Recognition of a New Serogroup of Legionnaires Disease Bacterium

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A strain of the Legionnaires disease bacterium (LDB) that was isolated by Joseph E. McDade from a postmortem lung specimen of a patient with fatal atypical pneumonia at the Veterans Administration Hospital in Togus, Maine was serologically different from 16 other strains of LDB that had been isolated previously from patients in other geographic locations. The serological differences of the Togus isolate were shown in results of direct and indirect fluorescent antibody staining and of immunoelectrophoresis with soluble antigen extracts. Seroconversion for the Togus strain of LDB in acute- and convalescent-phase sera from a second patient with atypical pneumonia at the Veterans Administration Hospital in Togus indicated that this patient had been infected with an LDB that was serologically similar or identical to the Togus isolate. The Togus serogroup of LDB should be considered when performing serological tests for Legionnaires disease.

The Legionnaires disease bacterium (LDB) is a gram-negative, non-acid-fast bacillus. The organism was first recognized as the etiological agent of Legionnaires disease (LD) by McDade et al. (5), who isolated it by infecting guinea pigs with a suspension of human lung tissue and by passaging the infected guinea pig spleen tissue in embryonated hens' eggs. Indirect fluorescent antibody (IFA) staining of yolk sac membrane suspensions of an LDB isolate was used in demonstrating seroconversion with acute- and convalescent-phase sera from LD patient. Cherry et al. (1a) prepared antisera and fluorescein isothiocyanate conjugates for five isolates of the LDB and demonstrated that direct fluorescent antibody (FA) staining was effective for detecting the LDB in lung tissue. Direct FA staining provided a relatively simple means of screening biopsy or postmortem tissue for the presence of the LDB and determining whether guinea pig isolation procedures should be used on a particular specimen. The usefulness of the technique was further documented for examining sputum and other respiratory system secretions for LDB. Cherry et al. (1a) used a conjugate prepared from rabbit antiserum against an LDB strain isolated from a patient in Knoxville, Tenn. (Knoxville strain) for their diagnostic FA reagent. High dilutions of this conjugate applied to the 10 isolates of LDB that were available at that time produced various intensities of FA staining. However, all isolates were satisfactorily

stained at a 1:80 dilution of the conjugate in phosphate-buffered saline (pH 7.6). The conjugate was remarkably specific for LDB in that it did not stain any of 59 other species from 25 genera of organisms tested. However, it did stain one strain of *Pseudomonas fluorescens* isolated from a commercial preparation of Evans' blue dye. Seven additional LDB isolates have been submitted to the Center for Disease Control from various sources since the report of Cherry et al. Six of these isolates also stain brightly with the diagnostic Knoxville conjugate. An isolate obtained from a postmortem lung specimen from a patient at the Veterans Administration Hospital in Togus, Maine did not stain with the Knoxville conjugate and differed serologically from the 16 other isolates. Results of IFA staining with acute- and convalescent-phase sera from a second patient with atypical pneumonia at the Veterans Administration Hospital in Togus suggest that this patient was also infected with an LDB of the Togus serogroup. In this report we describe the serological characteristics of this new LDB strain and emphasize the importance of considering this serogroup when performing diagnostic tests for LDB.

MATERIALS AND METHODS

Human tissue specimens. Both Formalin-fixed and fresh-frozen lung specimens from the patient who expired at the Veterans Hospital in Togus were examined by direct FA using the Knoxville conjugate

(1a). The tissues were reexamined using the conjugate prepared with the Togus isolate.

Strains used. Philadelphia 1, 2, 3, and 4; Pontiac; Knoxville; Albuquerque; Flint 1; Bellingham; and Burlington LDB strains were used by Cherry et al. (1a) in their study of direct FA staining of the LDB. We used these same strains and seven others in the study reported here (Table 1). The Togus strain, which is of primary interest in our study, was isolated from a postmortem lung specimen from a patient at the Veterans Hospital in Togus, Maine, who became ill on 2 April, 1978.

Characterization of the Togus isolate. DNA homology studies were performed by Don Brenner (1), β -lactamase activity and in vitro antibiotic sensitivity were determined by Clyde Thornsberry (7, 8), growth characteristics were described by Robert Weaver (9), and the gas-liquid chromatography profile of the cellular fatty acids was documented by one of us (C.W.M.) (6) by using methods that were outlined in previous reports on LDB.

