

Immunologic Characterization and Specificity of Three Monoclonal Antibodies against the 58-Kilodalton Protein of *Legionella pneumophila*

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Three monoclonal antibodies against the *Legionella pneumophila* 58-kDa protein were produced. By using immunoblot analysis, the percentages of reactivity against 47 serogroups of *Legionella* representing 29 species were determined to be 80.9, 87.2, and 95.6 for monoclonal antibodies GB5BE8, GB5AF6, and CA4AF5, respectively. Specificities obtained from testing 63 heterologous organisms representing 22 genera and 46 species were 90.7, 92.2, and 95.3% for monoclonal antibodies GB5BE8, GB5AF6, and CA4AF5, respectively. No single heterologous strain was reactive with all three monoclonal antibodies. These monoclonal antibodies successfully identified all 10 clinical isolates of *Legionella* examined in a dot blot assay and should be excellent reagents for use in genuswide diagnostic immunoassays.

Diagnosis of legionellosis has become complex and cumbersome because of the increasing number of *Legionella* species and serogroups (presently 48 serogroups representing 30 species) (2). This complexity in diagnosis can be attributed to the use of serogroup-specific reagents in most diagnostic testing for legionellae. The use of genuswide detection reagents in diagnostic tests would simplify this process; however, to date, only one assay that uses this approach (DNA probe test; Gen-Probe, San Diego, Calif. [23]) has been developed. In an earlier report, we identified a 58-kDa protein (also referred to as the 60-kDa protein [13]) which reacted with convalescent-phase sera from 15 legionellosis patients with culture-confirmed *Legionella pneumophila* (16). Various investigators have cloned (6) and sequenced (15) the gene coding for this protein and have shown that it is a heat shock protein which serologically cross-reacts with the GroEL protein of *Escherichia coli* (8, 9) and with the 65-kDa antigen of *Mycobacterium tuberculosis* (17). Studies by Plikaytis et al. (13) showed that this protein, which they designated the 60-kDa protein (but which gene sequence data have shown to be 58 kDa [15]), possessed epitopes common to other bacterial genera as well as epitopes specific for the genus *Legionella*. With this in mind, the current study was undertaken to produce monoclonal antibodies (MAbs) to the *Legionella*-specific epitopes of the 58-kDa protein which could be utilized in diagnostic tests. Here, we report the characteristics and specificities of three different hybridomas that we produced.

Legionella strains were from the stock cultures of the Immunology Laboratory, Division of Bacterial Diseases, Centers for Disease Control. They were grown on buffered charcoal-yeast extract (BCYE) plates for 48 h at 37°C. Non-*Legionella* strains were obtained from specialty laboratories in the Division of Bacterial Diseases.

The 58-kDa protein used for immunization was purified from *L. pneumophila* Philadelphia 1 as previously described

(12), with the following exceptions. After the initial extraction and ammonium sulfate precipitation by the procedures of Pau et al. (12), the enriched protein preparation was separated on a Sepharose CL-4B column (Pharmacia, Inc., Piscataway, N.J.) equilibrated with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5–50 mM NaCl at a flow rate of 2 ml/min. Fractions were monitored at 280 nm, and selected peak fractions were collected, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Pharmacia Phast system (Pharmacia), and silver stained as described previously (13, 14) to detect the *Legionella* 58-kDa protein antigen. Once the antigen was detected, pooled fractions containing the protein were purified by high-performance liquid chromatography on a Mono Q HR 5/5 ion-exchange column (Pharmacia). This column was equilibrated with 20 mM HEPES, pH 7.5 (buffer A), and fractions were eluted at a flow rate of 0.5 ml/min with 20 mM HEPES, pH 7.5–1 M NaCl (buffer B). The following step gradient was used: 16% buffer B for 15 min, 25% buffer B for 15 min, 30% buffer B for 15 min, and finally 100% buffer B for 3 min. Peak fractions were collected and analyzed for the *Legionella* 58-kDa protein antigen by SDS-PAGE as described above.

