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Distribution Of *Borreia Burgdorferi* Specific Antibody Among Patients With Juvenile Rheumatoid Arthritis In Korea

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Lyme disease, a multi-systemic infection occurring worldwide, has yet to be reported in Korea, although the spirochete B. burgdorferi, known as the causative organism of the disease, has recently been isolated from the vector tick Ixodes persulcatus in the region. To contribute to revealing whether Lyme disease exists in Korea or not, B. burgdorferi specific antibodies (IgG, IgM, and/or IgA) were measured by three individual enzyme-linked immunosorbent assays (ELISA) utilizing different antigens in 38 patients with juvenile rheumatoid arthritis (JRA) which shares a number of clinical features with Lyme arthritis. The antibody prevalence rates in patients with JRA were various depending on the antigens (21% for IgG and IgM antibodies to purified organisms, 0% for IgG antibody to purified native flagella, and 5% for IgG, IgM, and IgA antibodies to recombinant p39) and were not different compared to 39 controls (21%, 0%, and 0% respectively). The antibody prevalence rates compared in various subgroups of patients with JRA according to types of JRA, length of illness, age, and sex were not different. Comparing the three different antigens, the greatest number of positive responders were yielded by purified organisms followed by p39 and purified flagellin, however the possibility of nonspecificity with purified organisms remained. The data indicate that serologic tests using ELISA fail to illustrate Lyme disease among 38 patients with JRA in Korea.

Key Words: *Lyme disease, ELISA, anti-Borrelia burgdorferi antibody, juvenile rheumatoid arthritis*

INTRODUCTION

Lyme disease, a tick-borne infection caused by the spirochete *Borrelia burgdorferi,* was recognized in 1975 through the investigation for geographic clustering of children in Lyme, Connecticut, USA, who had been thought to have juvenile rheumatoid arthritis (Steer 1977, Burgdorfer 1982). The disease has since been reported in 6 continents and noticed as the most common tick-borne disease in the western hemisphere as the annual occurrence of cases is greater than the sum of all other tick-borne illness in the USA (Buchstein 1991). Lyme disease has yet to be reported in Korea; however, several facts indicate that the disease may occur in Korea. Firstly, Lyme disease has been reported in the neighbouring countries of China, Japan, and the USSR (Ai 1990, Masuzawa 1991, Sigal 1991). Secondly, *I. ricinus* and *I. persulcatus*, known as vectors for Lyme disease, have been reported to be in Korea (Ai 1990, Kang 1982, Noh 1972). Thirdly, antibody to *B. burgdorferi* has been detected in certain Korean populations (Chong 1989, Koo 1991, Que 1992). Fourthly,

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B. burgdorferi has allegedly been isolated from presumptive *I. persulcatus* in Korea (Park 1992). Therefore, to investigate whether Lyme disease occurs in Korea or not, the antibody response to *B. burgdorferi* in Korean patients with JRA was measured and analysed.

MATERIALS AND METHODS

Patients and sera: Thirty-eight patients with juvenile rheumatoid arthritis (JRA group) diagnosed and classified according to the established criteria (Cassidy 1986), and registered in the Department of Pediatrics and the Rheumatism Center, Hanyang University Hospital, were included in the study (Table 1) . Nine patients had rheumatoid factor (RF)-positive polyarticular type, 8 RF-negative polyarticular type, 2 pauciarticular type I, 13 pauciarticular type II, and 6 systemic type. Data with regard to rheumatoid factor (RF), antinuclear antibody (ANA) and VDRL are shown on table 2. Twelve healthy blood donors and 27 patients with minor illnesses (1 patient with asthma, 9 acute gastroenteritis, 2 hepatitis, 2 pneumonia, 3 transient hematuria/albuminuria, 9 upper respiratory tract infections, and 1 urinary tract infection) were included as controls (control group). All subjects in both JRA and control groups denied tick-bites or history of skin rash suggestive of erythema chronicum migrans (ECM). Serum samples were obtained from the patients and healthy volunteers and stored at -20°C until tested.

Lyme serology: All sera were measured for antibody to *B. burgdorferi* by enzyme-linked immunosorbent assay (ELISA) against three different antigens. Experiments were performed at room temperature in triplicates once with the Whittaker and in duplicate twice with the other two kits as follows.