Preparation of antisera. In our study we used the same antisera prepared by Cherry et al. (1a) for 10 strains of LDB. Antisera for the other isolates were prepared as follows. Whole-cell antigens were produced on Mueller-Hinton supplemented agar as described by Cherry et al. (1a). Cell suspensions in 0.85% NaCl containing 0.5% Formalin were adjusted to 40 international units of turbidity (4), or approximately 4×10^9 bacterial cells/ml. Young adult New Zealand white rabbits were immunized as described below. The first injection consisted of 2 ml of equal volumes of cell suspension and Freund complete adjuvant (Difco Bacto Adjuvant, Complete Freund; Difco Laboratories, Detroit, Mich.) distributed intracutaneously in approximately 40 sites along the shaved back of each rabbit. After 21 days, a second injection of 2 ml of equal volumes of cell suspension and Freund incomplete adjuvant was given in two deep-muscle injections in the hind quarters. Seven days later, 50 ml of blood was taken from the ear, and 2 ml of cell suspension was injected intravenously. Again after 7 days, 50 ml of blood was taken, and 2 ml of cell suspension was injected intravenously. The rabbits were exsanguinated 7 days later.

Preparation of conjugates. Immunoglobulin G (IgG) from Togus antisera was isolated by affinity chromatography on a column of staphylococcus protein A-Sepharose CL 4B (Pharmacia Inc., Piscataway,

N.J.) as described by Goding (2). IgG concentrations were estimated by absorption at 280 nm. Fluorescein isothiocyanate conjugates for the other LDB strains were prepared using methods described previously (3). All conjugates were adjusted to contain 10 mg of globulin per ml.

Conjugates prepared for our study or used in previous studies were available for 16 LDB isolates. A conjugate was not prepared for one isolate (Allentown) because it was received late in the course of the study.

Human serum specimens. Convalescent-phase serum, obtained from the patient from which the Detroit strain of LDB was isolated, was supplied by Thomas Neblett at Henry Ford Hospital, Detroit, Mich. The serum was taken on day 63 after the patient became ill. Hereafter, this serum will be referred to as the Detroit serum. IgG was isolated from the Detroit serum by affinity chromatography on a staphylococcus protein A-Sepharose column and was concentrated to approximately 100 mg/ml for use in immunoelectrophoresis (IE) studies.

Acute- and convalescent-phase sera from a patient who survived atypical pneumonia (suspected LD) at the Veterans Administration Hospital in Togus were sent to our laboratory. These sera were obtained on days 2 and 31 after the patient became ill on 22 May 1978, and are hereafter referred to as the Togus acute-phase and the Togus convalescent-phase sera.

Immunofluorescent staining. The conjugates prepared were tested on heat-fixed smears of all the LDB strains as described by Cherry et al. (1a). The staining titer was expressed as the reciprocal of the highest dilution of the conjugate that produced 4+ staining of the cells.

IFA tests on human sera were performed essentially as described by McDade et al. (5). The titers were expressed as the reciprocal of the highest dilution of the test serum producing 1+ IFA staining. Fluorescein isothiocyanate-labeled rabbit anti-human IgG was supplied by Knox Harrell, Biological Products Division, Center for Disease Control.

IE. Solubilized antigen extracts (SAE) were prepared with cells from the Togus and Knoxville LDB isolates. LDB were harvested after 4 days of growth at 35°C on modified Mueller-Hinton agar plates. The cells were washed once with 0.85% NaCl and packed by centrifuging at $1,200 \times g$ for 30 min. A 2-ml portion of packed cells was suspended in 10 ml of 0.85% NaCl and autoclaved at a temperature of 121°C (15 min). The autoclaved cell suspension was centrifuged for 30 min at $1,200 \times g$. The clear supernatant extract was removed, dialyzed against distilled water, and lyophilized. Solutions containing 50 mg of the SAE per ml were prepared in sterile distilled water. These SAE solutions were used in IE studies with antisera prepared in rabbits against the Knoxville and Togus strains of LDB and against human IgG from the Detroit convalescent-phase serum. Commercial IE plates (Behring Diagnostics, Somerville, N.J.) and Gelman IE apparatus (Gelman Instruments Co., Ann Arbor, Mich.) were used. Antigen solutions in the wells were subjected to electrophoresis at 150 V for 90 min. Antisera were added to the troughs, and the plates were allowed to develop for 16 h at room temperature before being refrigerated for 24 to 48 h at 4°C.

TABLE 1. LDB strains used in this study^a

Strain	Source	Isolated by
Berkeley	Lung tissue	Calif. State Health Dept.
Flint 2	Lung tissue	Morris Dumoff
Birmingham	Lung tissue	Joseph McDade
Detroit	Lung biopsy	Thomas Neblett
Miami Beach	Lung tissue	Joseph McDade
Allentown	Trans-tracheal aspirate	Gary Lattimer
Togus	Lung tissue	Joseph McDade

^a Additional strains of LDB used in this study and listed in a previous report are Philadelphia 1, 2, 3, and 4; Pontiac; Knoxville; Albuquerque; Flint 1; Bellingham; and Burlington.

