Characterization, Serological Specificity, and Diagnostic Possibilities of Monoclonal Antibodies Against *Legionella pneumophila*

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Hybridoma-producing monoclonal antibodies against *Legionella pneumophila* were produced by the fusion of nonsecreting mouse myeloma cells (NS-1) with splenocytes of BALB/c mice immunized by heat-killed *L. pneumophila* of serogroup I. Of 96 wells, 85 produced clones, of which 28 were positive as measured by an enzyme-linked immunosorbent assay technique. Ten of the hybridoma supernatants, remaining positive after 2 months of culture, were tested against the other *Legionella* serogroups and the atypical strains. None showed significant cross-reaction. Six of the positive clones were subcloned by limiting dilution, and two subclones were put into ascites in BALB/c mice. The monoclonal antibody obtained from the II-6-18 subclone was of the gamma-3 isotype. In this report, we describe the conditions for the use of this monoclonal antibody as a diagnostic tool for the detection of serogroup I *L. pneumophila*.

Since the first outbreak of Legionnaires disease in July 1976, the pathogen has been characterized as *Legionella pneumophila* (1). Epidemiologically, the disease has been reported as sporadic, community-acquired pneumonia, or as epidemic, especially in a hospital environment (3). The frequency of its identification has increased with the improvement of diagnostic procedures. According to a recent study, Legionnaires disease has been recognized in 15% of "adult, community-acquired pneumonia," placing *L. pneumophila* second to pneumococci as an etiological agent of pneumonia (9). Its severity varies from a benign pneumonia to a multilobar one resulting in death by respiratory failure (13). A rapid and fatal evolution is frequent in immunosuppressed patients (6), and an early diagnosis is required for this group of patients since the pathogen is insensitive to penicillins, the antibiotics currently used in the treatment of acute pneumonia.

The laboratory diagnosis of the disease is generally based on the increase in anti-*Legionella* antibodies in the serum of the patients (5). During the acute phase, diagnosis is very difficult, since the isolation and culture of the bacterium from expectorations or bronchic secretions are rarely successful. Recently, an enzyme-linked immunosorbent assay (ELISA) method has been described for the rapid detection of *Legionella* antigens in the expectorations and

the urine of patients (14), using a polyclonal rabbit antiserum. These antisera, however, display cross-reactions with other lung pathogens (2).

In this paper, we describe the characterization of a murine monoclonal anti-*Legionella* antibody and its diagnostic possibilities for the detection of the pathogen and antibodies in the serum of patients.

MATERIALS AND METHODS

Antigen preparation. *L. pneumophila* serogroup I (reference strain Philadelphia 1) was cultured in a medium consisting of agar (16 g/liter; Difco Laboratories, Detroit, Mich.), yeast extract (10 g/liter, Difco), active charcoal (2 g/liter), ACES buffer (10 g/liter; Sigma Chemical Co., St. Louis, Mo.), potassium ketoglutarate (1 g/liter), cysteine (0.5 g/liter) and ferrous pyrophosphate (0.25 g/liter). The medium was adjusted to pH 6.9 by adding potassium hydroxide and supplemented with 60 ml of horse blood per liter and 100 ml of globular extracts (heat-deproteinated serum from Pasteur production) per ml.

After 2 to 3 days of incubation at 37°C under aerobic conditions, *L. pneumophila* was harvested in a tube containing phosphate-buffered saline (PBS; pH 7.4). The suspension was homogenized by vortexing, and the bacteria were killed by boiling for 20 min. The killed suspension was checked for live bacteria by culture for another 7 days. The suspension of killed bacteria was adjusted to 2×10^9/ml by checking the turbidity against a MacFarland tube number 4.

Immunizations. BALB/c mice were injected intrave-

nously once per week for 3 weeks with 2×10^8 heat-killed bacteria. After a resting period of 3 months, they were boosted intravenously with the same amount of bacteria 3 days before the splenocytes were used for fusion.

