Immunoblot Analysis of Antibody Binding to Polypeptides of *Borrelia burgdorferi* in Children with Different Clinical Manifestations of Lyme Disease

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ABSTRACT. We analyzed by means of immunoblot technique the patterns of antibodies binding to polypeptides of *Borrelia burgdorferi* B31 in the sera of 21 children with different stages of Lyme disease. All sera but one recognized the flagellar protein 41 kD and all but two the 83-kD protein. The number of proteins recognized rose from clinical stage I to stage III. The polypeptides of the mol wt 55 and 31 kD were exclusively bound by IgM and the proteins 66, 58, 39, and 36 kD exclusively by IgG. Based on the number of proteins visualized by single sera, IgM was the predominant isotype in stages I and II peaking in stage II, whereas in stage III IgG predominated. Considering the number of proteins recognized and the corresponding antibody isotype, a serologic differentiation between the three stages of the disease is feasible: within stage I and within stage III patients with different clinical signs had distinct antibody patterns. No clearcut pattern could be discriminated in stage II for patients with different settings. Immunoblotting yields a possible distinction between active infection and serological scar by the detection of specific antibody patterns. (*Pediatr Res* 26:377-382, 1989)

Abbreviations

ACA, acrodermatitis chronica atrophicans
ECM, erythema chronicum migrans
IFA, immunofluorescence assay
NC, nitrocellulose
TBS, Tris-buffered saline

Lyme disease is a tickborne disorder caused by the spirochete *Borrelia burgdorferi* (1). It may involve various organ systems. Based on the chronologic relationship to the tick bite the disease can be subdivided into three stages.

Stage I. Characteristically, the disease begins 4 to 20 d after a tick bite with a local inflammatory reaction called ECM: around the bite wound a red spot develops (macule or papule) which will be surrounded by a gradually enlarging annular lesion. As general symptoms headaches and/or arthralgias may occur.

Stage II. Weeks to months later, some patients present signs

of dissemination with cardiac disease, migratory musculoskeletal pain, or neurologic involvement, *i.e.* meningitis, seventh nerve palsy, or polyradiculitis.

Stage III. Weeks to years later, a few patients may develop arthritis, which untreated may become chronic with pannus formation and even with destructive erosion of cartilage and bone. Untreated neurologic syndromes may also become chronic or intermittently recurring. For unknown reasons every sign or finding can occur in isolation (2). Similarly, ACA, a dermatologic condition with erythema, shift of pigmentation, and frail skin, is a sign of the third stage of the illness. These lesions are mostly localized on the extensor surface of one extremity of adults after the third decade (3), but we observed them also in children (4).

First and second stage manifestations may resolve spontaneously. Because of the potential progression to chronic disturbances, however, antibiotic treatment is recommended in all stages, in children preferably with penicillin, in adults also with tetracyclines (2). Recently, successful treatment of late Lyme disease with cephalosporins has also been reported (5).

A history of tick bite and/or previous or actual ECM strongly suggests the diagnosis of Lyme disease. It should immediately be confirmed by determination of antibodies against *B. burgdorferi* (6-9). In atypical patients serology is essential, with the reservation that a positive titer to *B. burgdorferi* may represent a "serologic scar" due to a previous infection without actual interest.

The purpose of the present study was 1) to analyze the antibody response to *B. burgdorferi* antigens in children with different manifestations of Lyme disease by means of the immunoblot technique, 2) to investigate possible correlations of the antibody patterns to the stage of the disease, and 3) to evaluate the potential of immunoblotting to distinguish between active infection and serological scar.

MATERIALS AND METHODS

Patients. All patients treated at the University Children's Hospital of Zurich because of Lyme disease during the period from June 1985 to December 1987 were included in the study. The clinical data were gathered by chart review. Sera collected before treatment were stored at -70°C.

Materials. All chemicals as well as the instruments for electrophoresis and immunoblotting were purchased from LKB (Dü-bendorf, Switzerland) with the following exceptions: 4-chloro-1-naphthol from Merck (Zurich, Switzerland), antisera for immunofluorescence assay from Behring (Marburg, FRG) and for immunoblotting from Dakopatts (IG-Instruments, Zurich, Switzerland), mol wt markers and NC paper from BioRad (Glatt-

Received February 20, 1989; accepted May 15, 1989.

