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Monoclonal Antibodies to *Legionella* Mip Proteins Recognize Genus- and Species-Specific Epitopes

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Monoclonal antibodies (MAbs) against the virulence-associated Mip protein of *Legionella* spp. were raised by immunizing BALB/c mice with (i) *Legionella pneumophila*, (ii) *Legionella micdadei*, and (iii) purified recombinant native Mip protein cloned from *L. pneumophila* Philadelphia 1. Following screening of seeded wells by immunoblot analysis with homologous antigens, eight Mip-specific MAbs were found. These MAbs were chosen to investigate the antigenic diversity of Mip proteins in the genus *Legionella*. Mip was detected in 82 *Legionella* strains representing all 34 species tested. One of these MAbs, obtained from immunization with *L. micdadei*, recognized an epitope common to all *Legionella* species tested by immunoblot analysis. Another MAb was discovered to be specific for the Mip protein of *L. pneumophila*. The remaining six MAbs recognized 18 to 79% of *Legionella* species included in this study. By making use of the MAbs introduced in this study, it could be shown that, based on Mip protein epitope expression, *Legionella* species can be divided into at least six antigenetically distinct groups. As demonstrated by 43 *L. pneumophila* strains representing all serogroups, no antigenic diversity of Mip proteins was found for this species. In addition, 18 non-*Legionella* species, including *Chlamydia trachomatis*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*, all of which are known to carry genes homologous to the *Legionella mip* genes, were reacted against all eight MAbs. No cross-reactivity was detectable in any of those strains.

Members of the genus *Legionella* normally inhabit natural aquatic environments and are capable of proliferating in freshwater protozoa (11, 23). When legionellae are inhaled in aerosol form by humans, the bacteria are able to survive and multiply in alveolar macrophages, causing severe pneumonia, termed Legionnaires' disease. Currently, the genus *Legionella* is known to include 39 species and multiple serogroups (8). It is speculated that the number of variants will increase in the future (9). Because of the high number of species and serotypes, it is desirable to set up a system for the differentiation of strains which starts at the genus level and specifies the diagnosis to the species level.

It has recently been shown that Mip (macrophage infectivity potentiator) and Mip-like proteins are produced by 29 of 30 *Legionella* species tested (3). The Mip protein is a surface-exposed protein necessary for full virulence of *Legionella pneumophila* (4, 6). Interestingly, Mip belongs to the substance class of immunophilins exhibiting peptidyl-prolyl-*cis-trans*-isomerase activity (12, 14). Mip molecules from different *L. pneumophila* isolates represent identical or nearly identical proteins of 24 kDa (18). The Mip-like proteins produced by species other than *L. pneumophila* vary in size from approximately 24 to 31 kDa, arguing for structural differences among this group of proteins (3). The heterogeneity in size is corroborated by the fact that the amino acid sequences of the Mip proteins of *L. pneumophila* and *Legionella micdadei* have only 71% homology (1). It is therefore suggested that a new and efficient system for the detection and differentiation of *Legionella* strains could be based on a pattern of reactivity with monoclonal antibodies (MAbs) raised against Mip proteins. As an initial approach, we established hybridoma cells to produce eight Mip-specific MAbs. It could be demonstrated that one antibody is able to recognize a Mip-specific epitope common to all *Legionella* species, while another MAb is specific for *L. pneumophila*. Different reaction patterns for individual MAbs may reflect the antigenic diversity of these virulence-associated proteins among members of the genus *Legionella*.