RESULTS

Immunofluorescent staining. On 13 April, a Formalin-fixed lung specimen from Togus was received in our laboratory. Smears prepared from tissue scrapings of this specimen were negative for fluorescent organisms when stained with the Knoxville conjugate. Subsequently, on 19 April, smears of a homogenate prepared from a frozen lung specimen of the same patient were examined by using the Knoxville conjugate and were found to contain organisms resembling LDB. Isolation procedures were initiated after this observation. The Togus organism was isolated from this tissue homogenate by Joseph McDade after it was passaged through guinea pigs and embryonated hens' eggs before being cultured on artificial media. Despite numerous attempts, we were unable to duplicate the positive FA results obtained on smears from the lung homogenate using the Knoxville conjugate.

Rabbits were immunized with the Togus isolate, and the IgG obtained from the first, second, and final bleedings was labeled with fluorescein isothiocyanate. The homologous titers by direct FA staining of the Togus LDB were 1,000, 1,000, and 2,000, respectively. These conjugates were completely negative when tested against the 16 heterologous strains of LDB. They also did not stain the same strain of *P. fluorescens* that Cherry et al. (1a) observed to stain brightly with the other LDB conjugates. Conversely, the conjugates for 15 other strains of LDB, including that of the diagnostic Knoxville strain, were completely negative in direct FA staining tests with the Togus strain.

When the Formalin-fixed lung tissue and the homogenate of the frozen lung from Togus were reexamined with the Togus conjugate, both contained a moderate number of brightly stained organisms resembling LDB.

IFA titers of the Detroit convalescent-phase serum and the Togus acute-phase and convalescent-phase sera against both Philadelphia 1 antigen and Togus antigen are shown in Table 2. Although an eightfold rise in titer (32 to 256) between the Togus acute-phase and convalescent-phase sera was observed with Philadelphia 1 antigen, the IFA titer of Togus serum with Togus antigen increased 256-fold (32 to 8,192) from acute-phase to convalescent-phase sera. No increase between the acute and convalescent phase was observed when Togus serum was tested against the Detroit antigen. A reverse relationship was observed for the Detroit convalescent serum, in that a titer of 262,144 was produced with both the Philadelphia 1 antigen and the homologous Detroit antigen, whereas a titer of only 128 was obtained with the Togus antigen.

Characteristics of the Togus isolate. The characteristics of the Togus isolate were consistent with those of the other LDB strains studied previously in terms of DNA homology, gas-liquid chromatography of cellular fatty acids, growth characteristics, production of β -lactamase, and in vitro antibiotic sensitivity (1, 6-9).

IE. The precipitin lines obtained with IE of the SAE of Togus and Knoxville cells against the homologous and heterologous rabbit antisera are shown in Fig. 1. Knoxville SAE in well no. 1 produced two major arcs near the well and two major cathodic and two anodic arcs near the troughs against homologous antisera in troughs A and B. Togus SAE in well no. 2 produced several precipitin arcs near the well and only one cathodic arc near trough C, which contained Togus antiserum. The Togus SAE produced no precipitin arcs near the well when reacted with Knoxville antiserum, indicating complete non-identity between Togus and Knoxville antigens in this area of the gel. However, three precipitin arcs were observed near the trough (one above the well, one cathodic, and one anodic). These apparently represent common or similar antigens in the Togus and Knoxville strains. Simi-

TABLE 2. IFA titers of human sera with the LD Philadelphia 1, Detroit, and Togus antigens

Antigen	Serum IFA titers ^a		
	Detroit	Togus	
		Acute	Convalescent
Philadelphia 1	262,144	32	256
Detroit	262,144	64	64
Togus	128	32	8,192

^a Serial twofold dilutions of the test sera were made with phosphate-buffered saline. Titers are expressed as the dilution factor of the highest dilution of the test serum that resulted in 1+ IFA staining.

FIG. 1. Immunoelectrophoresis patterns of SAEs of Knoxville and Togus strains of LDB with Knoxville and Togus antisera. Troughs A and B contain Knoxville antiserum, and trough C contains Togus antiserum. Wells 1 and 3 contain Knoxville SAE, and well 2 contains Togus SAE.

larly, a single cathodic precipitin arc was produced when Knoxville extract (well no. 3) was reacted with Togus antiserum (trough C). Non-reciprocity in the number of common antigens observed with the two antisera probably results from differences in antibody titer against these particular antigens. All major antigens observed were also present when Knoxville antiserum was reacted with SAE from 15 other LDB strains. Further, when Togus antiserum was tested against SAE from the 15 LDB strains, the common antigen that migrated toward the cathode was always present.