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Supported by the Federal Office of Public Health (Switzerland).

brugg, Switzerland), and human IgG from Schweizerisches Serum & Impfstoffinstitut (Bern, Switzerland).

Antigen preparation. *B. burgdorferi* B31 (ATCC 35210) was used for all antigen preparations. The spirochete was cultured in Barbour, Stonner, Kelly medium I (10) for 4–6 d at 37°C until a final density of at least 20 organisms per 400-fold magnification field was reached. Cells were harvested by centrifugation at $10\,000 \times g$ for 30 min at 4°C.

IFA. Global Ig-IFA was performed by standard methods, as previously described (7). In brief, the harvested *B. burgdorferi* cells were three times resuspended in PBS and centrifuged at $10\,000 \times g$ for 30 min at 4°C. The pellet was resuspended in PBS to a final concentration of 15–20 cells per 400-fold magnification field. Ten μ L of the suspension were pipetted onto each of 10 circles of a slide, air dried, and frozen at –75°C. Fixation with acetone was done before use. Twenty-five μ L of the patient's serum dilutions (2-fold steps beginning with 1:32) in PBS were incubated at 37°C for 30 min. After washing three times with PBS and air drying, the slides were stained with a 1:60 dilution of fluorescein-isothiocyanate-labeled goat antihuman γ -globulin fraction, incubated for 30 min, washed twice in PBS, and finally rinsed with deionized water. Titers $\geq 1:64$ were regarded as significantly elevated (compared with 100 sera collected from healthy blood donors).

Electrophoresis. SDS-PAGE was performed in a modified version of the method described by Laemmli (11). Pelleted spirochetes were resuspended three times in cold PBS with 5 mM MgCl₂ and centrifuged for 30 min at 4°C. The washed cells were resuspended in deionized water to reach a protein concentration of 0.5 g/L, measured according to the method of Lowry *et al.* (12). Electrophoresis of the spirochetal protein (0.5 μ g/mm gel) was done on a 10% acrylamide gel (18 cm plate, 1.5 mm gel thickness; 30:0.8 acrylamide:bisacrylamide) with 2-mercaptoethanol as reducing agent in the sample buffer. Two gels were run simultaneously in one chamber at a current of 60 mA at 12°C for approximately 5 h until a marker dye had traversed 10 cm of gel.

Separated polypeptides on the gels were stained with Coomassie Brilliant Blue R-250 or transferred to NC paper in a modified version of the method of Towbin *et al.* (13) using a Multiphor Novablot electrophoresis transfer unit (LKB). The transfer time was 1 h at a current of 150 mA. To assess the efficiency of transfer, one longitudinal strip was cut from the NC sheet and stained with amido black and compared to immunoblots. Prestained molecular weight markers were phosphorylase b (79 000), BSA (66 200), ovalbumin (42 622), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

Immunoblotting and detection of antigens. The NC paper containing transferred polypeptides was soaked for 1 h at room temperature in 2% BSA in TBS to saturate nonspecific protein-binding sites. The NC sheets were dried between filter paper and stored at 4°C. The NC was cut into 4-mm wide strips representing individual lanes from the gel. The strips were incubated individually with patient sera diluted 1:100 in TBS-0.05-Tween 20 overnight at room temperature on a rocking table. After washing the strips three times with TBS-0.05-Tween 20, they were incubated for 1 h at room temperature with a 1:200 dilution of horseradish peroxidase-conjugated goat antibody to human γ -chain. After three more washings the NC were stained with 4-chloro-1-naphthol for 20 min. Finally, the staining was stopped by washing the strips once with TBS and twice with deionized water. Control samples included serum from five normal individuals and from two patients with syphilis, and in addition the conjugate alone.

To identify spirochetal proteins, the mAb H5332 which reacts with the outer membrane protein subunit of *B. burgdorferi* B31 (31 000) (14) and H9724 which binds to *B. burgdorferi* flagellin (41 000) (15) were used (both kindly provided by A. G. Barbour, San Antonio, TX).

Rheumatoid factor. To avoid false-positive results in IgM detection, potentially present rheumatoid factor was removed by adsorption with latex particles coated with human IgG.