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MATERIALS AND METHODS

Strains. The strains used in this study are presented in Table 1. In addition to 57 *Legionella* reference strains representing 34 species and 53 serogroups, the following 25 *L. pneumophila* strains were included: (i) 10 patient isolates, (ii) 11 water isolates not associated with cases of legionellosis, and (iii) *L. pneumophila* strains which were previously tested for their ability to multiply intracellularly in U937 and *Acanthamoeba castellanii* cells at 37°C and to infect guinea pigs (2, 21). The *L. pneumophila* strains RIGP and Corby were able to multiply in U937 cells and protozoa and were infective for guinea pigs, whereas the *L. pneumophila* strains MH XXXV and U21S6 failed to grow intracellularly or to be infective for guinea pigs. *Legionella* strains were grown on buffered charcoal yeast extract (BCYE) agar for 48 to 72 h at 37°C (10). In addition, 18 organisms belonging to genera other than *Legionella* were tested.

Cloning and expression of the *Legionella* Mip proteins in *Escherichia coli*. Cloning of the Mip protein of *L. pneumophila* Philadelphia 1 was performed as described previously (12). The recombinant native Mip protein of Philadelphia 1 was purified from the *E. coli* clone HB101(pBLL106) as described before (12) and used for immunization of BALB/c mice. The *E. coli* clone JM109(pBA6004), which expressed the Mip protein of *L. micdadei* Tatlock, was generously provided by J. Bangsborg, Statens Seruminstitut, Copenhagen, Denmark.

MAbs. BALB/c mice were immunized by the method of Cianfriglia et al. (7) with living cells of either (i) *L. pneumophila* Jena 1 (water isolate), (ii) *L. pneumophila* W334 (serogroup 1, monoclonal subtype Bellingham; water isolate), (iii) *L. pneumophila* strain 421/88 (serogroup 3, patient isolate), (iv) *L. micdadei* ATCC 32218, or (v) purified recombinant native Mip protein cloned from *L. pneumophila* Philadelphia 1 (Table 2). Cellular fusions were carried out with P3X63-Ag8/653 mouse myeloma cells as described elsewhere (22). Supernatants of hybridoma cultures were screened for Mip-specific antibodies by immunoblot analysis. The antigens used were identical to those used for immunization. In cases of positive reactions, hybridoma cells were subcloned three

TABLE 1. Bacterial strains used

Strain (no. tested)	Strain no. or source[a] (no. tested) [reference]
Legionella spp.	
L. adelaidensis	ATCC 49625
L. anisa	ATCC 35292
L. birminghamensis	ATCC 43702
L. bozemanii	
SG 1	ATCC 33217
SG 2	ATCC 35545
L. brunensis	ATCC 43878
L. cherrii SG 1	ATCC 35252
L. cincinnatiensis	ATCC 43753
L. dumoffii	ATCC 33279
L. erythra	ATCC 35303
L. fairfieldensis	ATCC 49588
L. feeleii	
SG 1	ATCC 35072
SG 2	ATCC 35849
L. gormanii	ATCC 33297
L. gratiana	ATCC 49413
L. hackeliae	
SG 1	ATCC 35250
SG 2	ATCC 35999
L. israelensis	ATCC 43119
L. jamestowniensis	ATCC 35298
L. jordanis	ATCC 33623
L. lansingensis	ATCC 49751
L. longbeacheae	
SG 1	ATCC 33462
SG 2	ATCC 33484
L. maceachernii	ATCC 35300
L. micdadei	ATCC 33218
L. moravica	ATCC 43877
L. oakridgensis	ATCC 33761
L. parisiensis	ATCC 35299
L. pneumophila	
SG1 (20) strains	ATCC 33152, ATCC 43109, ATCC 43111, NCTC 11191, IMHD patient isolates (5), IMHD water isolates (8), RIGP (Philadelphia 1, virulent [2, 21]), MH XXXV (Philadelphia 1, avirulent [2, 21]), Corby (human isolate [16])
SG 2	ATCC 33154
SG 3 (4)	ATCC 33155, IMHD patient isolates (3)
SG 4 (2)	ATCC 33156, IMHD patient isolate
SG 5 (4)	ATCC 33216, NCTC 11417, IMHD patient isolates (2)
SG 6 (2)	ATCC 33215, U21S2 [2, 21]
SG 7	ATCC 33823
SG 8	ATCC 35096
SG 9	ATCC 35289
SG 10	ATCC 43283
SG 11	ATCC 43130
SG 12	ATCC 43290
SG 13	ATCC 43736
SG 14	ATCC 43703
Serotype Lansing 3	ATCC 35251
Jena 1[b]	
L. quinlivanii	ATCC 43830
L. rubrilucens	ATCC 35304
L. sainthelansi	
SG 1	ATCC 35248
SG 2	ATCC 43830
L. santicrucis	ATCC 35301
L. shakesspearei	ATCC 49655
L. steigerwaltii	ATCC 35302
L. tucsonensis	ATCC 49180
L. wadsworthii	ATCC 33877
Other species	
Chlamydia pneumoniae	TW-183 [13]
Chlamydia trachomatis	ATCC VR 902 B
Citrobacter diversus	IMIW
Enterobacter aerogenes	IMIW
Escherichia coli (5)	IMHD
Erwinia spp. (3)	IMIW
Hafnia alvei	IMIW
Klebsiella pneumoniae	IMIW
Neisseria meningitidis	IMHD
Proteus mirabilis	IMIW
Pseudomonas aeruginosa	
(2) strains	ATCC 49266, ATCC 49267
Pseudomonas fluorescens	
(2) strains	ATCC 49270, ATCC 49271
Saccharomyces cerevisiae	IMHD
Salmonella typhimurium	IMIW
Serratia spp.	IMIW
Shigella flexneri	IMIW
Shigella sonnei	IMIW
Yersinia pseudotuberculosis	IMIW
Escherichia coli K-12	
HB101(pBLL106)	IMIW [12]
JM109(pBA6004)	J. Bangsborg, Statens Seruminstitut [1]