ELISA with purified B. burgdorferi [Whittaker M.A. Bioproducts, Inc. (Walkersville, MD)]; One hundred μL of patient's serum diluted 1:20 with serum diluent was added to appropriate microtiter wells prepared with purified *B. burgdorferi,* strain B-31 and incubated for 15 min on plate shaker. Wells were washed twice with PBS and 100 μL of 1:10 dilution of alkaline phosphatase conjugated anti-human IgG/IgM was added to incubate for 15 min on plate shaker. Wells were washed as before and 100 μL of substrate (phenolphthalein monophosphate) was added to incubate for 15 min on plate shaker, followed by 200 μL of stop solution (2% sodium phosphate tribasic). The optical density (OD) of each well was determined at 550 nm using Titertech multiscan (Flow Laboratories, Helsinki, Finland). The measured OD values were transformed into the predicted index values by linear regression analysis using the test OD values and label index values of the controls provided by the manufacturer. The value greater than or equal to 1. 0 was considered positive, the value between 0.8 and 0.99 equivocal, and the value less than 0.8 negative. The sensitivity of the test is 85% and the specificity close to 100%.

ELISA with purified native flagella [Dako (Denmark)]; One hundred μL of patient's serum diluted 1:200 with sample diluent was added to appropriate microtiter wells prepared with purified native flagella of *B. burgdorferi,* strain DK-1 and incubated for 2 hr. Wells were washed 4 times with 0.1 M NaCl with 4% Tween and 100 μL of 1:5150 dilution of peroxidase-conjugated rabbit anti-human IgG was added to incubate for 1 hr. Wells were washed as before and 100 μL of chromogen substrate (0.04% 1,2-phenylenediamine dihydrochloride) was added to incubate covered from the light for 15 min, followed by 100 μL of stop solution (0. 85 M sulphuric acid). The OD of each well was determined at 492 nm. The measured OD value of the tested serum greater than that of the cut-off control was considered positive; the value below the cut-off control and above the borderline value, which was calculated by substraction of 0.05 from the cut-off control value, equivocal; and the value below the borderline value negative. To adjust the interassay variations, the measured OD values were transformed into the corrected values by linear regression analysis using the measured OD values of the controls and their corrected OD values provided by the manufacturer. The sensitivity of the test on patients with Lyme arthritis is close to 100% and the specificity is 98%.

ELISA with recombinant p39 [General Biometrics, Inc. (San Diego, CA)]; Patient's serum diluted 1:20 with PBS was incubated with *E. coli* protein in 1:20

Table 1. Characteristics of JRA and control groups

		JRA group (n=38)	Control group (n=39)
Sex			
	Male	22	22
	Female	16	17
Age (y)			
	Median	14	12
	range	7 - 21	7 - 19

Table 2. Lyme antibody, RF, ANA, and VDRL results in JRA and control groups

JRA group								Control group			
		LYME Assay							LYME Assay		
Serum No.	JRA type	(A)	(B)	(C)	RF	ANA	VDRL	Serum No.	(A)	(B)	(C)
1	poly+	–	–	–	+	w+	–	1	ND	–	–
2	pauci II	–	–	–	–	–	–	2	E	–	–
3	system	+	–	E	–	w+	–	3	ND	–	–
4	poly+		–	–	+	–	–	4	ND	–	–
5	system	–	–	–	–	–	–	5	–	–	–
6	system	–	–	–	–	–	–	6	–	–	–
7	pauci II	–		–	–	–	ND	7	–	–	–
8	poly+	E	E	E	+	w+	–	8	ND	–	–
9	pauci II	–	–	–	–	–	–	2	E	–	–
10	pauci II	–	–	–	–	–	–	2	E	–	–
11	poly+	+	–	+	+	–	–	11	ND	–	E
12	poly–	–	–	–	–	–	–	12	–	–	E
13	pauci II	–	–	–	–	–	–	13	ND	–	–
14	system	–	–	–	–	w+	–	14	–	–	–
15	poly+	–	–	+	+	–	–	15	ND	–	–
16	poly+	–	–	E	+	–	–	16	ND	–	–
17	poly+	E	–	E	+	+	–	17	–	–	–
18	pauci II	+	–	–	–	–	–	18	+	–	E
19	system	–	–	–	–	–	–	19	–	–	–
20	pauci II	–	–	–	–	–	–	20	ND	–	–
21	poly–	–	–	–	–	w+	–	21	–	–	E
22	pauci II	–	–	–	+	–	–	22	ND	–	–
23	pauci II	–	–	–	–	–	ND	23	+	–	–
24	poly–	–	–	–	–	w+	–	24	ND	–	–
25	pauci II	+	–	–	–	–	–	25	–	–	–
26	pauci II	–	–	–	–	–	–	26	–	–	–
27	system	E	–	–	–	–	–	27	–	–	–
28	poly+	+	–	–	+	w+	–	28	–	–	–
29	pauci II	–	–	–	–	–	ND	29	–	–	–
30	pauci II	+	–	–	w+	–	–	30	+	–	E
31	pauci II	–	–	–	–	–	–	31	–	–	–
32	pauci II	+	–	–	–	ND	–	32	+	–	–
33	poly–	–	–	–	–	–	ND	33	–	–	–
34	poly–	E	–	–	–	–	–	34	–	–	–
35	pauci II	–	–	–	–	–	ND	35	–	–	–
36	poly+	ND	–	–	–	–	–	36	–	–	–
37	poly–	–	E	–	–	–	–	37	–	–	–
38	poly–	+	–	E	–	w+	–	38	+	–	–
								39	–	–	–