RESULTS

During the period from July 1985 to December 1987, 21 patients aged 4–15 y were treated for Lyme disease at the University Children's Hospital, Zurich. Diagnosis was based on history and clinical and serologic findings (Table 1). Three stages of the disease were distinguished. None of the patients in the stages II and III remembered previous ECM and no patient in stage III had presented symptoms compatible with stage II.

Immunoblotting. Antibodies to *B. burgdorferi* antigens could in each case be identified as binding to individual polypeptides (Tables 2 and 3). Ig bound to as many as 17 distinct proteins with mol wt of 41, 83, 62, 47/48, 60/61, 29, 55, 66, 39, 22, 17/18, 58, 42, 31, 75, 36, and 23 kD (listed in order of frequency). Two of these polypeptides were bound exclusively by IgM (55 and 31 kD) and four others exclusively by IgG (66, 58, 39, and 36 kD) (Figs. 1 and 2).

Sera of the four patients with stage I (patients 1–4) contained Ig binding to three to 10 polypeptides. All sera had IgM reacting with two to seven polypeptides and IgG reacting with one to six. The 41-kD protein was bound by IgG from all sera and by IgM from all but one. Both patients with ECM had in common the binding of IgM to the 31-kD polypeptide and of IgG to the 29-kD protein, a pattern not found in the two children suffering from headaches.

The sera of the nine patients assigned to stage II (patients 5–13) reacted with four to 10 proteins, IgM with four to seven, and IgG to as many as six. Compared with stage I, six additional proteins were visualized. Furthermore, in stage II, IgM bound to more proteins per serum than in stage I (mean 5.7 bands *versus* 4.3), whereas the number of bands per serum visualized by IgG did not differ (mean 2.9 *versus* 3). A constant finding in all sera was the binding of the 83-kD protein by IgM and/or IgG and in all sera but one the reaction with the 41-kD polypeptide. The patient with polyneuropathy was the only one with no antibodies against the 41-kD protein and both patients with paresthesia were the only ones with IgM but no IgG reacting with this protein. Moreover, these latter two patients and the one with meningitis were unique with IgM binding to both proteins 60/61 and 47/48 kD.

Ig in the sera of the eight patients classified to stage III (patients 14–21) were found to react with eight to 13 polypeptides, IgM with as many as five, and IgG with six to 12. No additional polypeptides beside those also seen in stages I and II were visualized. Binding of IgM was detectable in all sera except in one from a patient with arthritis, but it involved a smaller number of proteins than in stage II (mean 2.5 bands per serum with reacting IgM *versus* 5.7). IgG, however, reacted with substantially more proteins than in stage II (mean 10 *versus* 2.9). In stage III every serum bound IgG to the four proteins 83, 60/61, 41, and 29 kD, a feature not found in stages I and II. Moreover, in stage III all sera but one contained IgG binding to the 58-kD polypeptide which was not visualized by stage II sera. Furthermore, antibodies binding to the 60/61-kD protein were exclusively IgG. Anti-55-kD protein antibodies, commonly found in stages I and II, were exceptional. All patients with arthritis except one differed from those with ACA by the absence of antibodies binding to the 75-kD polypeptide and all except two by the lack of Ig reacting with the 17/18-kD protein. Among the patients with arthritis one (patient 16) differed markedly from the others by absent antibodies reacting with the 58-, 47/48-, 41-, and 22-kD polypeptides.

No reactions were seen with the sera from the five normal individuals and with the conjugate alone. The two syphilis sera showed weak reactions: one with IgG against the proteins 41 and 29 kD, and IgM *versus* 60/61 kD, the other IgG *versus* 83 kD.

IFA titers. The titers of anti-*B. burgdorferi*-Ig ranged from 1:128 to 1:4096 (Table 1). Patients with Lyme disease in the second or third stage tended to have higher titers than patients in the initial stage, but there was no strict correlation between elevation of the titer and the stage of the disease.