MAbs were produced by the procedures of Kohler and Milstein (7) as modified by Zola and Brooks (24). BALB/c mice were initially immunized intraperitoneally with Freund's incomplete adjuvant with purified protein at a final concentration of 20 µg/ml in 0.01 M phosphate-buffered saline (PBS), pH 7.2. This was repeated in 6 weeks. One week later, each animal was given 10 µg of purified protein intravenously on two consecutive days; spleens were fused 3 days later. Sera from immunized mice and antibody from hybridized cells were screened for reactivity by immunoblot analysis of SDS-PAGE-separated proteins as described below, with a miniblitter apparatus (Immunetics, Cambridge, Mass.). Isotype determinations were made with the capture MonoAb-ID EIA kit (Zymed Laboratories, Inc., South San Francisco, Calif.) by the manufacturer's recommended procedure. Tissue culture supernatant fluids containing MAbs of known isotype were used as controls.

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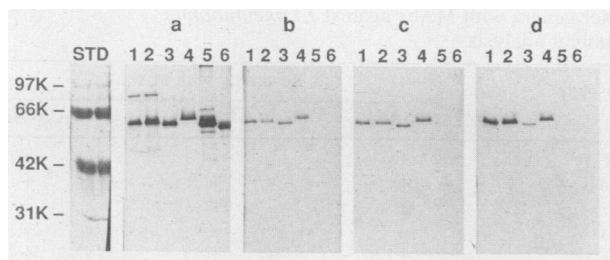


FIG. 1. Immunoblot analysis of polyclonal antibody and three MAbs with three species of *Legionella* and one species each of two heterologous genera. Blots were probed with polyclonal antiserum (13) (a) or monoclonal antibody GB5BE8 (b), GB5AF6 (c), or CA4AF5 (d). Lanes: STD, protein standards (in kilodaltons); 1, *L. pneumophila*, serogroup 1; 2, *L. pneumophila*, serogroup 2; 3, *L. bozemanii*, serogroup 1; 4, *L. micdadei*; 5, *B. pertussis*; 6, *P. fluorescens*. With the three MAbs, no reactivity with the species of the heterologous genera was seen, but all three species of the *Legionella* reacted.

The procedure for SDS-PAGE was basically as described by Tsang et al. (21), except that an 8% resolving gel with a 3% stacking gel was used. Sample preparation was done as described previously (14, 20), with each well receiving 4.8 μ g of protein (10). Immunoblots were done with the buffer system of Towbin et al. (19) as described previously (14). Transfer of polypeptide to nitrocellulose was performed at 90 V for 2 h. Sheets were washed three times in PBS containing 0.3% Tween-20 (PBS-TW) for 5 to 10 min, incubated overnight in the same solution, and blocked the next day for 30 min in PBS containing 20% fetal calf serum. After one wash in PBS-TW, the nitrocellulose sheets were incubated with MAbs diluted 1:10,000 in PBS-TW for 1 h, washed 3 times with PBS-TW, and then incubated for 1 h with peroxidase-labeled goat anti-mouse conjugate (Bio-Rad Laboratories, Richmond, Calif.) at a 1:2,000 dilution. After another set of three washes with PBS-TW, the sheets were developed with PBS containing 0.05% diaminobenzidine and 0.1% hydrogen peroxide. All reactions with visible bands at the 58-kDa position were scored as positive. Protein standards (Bio-Rad Laboratories) were used in all SDS-PAGE and immunoblotting experiments. Rabbit anti-standard sera (3) were used to visualize standard proteins on immunoblots.

Dot blot analysis was done as reported by Pau et al. (12). Cell lysates (protein concentration at 8 μ g per dot) were used since epitopes of the 58-kDa protein are not surface exposed (13). All incubations, buffers, and antibody and conjugate dilutions were the same as for the immunoblot described previously.

Ten hybridomas were expanded and stored. Three clones designated GB5BE8, GB5AF6, and CA4AF5 were chosen for further analyses because they were broadly reactive with *Legionella* species and because they did not react in immunoblot analysis (Fig. 1) with *Bordetella pertussis* and *Pseudomonas fluorescens*, two bacteria which show strong cross-reactivities with polyclonal *Legionella* anti-58-kDa serum (13) and often cross-react in serologic tests with *Legionella* reagents (1, 4, 11, 18). All three MAbs were immunoglobulin G1 isotype κ . The three clones have been deposited with the American Type Culture Collection under the above-mentioned designations.