[a] Abbreviations: IMHD, Institute of Medical Microbiology and Hygiene, University Hospital, Dresden, Germany; IMIW, Institute of Molecular Biology of Infections, University Würzburg, Würzburg, Germany; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, United Kingdom; SG, serogroup.

[b] Not typeable to serogroups 1 to 14 or serotype Lansing 3, as tested with absorbed rabbit hyperimmune sera.

times. Isotype determination of MAbs was made by ImmunoType (Sigma, St. Louis, Mo.). The concentration of mouse immunoglobulin G (IgG) in culture supernatants used for immunoblot analysis and enzyme-linked immunosorbent-assay (ELISA) was determinated by a mouse IgG ELISA (Boehringer, Mannheim, Germany).

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on slab gels was performed by the method of Laemmli (16) with 12.5% polyacrylamide gels. Bacteria grown on agar

TABLE 2. MAbs against *Legionella* Mip proteins: immunogen, antibody isotype, and reactivity with recombinant Mip proteins

Immunogen (strain)	MAb	Isotype	Reactivity[a] with recombinant Mip proteins from:	
			L. pneumophila Philadelphia 1	*L. micdadei* Tatlock
L. pneumophila (Jena 1)[b]	18/1	IgM	+	0
L. pneumophila SG 1 (water isolate)	20/2	IgG_1	+	0
	20/5	IgG_1	+	0
L. pneumophila SG 3 (patient isolate)	29/2	IgG_1	+	0
L. micdadei (ATCC 32218)	22/1	IgG_1	+	+
	23/1	IgG_1	+	+
	23/3	IgG_{2a}	0	+
Purified recombinant Mip protein from *L. pneumophila* Philadelphia 1	2D8	IgG_1	+	0

[a] +, positive reaction; 0, no reaction.