DISCUSSION

To our knowledge, our study is the first focusing on patterns of antibodies reacting with polypeptides of *B. burgdorferi* B31 in children with different stages of Lyme disease. Between the three

Table 1. Characteristics of 21 patients with different manifestations of Lyme disease

Stage	Patient No.	Sex	Age (y)	Symptoms*	Tick bite (wk before)	Borrelia burgdorferi IFA titer†
Stage I	1	F	8	Headache	None	256
	2	F	9	Headache	None	256
	3	M	10	ECM	-3	128
	4	F	4	ECM	-18	128
Stage II	5	M	6	PN	None	128
	6	F	10	Paresthesia	-?	512
	7	F	13	Paresthesia	None	128
	8	F	14	SNP	None	256
	9	M	6	SNP	-4	2048
	10	M	5	SNP	None	256
	11	F	15	SNP	-5	128
	12	M	9	SNP	-12	1024
Stage III	13	M	12	Meningitis	-5	512
	14	M	8	Gonarthrit	None	1024
	15	M	6	Gonarthrit	None	128
	16	F	7	Gonarthrit	-?	256
	17	M	15	Gonarthrit	None	4096
	18	F	7	Gonarthrit	None	1024
	19	F	11	Gonarthrit	None	1024
	20	M	10	ACA	-52	4096
	21	M	12	ACA	None	4096

* PN, polyneuropathy; SNP, seventh nerve palsy.

† Indirect immunofluorescence assay: global titer of specific immunoglobulins.

Table 2. Patterns of IgM antibody binding to polypeptides of *B. burgdorferi* in 21 children with different stages of Lyme disease as determined by immunoblotting

		determined by immunoblotting																
		Protein mol wt (kD)																
Stage	Patient	83	75	66	62	60/61	58	55	47/48	42	41	39	36	31	29	23	22	17/18
Stage I	1																	
	2																	
	3																	
	4																	
Stage II	5																	
	6																	
	7																	
	8																	
	9																	
	10																	
	11																	
	12																	
	13																	
Stage III	14																	
	15																	
	16																	
	17																	
	18																	
	19																	
	20																	
	21																	

Table 3. Patterns of IgG antibody binding to polypeptides of *B. burgdorferi* in 21 children with different stages of Lyme disease as determined by immunoblotting

Stage	Patient	Protein mol wt (kD)																
		83	75	66	62	60/61	58	55	47/48	42	41	39	36	31	29	23	22	17/18
Stage I	1																	
	2																	
	3																	
	4																	
Stage II	5																	
	6																	
	7																	
	8																	
	9																	
	10																	
	11																	
	12																	
	13																	
Stage III	14																	
	15																	
	16																	
	17																	
	18																	
	19																	
	20																	
	21

Fig. 1. Frequency of IgM and IgG binding to *Borrelia burgdorferi* B31 proteins in relation to stage of Lyme disease. Hatched areas of bars represent the percent of subjects positive for anti-protein antibody, while hatched plus open bars represent total number tested.

stages of the disease and in part also between clinical settings within the same stage we find considerable variations in number and nature of polypeptides bound and of binding isotypes.

The number of polypeptides detected in this study is similar (16, 17) or higher than in other reports (18–20). This might be due to several causes.

1) Technical differences—we did not add protease inhibitors during polypeptide separation by SDS-PAGE. Thus, insufficient breakdown of polypeptide dimers of *B. burgdorferi* might have resulted in supplementary or distinct bands (16). In addition, we transferred the polypeptides from SDS to NC by a semidry horizontal electrophoretic method which was not applied in other similar studies and which might also influence the results.

Fig. 2. Examples of immunoblot findings in respect to IgM and IgG to *B. burgdorferi* B31 in patients with different stages of Lyme disease. M_{AB} denotes MAb (H9724 binds to 41-kD and H5332 to 31-kD protein).

2) The strains of *B. burgdorferi* used as antigen might influence the detection of polypeptides of different molecular weight.

3) The geographical areas might be populated by antigenically different *B. burgdorferi* strains, which would elicit divergent host responses.

4) Stages of Lyme disease in our patients are different, and we compare them. Previous authors included patients with the same clinical setting or patients progressing through different stages. All the polypeptides visualized in our analysis, however, correspond to proteins previously observed in electrophoreses of the *B. burgdorferi* strain B31 (21).

In our study, IgM is the predominant isotype in stage I and even more pronounced in stage II, whereas in stage III the major isotype is IgG. False-positive results of IgM detection due to rheumatoid factor were ruled out. These findings are in agree-

ment with previous results in adults with early Lyme disease (16, 19). Inasmuch as we did not separate the isotypes before incubation of the sera, a competitive advantage of IgG over IgM in their binding to the polypeptide antigens cannot be excluded.