By using immunoblot analysis, the three MAbs were further tested for reactivity against 47 *Legionella* strains representing 29 species and against 63 heterologous organ-

isms representing 22 genera and 46 species (Table 1). The results with legionellae can be summarized as follows. All three MAbs were strongly positive with *L. pneumophila*, serogroups 1 to 11; *L. bozemanii*, serogroup 1; *L. sainthenlensis*; *L. parisiensis*; *L. spiritensis*; *L. quinlivanii*; *L. moravica*; *L. brunensis*; *L. tucsonensis*; and *L. gratiana*. Reactivity with remaining *L. pneumophila* serogroups 12 to 14 and the other *Legionella* species was variable, but all of the *Legionella* strains reacted with at least one of the MAbs (Table 1). Two species (*L. hackeliae* and *L. erythra*) did not react strongly with any of the three MAbs but did react weakly with one or more of them. Specifically, MAb GB5BE8 reacted with 38 of 47 serogroups (80.9%), and MAb GB5AF6 reacted with 41 of 47 serogroups (87.2%). Both MAbs yielded similar patterns; however, MAb GB5AF6 showed greater reactivity. MAb CA4AF5 had the highest percentage of reactivity, giving positive results with 44 of 47 (96%) *Legionella* serogroups tested.

Results of immunoblots with the 63 heterologous organisms show that there was very little cross-reactivity (Table 1). Again, MAbs GB5BE8 (specificity, 90.7%) and GB5AF6 (specificity, 92.2%) showed similar patterns. Both MAbs cross-reacted with *Yersinia enterocolitica* and the four strains of *Campylobacter jejuni*. In addition, MAb GB5BE8 reacted with *Campylobacter fetus*. MAb CA4AF5 showed the greatest specificity (95.3%), cross-reacting with only 3 of the 64 heterologous organisms: *Haemophilus influenzae* (2 serogroups) and *Pseudomonas diminuta*. No single heterologous strain was reactive with all three MAbs. All cross-reactions were very weak except for those seen with *Y. enterocolitica*.

Dot blot analysis was used to evaluate the reactivity of cell lysates from 10 randomly selected clinical isolates of *Legionella* and 14 heterologous gram-negative organisms (Fig. 2). These included *H. influenzae* and *Y. enterocolitica*, which cross-reacted in immunoblot analysis, as well as several *B. pertussis* and *Pseudomonas* strains. Results showed that all the clinical isolates of *Legionella* reacted strongly with all three MAbs and that only one of the 14 heterologous organisms, *Y. enterocolitica*, cross-reacted. *Y. enterocolitica* showed weak reactivity in the dot blot with MAbs GB5BE8 and GB5AF6, which was not surprising since it also reacted with these same MAbs in immunoblot analysis.

Data presented here indicate that we have produced MAbs to the *Legionella* 58-kDa protein which react with most of the *Legionella* serogroups tested. One hundred percent reactivity was not demonstrated with any single MAb, but all the *Legionella* strains reacted with at least one of the MAbs. This indicates that if used together in a pool, the MAbs have the capability of detecting all *Legionella* serogroups, which increases the sensitivity to 100% and lowers the specificity to 86%. Moreover, if the pool contained only GB5BE8 and CA4AF5, the *C. fetus* cross-reactivity would be eliminated. This would increase the specificity to 87.5% and still maintain 100% sensitivity. The reactivity patterns of the three MAbs were variable, which may indicate structural differences in epitopes with which each MAb reacts. The reactivities of the *Legionella* strains also varied with each of the MAbs. Similar results have been shown for MAbs to the 65-kDa common antigen in *M. tuberculosis* (17) and confirm the heterogeneity of the 58-kDa common antigen not only among gram-negative organisms, as was previously shown (5, 13), but within the genus *Legionella* itself.