JRA encompasses rheumatoid factor-positive polyarticular disease (poly +), rheumatoid factor-negative polyarticular disease (poly -), pauciarticular disease type I (pauci I), pauciarticular disease type II (pauci II), and systemic disease (system). Lyme assay measured the antibodies to *B. burgdorferi* by ELISA: +, positive results; -, negative results; E, equivocal results (details of the interpretation in MATERIALS AND METHODS), ND; not done, w+; weakly positive. (A) indicates Whittaker ELISA test, (B) Dako, and (C) General Biometrics.

Table 3. Comparison of antibody response to *B. burgdorferi* between patients with JRA and control groups

ELISA Test	JRA Group No.(%)				Control Group No.(%)			
	+	E	-	total	+	E	-	total
(A)	8(21)	4(11)	25(68)	37(100)	6(21)	1(4)	21(75)	28(100)
(B)	0(0)	2(5)	36(95)	38(100)	0(0)	0(0)	39(100)	39(100)
(C)	2(5)	5(13)	31(82)	38(100)	0(0)	5(13)	34(87)	39(100)

+ ; positive results, - ; negative results, E; equivocal results (details of the interpretation in MATERIALS AND METHODS). (A) indicates Whittaker ELISA test, (B) Dako, and (C) General Biometrics. p values in Chi-square analysis between JRA and control groups were all >.05 for (A), (B), and (C)).

Table 4. Comparison of antibody response to *B. burgdorferi* among patients with various types of JRA

	Lyme assay					
	(A)		(B)		(C)	
	+	E	+	E	+	E
JRA group (No.)						
poly + (9)*	2	2	0	1	2	3
poly - (8)	1	1	0	1	0	1
paucil (2)	0	0	0	0	0	0
paucill (13)	4	0	0	0	0	0
systemic (6)	1	1	0	0	0	1
Control group (No.)						
Total (39)@	6	1	0	0	0	5

+; positive results,
E; equivocal results (details of the interpretation in MATERIALS AND METHODS).
* One patient with RF-positive polyarticular JRA was not tested for Lyme assay (A).
@ The number of the controls tested for Lyme assay (A) was 28.

Table 5. Comparison of antibody response to *B. burgdorferi* between rheumatoid factor positive and rheumatoid factor negative patients

	Lyme assay					
	(A)		(B)		(C)	
	+	E	+	E	+	E
Rheumatoid factor (No.)						
positive (10)*	3	2	0	1	2	3
negative (28)	5	2	0	1	0	2
Controls (No.)						
Total (39)@	6	1	0	0	0	5

+; positive results,
E;equivocal results (details of the interpretation in MATERIALS AND METHODS).
* Patients with weakly positive RF were also included, and one patient with positive RF was not tested for Lyme assay (A).
@ The number of the controls tested for Lyme assay (A) was 28.

for 1 hr. One hundred μL of the serum treated with *E. Coli* was added to appropriate microtiter wells prepared with recombinant p39 of *B. burgdorferi*, strain B-31 and incubated for 30 min. Wells were washed three times with PBS and 100 μL of peroxidse-conjugated goat anti-human antibodies (IgG, IgM, and IgA) was added to incubate for 30 min. Wells were washed as before and 100 μL of substrate (ABTS) was added to incubate for 30 min, followed by 100 μL of stop solution (0.25 M oxalic acid). The OD of each well was determined at 405 nm. The interassay variations were adjusted by normalizing the measured OD values to the average of the positive control as follows:

$$A_N = \frac{A_S}{A_C} \times EV$$

where A_C = mean OD of the positive control obtained in the assay
A_N = normalized OD of the specimen
A_S = OD of the specimen
EV = expected value for the positive control provided by the manufacturer

The normal value greater than 0.21 was considered positive, the value between 0.12 and 0.21 equivocal, and the value less than 0.12 negative. The sensitivity of the test is 79% and the specificity is 99.7%.