[b] Not typeable to serogroups 1 to 14 or serotype Lansing, as tested by absorbed rabbit sera.

plates were washed twice with distilled water and adjusted to a concentration of 5×10^{10} cells per ml by nephelometry with the McFarland standard as a reference. After centrifugation at 15,000 × *g* for 3 min, the pellet was suspended in sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 5% β-mercaptoethanol, 2% SDS, 12.5% glycerol) at the same concentration. After boiling for 10 min, samples of 4 μl (equivalent to approx. 2×10^8 cells) were applied to each lane of the gel. For estimation of molecular weight, the LMW Calibration Kit MW 14.4-94 kDa (Pharmacia LKB, Piscataway, N.J.) was used. After electrophoresis, the antigens were transferred to nitrocellulose (0.45 μm; Schleicher & Schuell, Dassel, Germany) by the semidry electroblotting procedure at 0.8 mA/cm^2 for 90 min. The nitrocellulose sheets were saturated overnight with 10% fetal calf serum (FCS) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T-FCS). The MAbs were applied as culture supernatant diluted in PBS-T-FCS to a concentration of 50 ng of IgG per ml. For MAb 18/1, an IgM antibody, a dilution of 1:100 was chosen. The incubation times were 90 min for both MAbs and biotinylated anti-mouse Ig (Amersham, Braunschweig, Germany). After reaction with streptavidin-biotinylated horseradish peroxidase (HRPO) complex (Amersham) for 15 min, the blot was finished by adding a solution of the chromogen 4-chloro-1-naphthol (Serva, Heidelberg, Germany) and H_2O_2.

ELISA. Immuno-Plates (Greiner, Frickenhausen, Germany) were coated with living bacteria (McFarland no. 1, corresponding to approx. 3×10^8 cells per ml) in 0.05 M carbonate buffer (pH 9.5) overnight at 4°C. After being washed three times with PBS containing 0.05% Tween 20 (PBS-T), the wells were blocked with PBS-T containing 10% FCS (PBS-T-FCS) for 1 h at 37°C. The plates were washed once, and MAbs were added as culture supernatant diluted in PBS-T-FCS to a concentration of 50 ng of IgG per ml. A dilution of 1:100 was chosen for MAb 18/1 (isotype IgM). After incubation for 90 min at 37°C and three washes, the bound MAbs were detected by anti-mouse Ig (polyvalent)-HRPO (Sigma) diluted in PBS-T-FCS. The plates were incubated for 90 min at 37°C and washed three times. The substrate solution is 0.1 M phosphate-citrate buffer (pH 5.0) containing *o*-phenylenediamine (1 mg/ml) and H_2O_2 (0.003%). The optical densities at 492 nm (OD_{492}) of blanks (without antigen for coating and without MAb, respectively) did not exceed 0.075. An OD_{492} of ≥0.15 was judged to be a positive result.

RESULTS

Characterization of MAbs recognizing epitopes on recombinant *Legionella* Mip proteins. From the five cellular fusion experiments with spleen cells of mice immunized with living bacteria of *L. pneumophila*, *L. micdadei*, and purified native Mip protein of *L. pneumophila* Philadelphia 1, eight hybridomas secreting MAbs against Mip proteins were discovered. The Mip specificity of the MAbs was documented by immunoblot analysis. For this, whole-cell lysates of recombinant *E. coli* HB101(pBLL106) and JM109(pBA6004), which expressed the Mip proteins of *L. pneumophila* Philadelphia 1 and *L. micdadei* Tatlock, respectively, were used (Table 2). With the exception of MAb 23/3, all the MAbs recognized Mip epitopes expressed on recombinant Mip protein derived from *L. pneumophila*. MAbs 22/1 and 23/1 identified epitopes located on both recombinant Mip proteins tested, whereas MAb 23/3 recognized only the *L. micdadei* epitope. The controls with the vectors in *E. coli* HB101 and JM109 did not react with any of the MAbs in the ELISA or in the immunoblot analysis. MAb 18/1 was an IgM, MAb 23/3 was an IgG_{2a} type, and the remaining MAbs belonged to the IgG_1 class.

Detection of a genus- and a species-specific MAb. The eight MAbs recognizing the Mip proteins of the homologous strains were used to detect the Mip proteins in 82 *Legionella* strains representing 34 species. As demonstrated by immunoblot analysis (Fig. 1A and Table 3), MAb 22/1 recognized a Mip epitope common to all 34 *Legionella* species. These data argue for the presence of a genus-specific epitope in *Legionella* Mip proteins. With MAb 22/1 in the ELISA, the majority of *Legionella* species were shown to exhibit a strong positive reaction, indicated by ODs ranging from 1.0 to 2.2. However, the reactivity observed in the ELISA was weaker for *L. cherrii*, *L. gormanii*, *L. hackeliae*, *L. quinlivanii*, *L. wadsworthii*, and *L. tucsonensis* (see Table 4).

In contrast to MAb 22/1, MAb 20/2 reacted specifically with *L. pneumophila* strains in both serological assays (Fig. 1B and Table 3). In total, 43 *L. pneumophila* strains representing 15 serogroups, including serotype Lansing 3, were used in this study. Because Mip is a virulence-associated protein, the following strains were selected from the *L. pneumophila* serogroup type strains: (i) 10 patient isolates, (ii) 11 water isolates not associated with legionellosis cases, (iii) 2 strains capable of multiplying within U937 and protozoa cells as well as of infecting guinea pigs, and (iv) 2 strains not able to grow intracellularly or to infect guinea pigs. MAb 20/2 was able to recognize the Mip proteins of all 43 *L. pneumophila* strains but failed to recognize Mip-like proteins of strains other than *L. pneumophila*.

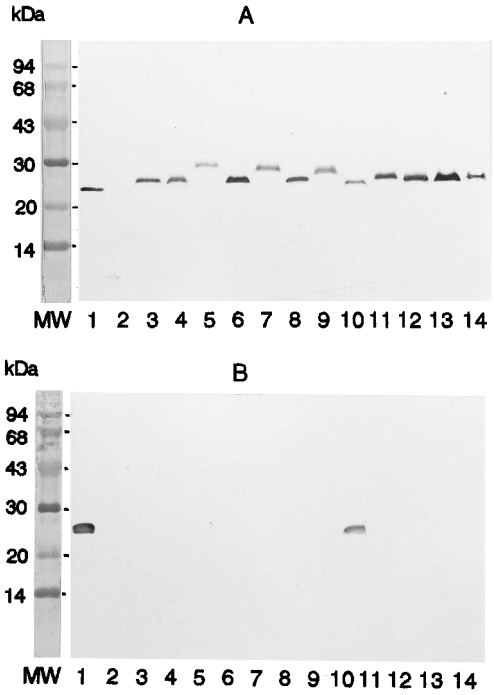

FIG. 1. Immunoblot analysis of recombinant *E. coli* HB101(pBLL106) and *Legionella* strains with (A) genus-specific MAb 22/1 and (B) *L. pneumophila*-specific MAb 20/2. Bacterial antigens were separated by SDS-PAGE and transferred to nitrocellulose. Lanes: MW, molecular mass standards (LMW-Kit; Pharmacia LKB); 1, *E. coli* HB101(pBLL106) expressing the Mip protein of *L. pneumophila* serogroup 1; 2, *E. coli* HB101(pBR322), not expressing the Mip protein; 3, *L. birminghamensis*; 4, *L. bozemanii* (serogroup 1); 5, *L. brunensis*; 6, *L. dumoffii*; 7, *L. jordanis*; 8, *L. longbeacheae* (serogroup 1); 9, *L. micdadei*; 10, *L. pneumophila* (serogroup 1, strain ATCC 43109); 11, *L. rubrilucens*; 12, *L. santicrucis*; 13, *L. shakespearei*; 14, *L. steigerwaltii*. In addition, 22 other *Legionella* spp. reacted with MAb 22/1, whereas no reactivity was found with MAb 20/2 (data not shown).

Characterization of MAbs by immunoblot analysis. In order to demonstrate the antigenic diversity of *Legionella* