisystemic disorder caused by infection with *B. burgdorferi* and associated species, which are transmitted by ixodid ticks. Ocular complications are not always evident; however, when they do arise, various ocular structures may be affected, sometimes with associated visual loss. Serological studies in animals in Hokkaido and Kanagawa have revealed that Lyme disease is endemic in Hokkaido (8–10, 18). In a recent study, we suggested that some cases of unclassified uveitis in Hokkaido were complications of Lyme disease (7) since the patients had high titers of antibody to *Borrelia* species. However, there was a possibility that their seropositivity may have been a nonspecific reaction (17).

In the present study, we demonstrated that the prevalence of antibody-positive cases of unclassified uveitis was much higher in patients from a region where Lyme disease is endemic (Hokkaido) than in those from a nonendemic region (Kanagawa), while the seropositivity rate was very low in healthy controls from the endemic region. If the difference in seropositivity rates in unclassified uveitis between Hokkaido and Kanagawa simply reflects the different chance of exposure to *Borrelia* species, then a high seroprevalence should be observed in the healthy controls from Hokkaido, as was observed in the patients with unclassified uveitis in Hokkaido. That a high seroprevalence was observed only in the patients with unclassified uveitis in Hokkaido strongly suggests a close relationship between *Borrelia* species and unclassified uveitis in Hokkaido. In addition, the antibody titers of the seropositive patients (Table 2) were as high as those of clinically diagnosed Lyme disease patients (optical density, 0.716 to 1.079) (7). Furthermore, the reaction pattern of antibodies in the sera of the patients with unclassified uveitis was the same as that in the sera of patients with Lyme disease. Therefore, we concluded that seropositive patients with unclassified uveitis should be classified as having Lyme borreliosis. Unfortunately, in cases of Lyme disease in which ECM is not manifested, the disease is often not diagnosed. Indeed, none of the patients with unclassified uveitis investigated here had been diagnosed with Lyme disease. This aspect is particularly important since the ocular manifestations of Lyme disease sometimes result in blindness.

TABLE 2. Levels of antibodies against *Borrelia* species in patients with unclassified uveitis

Region and type of patient	ELISA result[a]	ELISA level (OD[b] ± SD)			
		B. japonica		*B. garinii*	
		IgG	IgM	IgG	IgM
Kanagawa: unclassified uveitis	Positive	0.809	0.789 ± 0.064	0.667	0.666 ± 0.021
	Negative	0.281 ± 0.154	0.231 ± 0.074	0.336 ± 0.085	0.201 ± 0.073
	Total	0.294 ± 0.173	0.273 ± 0.164	0.344 ± 0.099	0.224 ± 0.124
Hokkaido					
Unclassified uveitis	Positive	0.793 ± 0.159	0.853 ± 0.178	0.830 ± 0.208	0.838 ± 0.192
	Negative	0.363 ± 0.156	0.213 ± 0.146	0.373 ± 0.135	0.269 ± 0.134
	Total	0.517 ± 0.259	0.322 ± 0.284	0.546 ± 0.277	0.408 ± 0.288
Healthy control	Positive	0.659	No[c]	0.601	No
	Negative	0.156 ± 0.126	0.156 ± 0.101	0.199 ± 0.112	0.160 ± 0.097
	Total	0.162 ± 0.137	0.156 ± 0.101	0.204 ± 0.120	0.160 ± 0.097

[a] Seropositive and seronegative patients were determined by ELISA.
[b] OD, optical density.
[c] No, no samples.

In the present study, we investigated only patients with uveitis, but we suggest that many cases of Lyme disease in which arthritis, carditis, or encephalitis is manifested without ECM may also fail to be diagnosed. It must be emphasized that Lyme disease should be taken into consideration when patients with inflammatory disease of unknown origin are treated, especially in regions where Lyme disease is endemic, even if it is reported only in animals.

The reaction patterns of antibodies in the sera of patients with neuro-Lyme disease, Lyme disease with ECM, or unclassified uveitis (ocular Lyme disease) were examined to determine their relationship to the clinical symptoms of Lyme disease. Although no specific patterns were observed for the types of Lyme disease, this finding does not exclude the possible existence of minor reaction pattern differences that could not be detected by the present Western blot systems. Of course there is another possibility, namely, that differences in the clinical symptoms are due to minor variations in *Borrelia* species that cannot be classified serologically. It is also possible that the variety of clinical symptoms of Lyme disease arises from complex interactions between host and parasite (1, 5, 6, 12, 13, 16). Since the reasons for the various clinical symptoms in Lyme disease are still unknown, we believe that the identification of factors of the host (such as HLA) and the parasite responsible for these differences is very important.