Specificity was another consideration when clones were screened and chosen for antibody production. Other investigations have implicated the genera *Bordetella* and *Pseudo-*

TABLE 1. Reactivity of legionellae and members of other genera with MAbs against *L. pneumophila* 58-kDa protein by immunoblot analysis

Bacterium	Serogroup	Strain or isolate no.	Reactivity with MAb		
			GB5BE8	GB5AF6	CA4AF5
<i>Legionella anisa</i>	1	WA-316-C3	+	+	+
<i>Legionella birminghamensis</i>	1	1407-AL-H	+	+	+
<i>Legionella bozemanii</i>	1	WIGA	+	+	+
<i>Legionella bozemanii</i>	2	Toronto 3	-	+	+
<i>Legionella brunensis</i>		411-1	+	+	+
<i>Legionella cherri</i>	1	ORW	+	+	+
<i>Legionella cincinnatiensis</i>	1	72-OH-H	+	+	+
<i>Legionella dumoffii</i>	1	NY-23	+	+	-
<i>Legionella erythra</i>	1	SE-32A-C8	+	+	-
<i>Legionella feeleii</i>	1	WO-44C-C3	-	-	+
<i>Legionella feeleii</i>	2	691-W1-H	-	-	+
<i>Legionella gormanii</i>	1	LS-13	+	+	+
<i>Legionella gratiana</i>		Lyon 8420412	+	+	+
<i>Legionella hackeliae</i>	1	Lansing 2	-	-	+
<i>Legionella hackeliae</i>	2	798-PA-H	-	-	+
<i>Legionella israelensis</i>	1	Bercovier 4	-	-	+
<i>Legionella jamestowniensis</i>	1	JA-26-G1-E2	+	+	+
<i>Legionella jordanis</i>	1	BL-540	+	+	+
<i>Legionella longbeachae</i>	1	Long Beach 4	+	+	+
<i>Legionella longbeachae</i>	2	Tucker 1	+	+	+
<i>Legionella maceachernii</i>	1	PX-1-G2-E2	+	+	+
<i>Legionella micdadei</i>	1	TATLOCK	+	+	+
<i>Legionella moravica</i>		316-36	+	+	+
<i>Legionella oakridgensis</i>	1	Oak Ridge 10	-	-	+
<i>Legionella parisiensis</i>	1	PF-209C-C2	+	+	+
<i>Legionella pneumophila</i>	1	Philadelphia 1	+	+	+
<i>Legionella pneumophila</i>	2	Togus 1	+	+	+
<i>Legionella pneumophila</i>	3	Bloomington 2	+	+	+
<i>Legionella pneumophila</i>	4	Portland 1	+	+	+
<i>Legionella pneumophila</i>	5	Cambridge 2	+	+	+
<i>Legionella pneumophila</i>	6	Chicago 2	+	+	+
<i>Legionella pneumophila</i>	7	Chicago 8	+	+	+
<i>Legionella pneumophila</i>	8	Concord 3	+	+	+
<i>Legionella pneumophila</i>	9	In-23-G2-C2	+	+	+
<i>Legionella pneumophila</i>	10	Leiden 1	+	+	+
<i>Legionella pneumophila</i>	11	797-PA-H	+	+	+
<i>Legionella pneumophila</i>	12	570-CO-H	-	+	+
<i>Legionella pneumophila</i>	13	82A3105	+	+	+
<i>Legionella pneumophila</i>	14	1169-MN-H	+	+	+
<i>Legionella quinlivanii</i>		1442-AUS-E	+	+	+
<i>Legionella rubrilucens</i>	1	WA-270A-C2	+	+	+
<i>Legionella sainthelensi</i>	1	Mt.St.Helen 4	+	+	+
<i>Legionella santicrucis</i>	1	SC-63C7	-	+	+
<i>Legionella spiritensis</i>	1	Mt.St.Helen 9	+	+	+
<i>Legionella steigerwaltii</i>	1	SC-18-C9	+	+	+
<i>Legionella tucsonensis</i>		1087-A2-H	+	+	+
<i>Legionella wadsworthii</i>	1	81-716	+	+	-
<i>Acinetobacter lwoffii</i>		mima	-	-	-
<i>Alcaligenes faecalis</i>			-	-	-
<i>Bordetella bronchiseptica</i>		F6286	-	-	-
<i>Bordetella bronchiseptica</i>		F6287	-	-	-
<i>Bordetella parapertussis</i>		E1142	-	-	-
<i>Bordetella pertussis</i>		F6324	-	-	-
<i>Bordetella pertussis</i>		F6323	-	-	-
<i>Campylobacter fetus</i>		D223	-	-	-
<i>Campylobacter fetus</i>		D373	-	+	-
<i>Campylobacter fetus</i>		D406	-	-	-
<i>Campylobacter fetus</i>		D411	-	-	-
<i>Campylobacter jejuni</i>		D133	+	+	-
<i>Campylobacter jejuni</i>		L1	+	+	-
<i>Campylobacter jejuni</i>		L2	+	+	-
<i>Campylobacter jejuni</i>		L9	+	+	-
<i>Clostridium difficile</i>		880-221	-	-	-
<i>Clostridium perfringens</i>		860386	-	-	-