Experiments were considered valid when corrected OD values of the various controls fall in their respective label ranges provided by the manufacturers. When experiments were done twice,

discordant results occurred on three occasions (3.9 %); two samples with positive and equivocal results on the Dako were considered equivocal and one sample with equivocal and negative results on the General Biometrics was considered negative.

Statistical analysis: Statistical analysis was done by Chi-square test. p values equal to or less than 0.05 were considered significant.

RESULTS

The antibody prevalence rates to *B. burgdorferi* in the JRA group were various depending on the antigens utilized and the antibodies detected; 21% for IgG and IgM antibodies to purified organisms, 0% for IgG antibody to purified flagella, and 5% for IgG, IgM,and IgA antibodies to recombinant p39, and were not different from those in controls (21%, 0%, 0% respectively) (Table 3). Among the three antigens, purified organisms yielded the greatest number of positive responders; however the difference between JRA and control groups was not significant and the OD values of the positive specimens were low (ranged from 1.06 to 1.94) in comparison with 1.0, the lowest OD value to be positive. Purified native flagella was able to detect antibodies in none of the JRA or control group, although two patients in the JRA group responded equivocally. Recombinant p39 detected antibodies in two patients with JRA but none of the control group, whereas a similar portion responded equivocally in both JRA and control groups. However, the OD values of the two positive specimens were not high (0.292 and 0.217) in comparison with 0.21, the lowest OD value to be positive.

The antibody response to *B. burgdorferi* was also compared among subgroups divided according to the JRA types (Table 4), age, sex, and the duration from onset to the study entry, and the only difference was in that more female patients with JRA responded positively to p39 than male patients (p value 0.031), although the difference between female patients and female controls was not significant. Comparison of the antibody response to *B. burgdorferi* between 10 patients reactive to RF including one patient with weakly positive RF and 28 patients with nagative RF revealed no difference (Table 5). None of the positive responders to purified organisms or p39 were positive for ANA, but two positive responders to purified organisms were weakly positive for ANA. One patient with positive ANA responded equivocally to purified organisms and p39. All the positive or equivocal responders in the JRA groups were negative for VDRL (Table 2).

DISCUSSION

With growing awareness since 1975, Lyme disease has drawn much attention worldwide. The onset of the disease is usually heralded by constitutional symptoms (fever, headache, myalgia) and a pathognomonic skin lesion, known as erythema chronicum migrans (ECM) (Berger 1989, Malane 1991). Unless treatment is initiated early, the disease usually disseminates, often resulting in cardiac, neurologic, and joint manifestations (Ciesielski 1989). Lyme arthritis is clinically indistinguishable from JRA, nevertheless it is important to discriminate one from the other bacause the optimal therapy is quite different (Buchstein 1991).

Currently the diagnosis of Lyme disease depends primarily on clinical manifestations, particularly ECM rash and epidemiologic factors, and secondarily on laboratory analysis for the immune response to *B. burgdorferi* (Buchstein 1991, Duffy 1990). The classification and staging of Lyme disease have recently been proposed (Sigal 1991). In endemic areas, in cases without evidence of ECM, diagnostic confirmation of Lyme disease may be difficult and need a positive serologic test. Even with laboratory aid, the clinical dilemma remains whether the clinical manifestations suggestive of Lyme disease, without ECM but with reactive ELISA, are sufficient to diagnose and treat Lyme disease, since the majority of patients without ECM, who had positive ELISA but negative Western blot, had rheumatic or inflammatory conditions other than Lyme disease (Rose 1991). Therefore, the results of serologic testing should not be relied on as the sole criteria in making the diagnosis of Lyme disease. To make things worse, there is a substantial number of asymptomatic seropositive individuals in groups at high risk for exposure to *B. burgdorferi* in that the significance and the natural history remain unanswered (Huycke 1992). Therefore, it is not difficult to imagine that diagnostic dilemmas of Lyme disease in nonendemic areas would be greatly multiplied.