REFERENCES

1. **Azuma, Y., E. Isogai, H. Isogai, and K. Kawamura.** 1994. Canine Lyme disease: clinical and serological evaluations in 21 dogs in Japan. Vet. Rec. **134:**369–372.
2. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. D. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. **42:**378–383.
3. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216:** 1317.
4. **Canica, M. M., F. Nato, L. du Merle, J. C. Mazie, G. Baranton, and D. Postic.** 1993. Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand. J. Infect. Dis. **25:**441–448.
5. **Cinco, M., E. Banfi, D. Balanzin, C. Godeas, and E. Panfili.** 1991. Evidence for (lipo) oligosaccharides in *Borrelia burgdorferi* and their serological specificity. FEMS Microbiol. Immunol. **3:**33–38.
6. **Habicht, G. S., L. I. Katona, and J. L. Benach.** 1991. Cytokines and the pathogenesis of neuroborreliosis: *Borrelia burgdorferi* induces glioma cells to secrete interleukin-6. J. Infect. Dis. **164:**568–574.
7. **Isogai, E., H. Isogai, S. Kotake, K. Yoshikawa, A. Ichiishi, S. Kosaka, N. Sato, S. Hayashi, K. Oguma, and S. Ohno.** 1991. Detection of antibodies against *Borrelia burgdorferi* in patients with uveitis. Am. J. Ophthalmol. **112:**23–30.
8. **Isogai, E., H. Isogai, T. Masuzawa, Y. Yanagihara, N. Sato, S. Hayashi, T. Maki, and M. Mori.** 1991. Serological survey for Lyme disease in sika deer (*Cervus nippon yesoensis*) by enzyme-linked immunosorbent assay (ELISA). Microbiol. Immunol. **35:**695–703.
9. **Isogai, E., H. Isogai, N. Sato, M. Yuzawa, and M. Kawakami.** 1990. Antibodies to *Borrelia burgdorferi* in dogs in Hokkaido. Microbiol. Immunol. **34:**1005.
10. **Isogai, H., E. Isogai, T. Masuzawa, Y. Yanagihara, M. Matsubara, M. Shimanuki, T. Seta, K. Fukai, N. Kurosawa, M. Enokidani, T. Nakamura, M. Tajima, K. Takahashi, K. Takahashi, and N. Fujii.** 1992. Seroepidemiological survey for antibody to *Borrelia burgdorferi* in cows. Microbiol. Immunol. **36:**1029–1039.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
12. **Lahesmaa, R., M. C. Shanafelt, A. Allsup, C. Soderberg, J. Anzola, V. Freitas, C. Turck, L. Steinman, and G. Peltz.** 1993. Preferential usage of T cell antigen receptor V region gene segment V beta 5.1 by *Borrelia burgdorferi* antigen-reactive T cell clones isolated from a patient with Lyme disease. J. Immunol. **150:**4125–4135.
13. **Ma, Y., and J. J. Weis.** 1993. *Borrelia burgdorferi* outer surface lipoproteins OspA and OspB possess B-cell mitogenic and cytokine-stimulatory properties. Infect. Immun. **61:**3843–3853.
14. **Masuzawa, T., Y. Okada, Y. Yanagihara, and N. Sato.** 1992. Antigenic properties of *Borrelia burgdorferi* isolated from *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan. J. Clin. Microbiol. **29:**1568–1573.
15. **Postic, D., J. Belfaiza, E. Isogai, I. S. Girons, P. A. D. Grimont, and G. Baranton.** 1993. A new genomic species in *Borrelia burgdorferi* sensu lato isolated from Japanese ticks. Res. Microbiol. **144:**467–473.
16. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. Analysis using a CAT reporter construct. J. Immunol. **147:**1968–1974.
17. **Rosenbaum, J. T., and D. W. Rahn.** 1991. Prevalence of Lyme disease among patients with uveitis. Am. J. Ophthalmol. **112:**462–463.
18. **Takahashi, K., E. Isogai, H. Isogai, T. Takagi, K. Sasaki, N. Fujii, and K. Kimura.** 1993. Serological survey for *Borrelia burgdorferi* infection in cattle in southern Hokkaido. J. Vet. Med. Sci. **55:**921–924.