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Detection of Antigens in Urine of Mice and Humans Infected with *Borrelia burgdorferi*, Etiologic Agent of Lyme Disease

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Lyme disease is a seasonal tick-borne malady which has worldwide distribution. Early and accurate diagnosis of Lyme disease is essential for successful antibiotic therapy. Symptoms are too vague to make an early diagnosis based on conventional criteria. We report the detection of antigens of *Borrelia burgdorferi*, causative agent of Lyme disease, in the urine of infected mice and humans. This technique may eventually provide a rapid diagnostic test for the early and accurate detection of this illness.

Lyme disease is a collection of chronic and acute syndromes caused by the bacterium *Borrelia burgdorferi* (14). The disease is transmitted chiefly by ticks of the genus *Ixodes*; the primary reservoir for the organism is the white-footed mouse (2, 4). Infection of reservoir animals apparently results in chronic infection (14) with few side effects, but in other animals including dogs (3) and horses (5) and in humans, this chronic infection is accompanied by a number of neurological, cardiac, and arthritic sequelae of intermittent duration which are exacerbated over time (14). All stages of the disease in humans may be treated with antibiotics; however, the success of chemotherapy is decreased in patients with long-standing disease.

Primary Lyme disease is characterized by general malaise or flulike symptoms, often accompanied by a distinctive skin lesion, erythema chronicum migrans. Weeks to months later, some patients experience neurological and cardiac symptoms; a somewhat vague migratory arthritis may also occur during this second phase of the disease. Tertiary Lyme disease in the United States is typified by intermittent bouts of arthritis which may be as destructive as chronic inflammatory arthritides (14). In Europe, this stage more commonly results in severe neurological disease (14).

The diagnosis of Lyme disease is made by clinical examination combined with a history of tick bite or exposure in endemic areas, usually with evidence of seroreactivity to the organism. The erythema chronicum migrans rash may be absent in early disease, and antibody titers are usually low, which can make the diagnosis problematic. In later stages of the disease, serum titers are often helpful, but allowances must be made for possible cross-reactivity to other bacteria (11). The spirochete has been cultivated from several patients, but this is impractical for diagnostic purposes (13).

We examined the urine of mice and humans for the presence of *B. burgdorferi* antigens using two different immunological techniques. We found that at least three of the major antigenic components of the spirochete can be detected in these urine samples with monoclonal antibodies. The presence of these antigens in the urine offers the possibility of a rapid diagnostic method for Lyme disease.

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In this communication, we describe a monoclonal antibody-based detection method for *B. burgdorferi* antigens in the urine of mice and humans with Lyme disease.

MATERIALS AND METHODS

Collection of urine specimens from humans and mice. Urine specimens were collected from humans by a clean-catch technique and stored at −70°C until assayed. Samples were collected from patients diagnosed as having Lyme disease by the Minnesota Department of Public Health or the University of Minnesota; normal samples were collected from randomly chosen individuals with no history of Lyme disease at the 3M Center in St. Paul, Minn. Pooled urine samples were collected from mice which had been experimentally infected with *B. burgdorferi* 297 (9). Samples from these mice were collected on days 7, 11, 15, 18, and 22 postinfection into sterile tubes and stored until assayed as described above. Portions of 1 ml of each urine sample were placed into Barbour-Stoenner-Kelly (1) medium in an attempt to cultivate spirochetes from the urine specimens and placed at 34°C. The tubes of medium were examined weekly by dark-field microscopy.

Construction and analysis of monoclonal antibodies to *B. burgdorferi*. Monoclonal antibodies to *B. burgdorferi* surface components were constructed by the method of Galfre and Milstein (7). Briefly, BALB/c mice were immunized with 10^6 spirochetes per ml per injection over a course of 3 months. Mice were sacrificed by cervical dislocation, and the spleens were removed. Splenic leukocytes were harvested from the spleen sac and fused with an immortal mouse cell line (P3-X63-Ag8.653) by a polyethylene glycol technique as described previously (7). Clones producing antibodies specific for *B. burgdorferi* were injected intraperitoneally into BALB/c mice for ascites production. Monoclonal antibodies were purified from contaminating immunoglobulin and other mouse protein by protein A (Pierce Chemical Co., Rockford, Ill.) purification. Antibodies to *B. burgdorferi* were characterized by the Western blot (immunoblot) method of Towbin et al. (15) for specific binding to *B. burgdorferi*.

Immunoassay of urine specimens. Proteins from urine specimens were immobilized onto nitrocellulose membranes in Millititer plates (Millipore Corp., Bedford, Mass.) by applying 150 μl of urine to each well and drawing the liquid

through under vacuum. A 50-μl sample of monoclonal antibody (100 μg/ml in phosphate-buffered saline with 0.05% Tween 20 [PBS-T]) was added to each well and incubated at room temperature for 30 min. Each well was washed with 600 μl of PBS-T. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was added to each well, followed by incubation and washing as before. Substrate (0.1 M sodium citrate [pH 4.5] containing 1 mg of *o*-phenylene diamine per ml and 0.03% H_2O_2) was added to each well and allowed to develop for 30 min. The plates were read on a ELISA plate reader at 490 nm (Dynatech Laboratories, Inc., Alexandria, Va.). Optical density values greater than 3 standard deviations above the mean of the normal control urine samples were considered positive. Normal urine inoculated with 10^5 *B. burgdorferi* per ml was used as a positive control.

Western blot analysis of urine samples. Samples (1 ml) of urine were lyophilized and reconstituted in 50 μl of sodium dodecyl sulfate sample buffer (62 mM Tris hydrochloride [pH 6.8], 10% [vol/vol] glycerol, 5% 2-mercaptoethanol, 2% [wt/vol] sodium dodecyl sulfate, 0.0013% bromophenol blue). The samples were applied to a sodium dodecyl sulfate-12.5% polyacrylamide gel and electrophoresed by the method of Laemmli (10) to separate the components of the urine. The proteins in the gel were transferred to a sheet of nitrocellulose by the method of Towbin et al. (15). The transferred proteins were probed with a mixture of monoclonal antibodies generated to specific surface antigens of *B. burgdorferi* (OspA, OspB, and flagellar proteins). The blots were washed with PBS-T, after which alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G in PBS-T was added and incubated as described above. The blots were developed with a Nitro Blue Tetrazolium substrate (1 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml, 5 mg of Nitro Blue tetrazolium [Sigma Chemical Co., St. Louis, Mo.] per ml in 0.1 M Tris hydrochloride [pH 9.5]). Whole solubilized bacteria diluted in normal urine were used as positive controls, and normal lyophilized urine was used as a negative control.

RESULTS

Characterization of monoclonal antibodies to *B. burgdorferi*. Hybridoma clones were selected from positive fusion wells which reacted with whole cell lysates of *B. burgdorferi* B31. These clones were screened against *B. burgdorferi* B31 (ATCC 35210), G25 (1), and 297 (1) as well as several unrelated species of bacteria (*Bacteroides gingivalis*, *Treponema denticola*, *Streptococcus salivarius*) and human lymphoid cells (Daudi, ATCC CCL 213). The antibodies were also screened against *Borrelia parkeri*, an agent of relapsing fever which shows significant DNA homology with *B. burgdorferi* (8). Three clones were selected as being reactive with representative surface epitopes of the Lyme disease spirochete. These antibodies were designated LYB008, LYM039, and LYM065 and reacted with the 31,000-, 34,000-, and 41,000-dalton antigens (OspA, OspB, and flagellar proteins), respectively.

Immunoassay of urine specimens for presence of *B. burgdorferi* antigen. Urine from mice experimentally infected with *B. burgdorferi* were positive for the 31,000-dalton surface antigen (OspA) as determined by dot-blot enzyme-linked immunosorbent assay analysis with antibody LYB008. The dilutions of urine found to be positive in the day 22 sample (Table 1) are typical of the results seen in all samples from these animals. The normal control samples from uninfected mice yielded results barely above the background level as determined by using a blank (PBS). Attempts to cultivate *B. burgdorferi* from the positive samples were unsuccessful.

Urine from the 10 patients with Lyme disease and 10 normal controls were assayed by dot-blot enzyme-linked immunosorbent assay and by Western blot as described above with LYB008, LYM039, and LYM065 (Table 2). Only those samples collected from patients with Lyme disease were positive; however, the reactivity between the urine samples and the monoclonal antibodies in the positive cases was highly variable. Some urine samples reacted only with one antibody; others reacted with two or all three used in the assays. The results of the Western blots are shown in Fig. 1. The wavy bands in the blot were also observed in the gels. The histories of the patients who contributed positive samples are also shown in Table 2. There was apparently no correlation between reactivity of the urine samples and the immunofluorescence assay titer of these individuals. The antigen was detected in the urine of patients in different stages of the disease; however, several samples from patients with confirmed cases of Lyme disease were negative in both the dot-blot assay and by Western blot (data not shown).

TABLE 1. Detection of *B. burgdorferi* antigen in mouse urine with monoclonal antibody LYB008 (anti-OspA)

Dilution of urine	Optical density at 490 nm	
	Control	Infected
Undiluted	0.128	0.569
1:2	0.031	0.497
1:4	0.015	0.351
1:8	0.000	0.120
1:16	0.000	0.031
1:32	0.000	0.000
1:64	0.000	0.000

TABLE 2. Detection of *B. burgdorferi* antigens in human urine with monoclonal antibodies[a]

Patient no.	Disease status	ECM history	IFA titer	Optical density at 490 nm[b]		
				LYB008	LYM039	LYM065
1	Early	+	ND[c]	0.510	0.291	0.421
2	Early	+	1:8	0.336	0.321	0.163
3	Early	+	1:8	0.310	0.196	0.271
4	Early	+	1:16	0.270	0.529	0.634
5	Late	−	1:64	0.495	0.129	0.655
6	Late	−	1:64	0.420	0.311	0.109
7	Late	+	1:128	0.393	0.181	0.419
8	Late	+	1:16	0.388	0.162	0.174
9	Late	+	1:64	0.302	0.411	0.203
10	Late	+	1:16	0.276	0.373	0.578
Control		−	1:2	0.063	0.020	0.041

[a] ECM, Erythema chronicum migrans; IFA, immunofluorescence assay.
[b] Values are the means of three experiments. A positive was considered to be the mean plus 3 standard deviations above the mean of the control urine specimens. The controls were 10 individuals with no history of Lyme disease. The specificities of the monoclonal antibodies are described in the text.
[c] ND, Not determined.

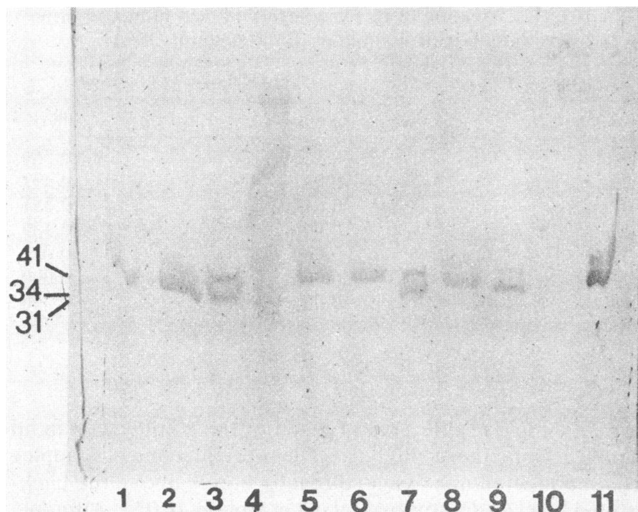

FIG. 1. Western blot of sera from individuals with suspected cases of Lyme disease which tested positive in the enzyme-linked immunosorbent assay-based assay for *B. burgdorferi* antigenuria. Lanes 1 through 3 correspond to patients 1 to 3 listed in Table 2. Lane 4 contains molecular weight standards. Lanes 5 through 11 correspond to patients 4 through 10 in Table 2. The urine samples were boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and electrophoresed as described in the text, followed by transfer to nitrocellulose for probing with monoclonal antibodies LYB008, LYM039, and LYM065. The 31,000-dalton antigen is OspA, the 34,000-dalton antigen is OspB, and the 41,000-dalton antigen is the flagellar antigen. The numbers on the left show the locations of the three antigens. The specificities of the monoclonal antibodies are described in the text.

DISCUSSION

Diagnosis of Lyme disease is currently based on epidemiological and clinical criteria. The presence of the erythema chronicum migrans skin rash, history of a tick bite, travel in an endemic area, and a convalescence antibody titer of greater than 1:256 by immunofluorescence assay are the standard criteria for a confirmed case of Lyme disease. Detection of specific antigen in the urine of infected individuals may be a more convenient and faster method of diagnosing the disease, especially if used in conjunction with clinical findings. Antigenuria has been previously demonstrated in hamsters infected with *B. burgdorferi*; a monoclonal antibody prepared by Benach et al. (2) was shown to bind to an OspA determinant found in the urine of hamsters up to 72 h postinfection with the spirochete.

Of the 10 patients with Lyme disease who tested positive for the antigen in the urine assay, 8 had a history of erythema chronicum migrans; 2 of the patients with early primary disease had the rash at the time that the urine and serum samples were taken. Neither of these patients had elevated serum antibody titers (>1:256). All the patients with secondary disease had at least a 1-year history of Lyme disease with either arthritic, neurological, or cardiac complications. Although the serum titers were higher for the late group than for the early group, none of these individuals had titers >1:256 at the time of sampling. Individuals with nonreactive urine assays may shed spirochetes intermittently and consequently were not detected in our assay. Several of these individuals had titers of >1:256.

Serological assays for infectious disease suffer from a number of shortcomings which may complicate the interpretation of their results. Single serum antibody titers obtained between exposure to the infecting agent and the response to that agent are affected by a number of factors, including the immune status of the patient, dose of antigen, and route of exposure. Often, sequential serum samples are necessary to detect secondary infections or relapses in chronic cases (11, 12). In Lyme disease, there may be little or no correlation between serum titer to the spirochete and the clinical symptoms, possibly owing to a poor immune response to the microbe. Negative serological titers are common in early stages of Lyme disease; in secondary and tertiary stages, the titers may be quite high but may not correlate with exacerbations of the disease (12). The specificity of monoclonal antibodies can preclude any cross-reactivity with other microorganisms; this is a problem with the currently used serological tests (11). Clinical trials are necessary to determine the utility of this new assay and the relevance of antigenuria in the progression of Lyme disease to the more severe secondary manifestations. Assays for the presence of the antigens or antigenic components of suspected pathogens such as *B. burgdorferi* may be more sensitive indicators of infection and for the need for therapeutic intervention.

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