In our study with cross-sectional character, the scope of proteins bound by antibodies enlarged remarkably from stage I to stage III. A similar finding was reported from a longitudinal analysis in adults progressing through the different stages of Lyme disease (18). This appearance of new protein bands during the course of the illness suggests the persistence of *B. burgdorferi* within the host: the longer the bacterium is not eliminated, the more additional antigens, corresponding to protein bands, will be recognized by the host's immune system. Indeed, viable *B. burgdorferi* has been isolated from individuals with different clinical stages and settings of Lyme disease (2).

We and others (16) have demonstrated that the binding of antibodies in stage I is not restricted to the 41-kD protein, which is associated to the borrelial flagellum (22). This contrasts with findings in previous studies pointing out the ability of the 41-kD protein to suppress the immune response in early Lyme disease (18, 19). Furthermore, we have shown that patients with stage I may exhibit distinct antibody patterns, depending on their clinical setting: sera of patients with ECM recognize the proteins 31 and 29 kD, but patients with headaches do not. The antibody pattern of the only child with meningitis is similar to that of reported patients (17, 20). Despite divergences between the patients with different manifestations, we cannot discriminate a clear-cut antibody pattern for each setting in stage II. In stage III, however, we find serologic patterns in children with arthritis which differ from those in patients with ACA. The antibody panels in children with arthritis are analogous to those of the few reported patients with the same setting (18). In contrast to a previous study (22), our patients with ACA recognized more polypeptides than patients with arthritis.

In our study, none of the children with stage III had previous ECM or neurologic involvement and none with stage II had a history of ECM, and this is consistent with recent reports in the literature. Thus, the question arises, whether the classification of Lyme disease into three stages is justified. Applying this classification, we and others (18) find, as already mentioned, an increase in the number of proteins recognized from stage I to III. This has been explained by the different duration of persistence of *B. burgdorferi* within the host. Therefore, classification into three stages seems appropriate. But taking into account the patients with an atypical history, another question arises, *i.e.* whether the clinical expression of Lyme disease is determined by properties of the infecting *B. burgdorferi* strain with particular antigenic components, or rather by the individual immune response of the host.

The clinical presentation of *B. burgdorferi* infection varies geographically. In northern Europe neurologic disorders and ACA predominate, whereas in the United States joint involvement seems to be more frequent. These observations could be explained by a hypothesis of different strains causing different diseases. In fact, the polypeptides of Scandinavian and North American *B. burgdorferi* isolates are not identical (23), and strains from central Europe have recently been described in correlation with diverse clinical manifestations (24).

However, it is still obscure why some individuals overcome infection with *B. burgdorferi* without symptoms, and why in some patients Lyme disease is restricted, *e.g.* to ECM, and why in others, with or without a typical history, it develops into a chronic disorder. Individual proneness of the host to such different evolution, analogous to HLA-B27-associated arthritis, could provide an explanation. In fact, the HLA-phenotype DR2 has been reported with significant preponderance in patients with chronic Lyme disease (25, 26).

In patients lacking the typical history of tick bite and/or of ECM, the diagnosis of Lyme disease has to rely on serologic

findings. Antibodies against *B. burgdorferi*, however, may also be detected in asymptomatic individuals. In endemic areas up to 9% of the population were found to be seropositive (27). So far, no definite laboratory test differentiates between specific IgG in active disease from the asymptomatic antibody response. Such a distinction would be of therapeutic relevance to avoid unnecessary antibiotic treatment.

Indirect indicators for recent or actual infection are seroconversion or presence of specific IgM. But early seroconversion in Lyme disease is rarely detected because first symptoms may considerably lag behind the infection. Moreover, detection of IgM by IFA or ELISA test is enigmatic (6). Immunoblotting could facilitate the distinction between active infection and serological scar: first by detection of specific IgM and second by visualization of the complex pattern of antibodies binding to many polypeptides. Several investigators (16, 18) have stressed the superiority over other serologic methods of immunoblotting in detection of IgM. All these data are quite suggestive for the value of antigen-antibody patterns in close correlation with the clinical evolution.

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Announcements

Abstract Deadline

The American Pediatric Society and The Society for Pediatric Research announce the abstract deadline for the 1990 Annual Meeting (May 7-11, 1990, Anaheim Hilton & Convention Center, Anaheim, CA) has been set as January 4, 1990.

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