In this study, the antibody response to *B. burgdorferi* in 38 Korean patients with JRA were measured to demonstrate whether the illness in some patients might have been associated with *B. burgdorferi* infection. Since the serologic diagno-

sis of Lyme disease is difficult as described above, three commonly used individual ELISA tests were utilized to detect various antibodies (IgG, IgM, and/or IgA) against various antigens (purified *B.burgdorferi* of strain B-31, purified flagella of strain DK-1, and recombinant p39 of strain B-31). Experiments revealed no difference in the prevalence of antibody among the JRA group compared to the contol on all three ELISA tests with high specificity and sensitivity indicating that JRA in Korean patients may not be associated with *B. burgdorferi* infection and the low OD values of the responders again support this notion further. The implication of the results is comparable to the recent reports in that the prevalence for antibodies to *B. burgdorferi* among *Apodemus agrarius*, a known reservoir for *B. burgdorferi,* in several provinces in Korea was not high and among 253 individuals with serologically proven or clinically suspected tsutsugamushi diaease, the subjects with high risk for tick exposure, only 18 (7.2%) had indirect immunofluorescent antibody titers greater than 16, although the same study group previoulsy reported higher seroprevalence (Lee 1992, Chong 1992). Two other study groups also reported a similar antibody prevalence rate among some Korean populations (Koo 1991, Que 1992). Our results are also comparable to the previous report demonstrating the low prevelance of antibody to *B. burgdorferi* in children with JRA residing in a nonendemic area (Saulsbury 1990). Although the antibody prevalence rates were not significantly different among the JRA types in this study, more positive responders among patients with RF-positive polyarticular disease and pauciarticular type II disease were comparable to a previous report (Saulsbury 1990). The rate among the JRA group younger than 14 years of age (median age) was also not different than that among the rest of the group, although children aged 5 to 14 years reportedly have the highest incidence of Lyme disease (Williams 1990). The antibody prevalence rate among patients with JRA who had been diagnosed for less than 2 years (median duration from the diagnosis of JRA to the study entry) were not different compared to the rest of the JRA group. Comparing the antibody prevalence rate between males and females, more positive responders were among female JRA patients compared to male patients when measured to p39, although Lyme disease has male preponderance (Williams 1990). Furthermore, the difference between female patients and female controls was not significant in this study.

The possibility of failure to detect the difference between the JRA and control groups originating from false negative results due to the lack of the antibody response to the pathogen during the first several weeks of infection is unlikely, since the study patients had JRA (Schutzer 1991). However, the possibility of seronegative Lyme disease following antibiotics administration early in the disease must be considered, since it has been suggested that early treatment of Lyme disease may abrogate the antibody response without eliminating infection and frequent antibiotics usage over the counter and by doctor's prescription is a common practice in Korea (Berg 1991). It has well been known that various strains from Europe, North America, and Japan are heterogeneous (Masuzawa 1991, Fattorini 1991). Therefore, the possible heterogeneity of Korean strains from foreign ones can account for the unequivalent study results.

The fact that two patients with JRA but none of the controls responded positively in the General Biometrics, although not significantly different, may have been due to utilization of recombinant p39 as antigen, inclusion of adsorption procedure with *E. coli* protein, and measurement of IgA antibody along with IgG and IgM antibodies. Previously, reactivity with p39 by immunoblots on sera from patients having Lyme borreliosis has been reported (Simpson 1990). Greater specificity of ELISA but with no change in sensitivity by inclusion of adsorption procedure with *E.coli* antigen fraction has also been reported (Fawcett 1989, Fawcett 1991). The detection rates of the antibody reacted to purified native flagella in both the JRA and control groups were lower compared to purified organisms in this study. This is contrast to previous reports in that the 41kd flagellar protein has been demonstrated significantly more sensitive than sonicated whole cell antigen in detecting the antibody (Hansen 1988). However, the flagellin, highly conserved and shared among borrelia and treponemes, has been recognized less reliable as a marker for *B. burgdorferi* than originally thought, owing to illiciting cross-reaction to reduce the specificity of serological asays (Collins 1991).

Greater efforts to verify whether Lyme disease occurs in Korea or not have to be made and maintained; attention to a high index of suspicion for Lyme disease is required, which can only be drawn by greater awareness of the disease by physicians of various specialties, considering Ly-

me disease is a multi-systemic illness; application of standardized serological assays with better sensitivity and specificity needs to be sought; a recent report of *B. burgdorferi* isolation from ticks of presumptive *I. persulcatus* needs to be confirmed with better delineation of heterogeneity from foreign strains; and the geographical distribution of *I. persulcatus* and *I. ricinus* in Korea also needs to be investigated.

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