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TABLE 1—Continued

Bacterium	Serogroup	Strain or isolate no.	Reactivity with MAb		
			GB5BE8	GB5AF6	CA4AF5
<i>Clostridium septicum</i>		70426	—	—	—
<i>Enterobacter aerogenes</i>		1942-81	—	—	—
<i>Escherichia coli</i>		16	—	—	—
<i>Escherichia coli</i>		013	—	—	—
<i>Escherichia fergusonii</i>		1295-83	—	—	—
<i>Escherichia hermannii</i>		460-84	—	—	—
<i>Flavobacterium meningosepticum</i>		698	—	—	—
<i>Haemophilus influenzae</i> type a		KC818	—	—	—
<i>Haemophilus influenzae</i> type b		1179-85	—	—	+
<i>Haemophilus influenzae</i> type e		KC528	—	—	+
<i>Klebsiella ascorbata</i>		426-84	—	—	—
<i>Klebsiella oxytoca</i>		4698-84	—	—	—
<i>Klebsiella pneumoniae</i>		4809-84	—	—	—
<i>Listeria innocua</i>		KC1783	—	—	—
<i>Listeria innocua</i>		KC1784	—	—	—
<i>Listeria monocytogenes</i>		KC1775	—	—	—
<i>Listeria monocytogenes</i>		Kc2380	—	—	—
<i>Neisseria meningitidis</i>		KC792	—	—	—
<i>Providencia rettgeri</i>		5317-81	—	—	—
<i>Providencia stuartii</i>		4007-83	—	—	—
<i>Pseudomonas acidovorans</i>		KC1769	—	—	—
<i>Pseudomonas aeruginosa</i>		5	—	—	—
<i>Pseudomonas aeruginosa</i>		2	—	—	—
<i>Pseudomonas aeruginosa</i>		8	—	—	—
<i>Pseudomonas alcaligenes</i>		ABB50	—	—	—
<i>Pseudomonas cepacia</i>		KC1766	—	—	—
<i>Pseudomonas diminuta</i>		KC1797	—	—	+
<i>Pseudomonas fluorescens</i>		CDC93	—	—	—
<i>Pseudomonas fluorescens</i>		E.B.	—	—	—
<i>Pseudomonas maltophilia</i>		KC1768	—	—	—
<i>Pseudomonas paucimobilis</i>		B3271	—	—	—
<i>Pseudomonas testosteroni</i>		KC1765	—	—	—
<i>Salmonella typhimurium</i>		2489-88	—	—	—
<i>Serratia marcescens</i>		4391-83	—	—	—
<i>Shigella flexneri</i>		37	—	—	—
<i>Shigella sonnei</i>		85	—	—	—
<i>Staphylococcus aureus</i>		42BP	—	—	—
<i>Streptococcus pneumoniae</i>		Pn-1	—	—	—
<i>Streptococcus pyogenes</i>		SS482	—	—	—
<i>Streptococcus pyogenes</i>		SS91	—	—	—
<i>Streptococcus salivarius</i>		SS908	—	—	—
<i>Streptococcus salivarius</i>		SS1062	—	—	—
<i>Vibrio cholerae</i>		01 Inata	—	—	—
<i>Yersinia enterocolitica</i>		1149-84	+	+	—
<i>Yersinia pseudotuberculosis</i>		514-84	—	—	—