Hybridization procedures. The myeloma cell line NS-1 was obtained from P. Goodfellow (Imperial Cancer Research Fund Laboratories, London). It was selected for aminopterin sensitivity and grown in RPMI (Boehringer Mannheim Corp., New York, N.Y.) containing 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml (complete RPMI).

Splenocytes were dispersed by the injection of serum-free RPMI into the spleen, and the cells were washed three times in serum-free RPMI before fusion.

NS-1 cells and the splenocytes of two spleens were fused at a 1:10 ratio in the presence of 41% polyethylene glycol (E. Merck AG, Darmstadt, Germany) by the method of Köhler and Milstein (8), washed in RPMI, and suspended in 100 ml of the complete medium. The cells (22×10^6 cells per ml) were redistributed in Nunclon plates (24 wells per plate) at 1 ml/well. After 24 h, 1 ml of selective medium (complete RPMI containing 0.1 mM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) per well was added to each well. Additional portions of selective medium were added on days 3, 6, and 10 after fusion. After 15 days, the remaining hybridomas were cultured in complete RPMI, supplemented with hypoxanthine and thymidine; at 28 days after fusion, the supernatants were screened for the first time for the presence of anti-*Legionella* antibodies. The clones were then progressively cultured in normal complete RPMI.

Six positive cultures were subcloned by limiting dilution (11), suspending the cells of one well to a final concentration of 10 cells per ml in 5 ml of complete medium. The cells were distributed at 0.05 ml/well, together with 0.3×10^6 splenocytes as feeder cells in 96 Nunclon microculture wells. After a week, wells containing single clones were selected, and 15 days later, the supernatants were tested for anti-*Legionella* antibodies.

Two subclones, issued from two different clones, were selected for amplification in BALB/c mice. Old BALB/c females were primed intraperitoneally with 0.3 ml of tetramethylpentadecane (Aldrich Chemical Co., Milwaukee, Wis.). After at least 4 days, 2×10^7 hybrid cells were injected. Finally, after at least 15 days, the first ascites were harvested and tested for anti-*Legionella* activity.

ELISA technique for screening of the anti-*Legionella* antibodies. Microwells on a NUNC-ELISA plate were coated with 0.05 ml of a polylysine (Sigma) solution (10 μg/ml). After 1 h, the wells were shaken dry, and 0.05 ml of a heat-killed or formolized bacterial suspension (2×10^9/ml) was allowed to evaporate at 50°C in each well. To avoid the nonspecific adsorption of proteins, we further saturated the wells with 10% horse serum in PBS or an egg white solution prepared as described previously (12) at room temperature for 1 h. The wells were then washed once in PBS containing 0.1% bovine serum albumin. The antisera, hybridoma supernatants, or ascitic fluids to be tested were diluted in PBS–0.1% bovine serum albumin, and 0.05-ml

portions were incubated in the wells for 1 h at 37°C. After three washings in PBS–0.1% bovine serum albumin, 0.05 ml of a sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (Pasteur Production, Paris, France) at a 1:500 dilution was added to each well. Blanks were prepared by incubating the conjugate in wells which had not been incubated previously with anti-*Legionella* antibodies or in wells incubated with mouse immunoglobulins or ascitic fluids nonspecific for *Legionella*. After 1 h of incubation with the conjugate, the wells were washed three times in PBS, and the substrate (2.5 mM H_2O_2 and 2 mM 2,2'-azino-di(3-ethyl-benzothiazolin-sulfonate [Sigma]) was added to a 0.1 M acetate-phosphate buffer (pH 6.5). After 30 min the optical density at 405 nm of each well was read in an ARTEK-scanner.

Indirect immunofluorescence. Bacterial suspensions of all of the serogroups were dried on microslides and fixed with acetone at room temperature. The slides were incubated with the ascites at serial dilutions, beginning with 1:8, for 1 h at room temperature. The antibodies were detected by a sheep anti-mouse immunoglobulin antibody labeled with fluorescein isothiocyanate at a 1:40 dilution (Pasteur Production).

RESULTS

Isolation of hybrid cell lines producing anti-*Legionella* antibodies. Using the ELISA, which was developed to detect anti-*Legionella* antibodies, we detected 28 positive wells of 85 which contained hybrids, with a criterion of three times the background (optical density, 0.05) to define a positive reaction (Fig. 1). Of these 28 wells, 10 were tested to verify the serospecificity. Although the 10 hybridoma supernatants displayed a strong positive reaction to serogroup I, they reacted significantly less with a mixture of serogroups I, II, and III and not at all with a mixture of serogroups IV, V, and VI or with the atypical strains NY-23 (ATCC 33279), Tatlock (ATCC 33218), or Wiga (ATCC 33217) (Fig. 2).

Before attempting to develop the production of monospecific anti-*Legionella* antibodies, we performed the subcloning of six hybrid cell cultures. Of the six cultures, only three gave stable hybrids after subcloning. Two of the subclones (II-6-18 and III-1-12) were put into ascites and resulted in the production of anti-*Legionella*-positive ascitic fluids.

Serological characterization of the two monoclonal antibodies obtained from the ascitic fluids. We tested the isotypes of both monoclonal antibodies by an indirect ELISA, using monospecific polyclonal rabbit antibodies against the different murine immunoglobulin isotypes. The ascitic fluid obtained after the injection of the II-6-18 subclone yielded gamma-3 antibodies, whereas that of the III-1-12 subclone yielded gamma-2b antibodies.

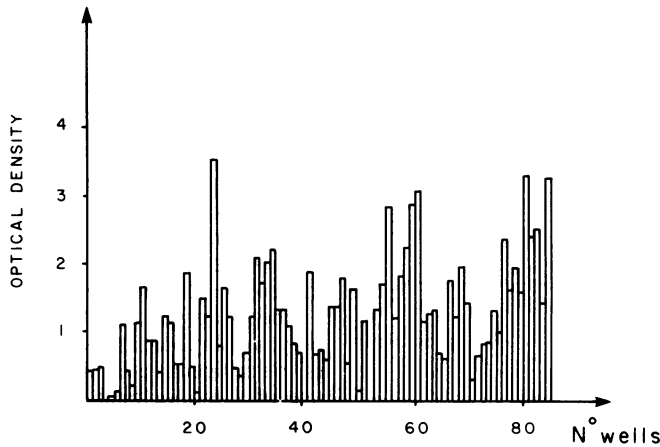Both antibodies were tested by ELISA and by indirect immunofluorescence against nine differ-

FIG. 1. Screening of the wells for positive anti-*Legionella* clones by the ELISA technique (y axis = 10× optical density). Wells were considered positive when the optical density was three times higher than background (optical density, 0.05).

ent *Legionella* strains. Only serogroup I was recognized by both tests. To check for interspecific cross-reactions, we performed an ELISA, using *Klebsiella pneumoniae* and *Mycoplasma pneumoniae* as antigens. No cross-reaction could be shown for either antibody.

Titration of the II-6-18 ascitic fluids for serodiagnostic purposes. To study the possible use of the anti-*Legionella* monoclonal antibody for immunodiagnosis, we performed titrations on the II-6-18 ascitic fluid. The minimal amount of bacteria giving a positive result by the ELISA was assessed. The amount of bacteria giving 50% of the maximal response was 2×10^6, and even amounts as small as 30,000 bacteria could be detected with significant accuracy, depending on the second conjugate-antibody (Fig. 3A). To verify that the sensitivity of the test was high enough to detect the antigen in clinical samples, we performed an ELISA on bronchoscopic samples from a patient giving a positive *L. pneumophila* serotype 1 culture. The sample was found

to be positive, whereas that of a patient without a detectable legionellosis (negative bacterial culture and serology) did not react with the antibody (Fig. 4).

The titration of the ascitic fluid upon 2×10^6 bacteria is illustrated in Fig. 3B. Fifty percent of the maximal response was obtained at a 1:1,000 dilution. The competition of the binding of the monoclonal anti-*Legionella* antibody at this concentration to the bacteria with a polyclonal rabbit antiserum resulted in a maximal 80% inhibition with a half-maximal response at a 1/64 rabbit antiserum dilution as detected by the ELISA technique.

DISCUSSION

The introduction of serodiagnostic tests to study the etiology of atypical pneumonias has shown that the major pathogen of this disease is *L. pneumophila* (9). It seemed interesting, therefore, to develop a standardized methodology for
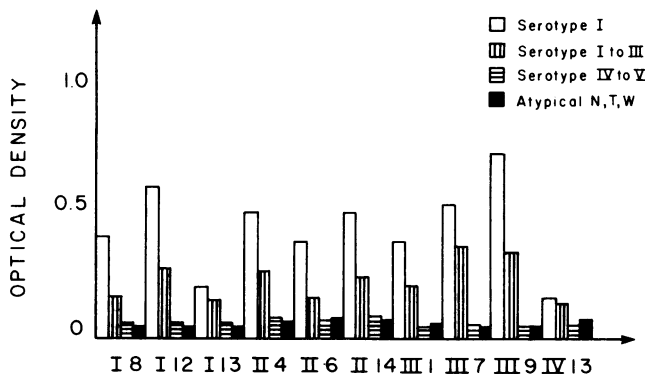

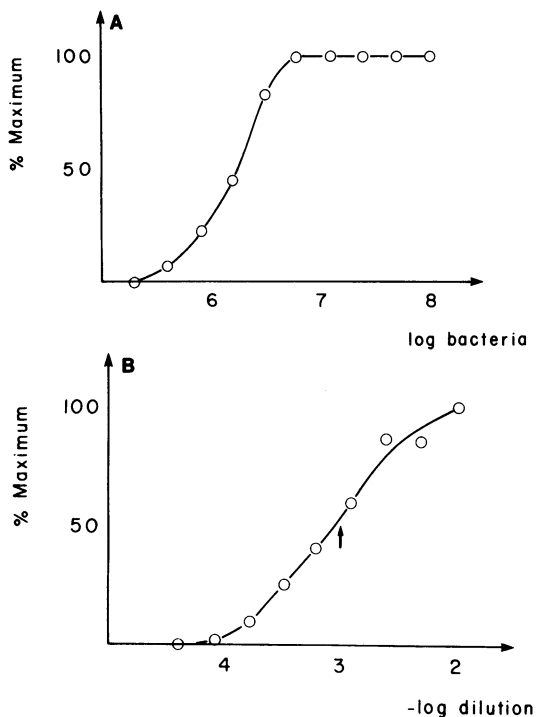FIG. 2. Serospecificity of the 10 most positive clones tested by the ELISA technique.

FIG. 3. Titration of the II-6-18 ascitic fluid. (A) Titration as a function of the number of bacteria. The dilution of the ascitic fluid used was 1:100. (B) Titration as a function of the ascitic fluid dilution. The number of bacteria used per well was 2×10^6.

a serodiagnosis which would be able to detect not only human anti-*Legionella* antibodies (occurring late in the infection) (4), but also minute amounts of the bacteria themselves, harvested from sputum or broncho-tracheal washings. The tool of choice for this standardized technique could be a monoclonal antibody with a constant, stable, and high affinity for one of the *Legionella* antigens.

To produce monoclonal anti-*Legionella* antibodies, we immunized BALB/c mice with serogroup I bacteria, since this strain is the most commonly found infectious agent in the human disease (10). In addition, we wished to investigate the possibility of finding an antibody directed against an antigen common to the species but one which would not cross-react with other bacteria. An immunization schedule was used to stimulate memory cells for antibody synthesis to avoid hybridomas producing IgM, which has been shown to be more labile than IgG. Therefore, we performed the 'fusion of the splenocytes from immunized mice, which were given an intravenous boost 3 days before fusion, 3 months after the priming immunizations. The high percentage of positive clones (28 of 85)

(Fig. 1) indicates that the mice responded well to the *Legionella* bacteria.