The IE patterns of concentrated IgG from the human Detroit convalescent-phase serum with SAE of cells of three LDB strains are shown in Fig. 2. Two precipitin arcs were produced against both Detroit and Knoxville SAE, but no precipitin arcs were formed against the Togus SAE.

DISCUSSION

The fact that the original lung homogenate from the Togus patient was positive by a direct FA stain with the Knoxville conjugate cannot be readily explained. The possibility that the patient had a mixed infection caused by members of two serogroups of LDB and that the Togus serogroup was selected by the isolation procedures cannot be absolutely ruled out. However, except for the initial procedure in which fluorescent organisms were observed in the lung homogenate, all FA tests using the Knoxville conjugate were negative. The possibility of antigenic shift on passage of the organism through guinea pigs and hens' eggs is not compatible with the observations, because the conjugate prepared from Togus antisera stained organisms in the original homogenate as well as in scrapings and tissue sections of the Formalin-fixed lung specimen. The recognition of a strong seroconversion to the Togus serogroup in a second patient from

the same hospital is further evidence against antigenic shift. The selection of the Togus homogenate for isolation procedures based on FA staining with the Knoxville conjugate fortuitously led to the isolation and recognition of a new serogroup of LDB.

Direct FA staining with the Knoxville and Togus conjugates resulted in complete distinction between the Togus strain and 16 other LDB strains tested. Thus, the rabbit did not respond to surface antigens of the Togus group in immunogenic preparations of the Knoxville strain. However, both common and specific antigens are evident in the IE patterns (Fig. 1), indicating that hidden or nonsurface antigens are also involved in IE. We must assume that common or related surface antigens of the two groups of LDB evoked a response from the human immunological system, to account for the observed IFA titers of Detroit convalescent-phase serum with Togus antigen and of Togus convalescent-phase serum with Philadelphia 1 antigen (Table 2). Speculation as to whether this represents a species difference in response to the two immunogens would not be justified on the basis of the data presented here because of differences in immunization routes and history of the immunogen and because of possible discrepancies resulting from comparing direct versus indirect FA staining.

Since the *in vitro* antibiotic sensitivity of the Togus strain is similar to those of the other LDB isolates, serogroup identification is probably irrelevant to decisions concerning treatment of patients. However, being able to identify serogroups could be valuable in epidemiological studies, in that a given type may be associated with certain geographic areas.

A most important aspect in recognizing serological differences is concerned with diagnostic serological tests for LD. Although the greater than fourfold rise in IFA titer to Philadelphia 1 antigen observed between the acute- and convalescent-phase Togus sera was indicative of a recent infection with LDB (Table 2), the far greater rise in titer against Togus antigen (32 to 8,192 versus 32 to 256) strongly suggests that this patient was infected with LDB of the Togus serogroup. Further evidence of the effects of serogroup on IFA staining is obtained from the titer of Detroit convalescent-phase serum. The titer of this serum with the homologous Detroit antigen and with the standard Philadelphia 1 antigen currently being used in IFA tests was 262,144 in both cases. The IFA titer of this serum with the Togus antigen was only 128. The Philadelphia 1 antigen and the Detroit antigen are immunologically similar in this test, whereas the Togus antigen is distinctly different. These data

FIG. 2. Immunoelectrophoresis of SAEs of Togus, Knoxville, and Detroit strains of LDB with concentrated IgG from the Detroit patient. Wells 1 through 3 contain Togus SAE, Knoxville SAE, and Detroit SAE, respectively. The Detroit convalescent-phase IgG is in the troughs.

indicate that cases of LD caused by the Togus group of LDB may go undetected if the Togus group antigen is not included in IFA tests.

The identification of the Togus serogroup obviously affects currently accepted diagnostic direct FA tests, because the Knoxville conjugate, which has been used in screening tissue specimens and respiratory secretions, completely fails to recognize LDB of the Togus group. Diagnostic direct FA screening for the currently known LDB serogroups requires conjugates prepared from antisera raised against both the Knoxville and the Togus strains of LDB. The Togus strain represents a newly recognized serogroup of LDB that should be included in any diagnostic procedure for LD that is based on immunological reactions. The existence of other, as yet unrecognized serogroups of LDB is also a distinct possibility; therefore, lung tissue obtained aseptically from suspected cases of LD should be quick frozen and submitted to reference laboratories for isolation procedures even though the FA tests with currently available conjugates are negative.

ADDENDUM

Since the submission of this manuscript, the Togus serogroup of LDB has been isolated from two fatal cases of LD in the Atlanta area.

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