monas particularly as sources of cross-reactivity that could lead to misinterpretation of diagnostic tests (1, 4, 11, 18). The genus *Bordetella* could especially be a problem since the conditions for its growth are similar to those for the genus *Legionella* (grows on BCYE but not on BCYE without cysteine and grows poorly or not at all on blood agar). To circumvent the possibility of cross-reactivity with members of these genera, MAbs which did not react with *B. pertussis* and *P. fluorescens* were selected. As a result, the MAbs selected were highly specific, cross-reacting only with organisms that are easily discriminated from legionellae on the bases of growth characteristics and morphology. More important, the cross-reactions that occurred were very weak (especially those with *Campylobacter* strains) and most likely can be eliminated by adjusting the dilutions of test reagents. Therefore, the specificities reported here (91, 92, and 95% for GB5BE8, GB5AF6, and CA4AF5, respectively) are exceptionally good, particularly in light of the highly

conserved sequence of this protein among other bacteria (17, 22).

Our studies show that these MAbs have genuswide specificity and sensitivity. Despite the fact that these levels of sensitivity and specificity cannot be directly extrapolated to what may occur in any clinical assay without extensive testing within the assay system chosen, they are nevertheless promising. The individual MAb reactivity profiles suggest that sensitivity levels can be increased to 100% through utilizing the MAbs in a pool. Results from the dot blot assay indicate a possible use in culture identification if further evaluation substantiates the data shown here. Other potential uses for these MAbs that one might investigate include their use in antigen detection and as components in a latex agglutination assay for rapid genus identification. Finally, these MAbs could be used to identify *Legionella*-specific portions of the 58-kDa protein, which could lead to the development of a polymerase chain reaction assay or to the

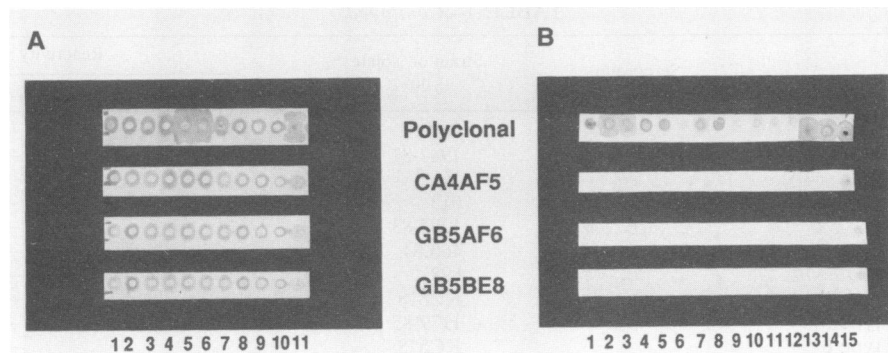


FIG. 2. Dot blot analysis of clinical cultures and cultures that cross-reacted in immunoblot analysis. (A) Reactivity of clinical isolates with MAbs GB5BE8, GB5AF6, and CA4AF5 and with polyclonal anti-58-kDa protein sera. Numbers represent clinical isolates of *Legionella* species as follows: 1, *L. feeleii*, serogroup 1; 2, *L. gormanii*; 3, *L. jordanis*; 4, *L. pneumophila*, serogroup 6; 5, *L. pneumophila*, serogroup 1, D2397; 6, *L. pneumophila*, serogroup 1, D2434; 7, *L. sainthelensi*; 8, *L. anisa*; 9, *L. micdadei*; 10, *L. tucsonensis*; 11, *L. pneumophila*, serogroup 1, Philadelphia 1 (positive control). (B) Reactivity of selected heterologous organisms with the three MAbs. Numbers represent isolates as follows: 1, *P. aeruginosa*; 2 and 3, *B. bronchisepta*, F6286 and F6287, respectively; 4, *P. diminuta*; 5, *P. cepacia*; 6, *H. influenzae* a; 7, *P. fluorescens*; 8, *Y. enterocolitica*; 9 and 10, *H. influenzae* e and b, respectively; 11 and 12, *P. aeruginosa* 2 and 8, respectively; 13 and 14, *B. pertussis* F6324 and F6323, respectively; and 15, *L. pneumophila*, serogroup 1.

production of *Legionella*-specific peptides for use in diagnostic immunoassays.

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