The most positive and stable hybridomas were tested for their serospecificity. None of the positive clones cross-reacted with any of the serogroups other than serogroup I. This confirms that, as in humans (10) or in rabbits (7), mice respond to a major or at least very immunogenic antigen, determining the serospecificity of the bacterial strain in all species. The fact, moreover, that the binding of the monoclonal antibody was practically totally inhibited by the polyclonal rabbit antiserum suggests the identity of the antigens recognized by both species. The use of a monoclonal serospecific antibody for the purification and characterization of that antigen should greatly advance our knowledge of its structure.

The use of a monoclonal antibody for serodiagnostic purposes requires an abundant supply of a stable antibody calibrated against the antigen. For this reason, we transferred the highest secreting subcloned hybridomas into pristane-primed BALB/c mice. Each mouse yielded 5 to 10 ml of ascites, which was tested for the isotype of the secreted antibody and for its activity. The two selected subclones secreted antibodies of the gamma-3 and the gamma-2b isotypes.

The serospecifity for serogroup I *L. pneumophila* was confirmed both by indirect immunofluorescence and by the ELISA technique. Moreover, no cross-reactions were found with two other lung pathogens, *M. pneumoniae* and *K. pneumoniae*, confirming the discriminatory potential of the monoclonal antibody in lung disease. The stability of the antibodies in the
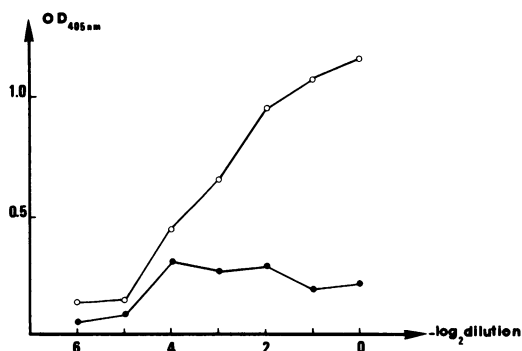

FIG. 4. Titration of bacterial antigen on bronchoscopic samples. The samples were dried at different dilutions in the wells of the microtiter plates. The plates were further processed as described in the text. The II-6-18 ascites was diluted at 1/100 for the detection. A sample yielding a positive culture (○) was compared with a sample from a patient found to be negative by the culture and indirect immunofluorescence of his serum (●). $OD_{405 nm}$, Optical density at 405 nm.

ascitic fluids was tested over several months. They were stored at $-20°C$, with frequent freezings and thawings, and no decrease in titer could be found. Since the titer of the gamma-3 isotypic monoclonal was at least 10 times higher than that of the gamma-2b isotypic monoclonal, we decided to test the former for diagnostic purposes.

The calibration of this antibody with the ELISA technique showed it to be sensitive to a minimal amount of bacteria (Fig. 3). The calibration of the antibody showed a half-maximum response at a dilution of 1:1,000. The sensitivity of the ELISA technique was high enough to detect the antigen in clinical samples. A comparison of the results obtained from the clinical sample with a calibration curve on intact bacteria showed that 0.05 ml contained the amount of antigen corresponding to 1.25×10^5 bacteria. This high amount suggests that the ELISA technique mainly reveals killed bacteria or shed antigen since the number of colonies obtained from this sample was very low.

To summarize, we have produced a monoclonal serotype I-specific anti-*Legionella* antibody in high quantities and with the qualities necessary for use in the direct quantitation of a specific bacterial antigen in clinical samples. This antigen seems to be identical to one inducing an immune response in other species. The monoclonal antibody described should, therefore, be suited for the diagnosis of legionellosis in the early stages of the disease, as well as for detection of the nature of the bacterial antigens responsible for the immune response.

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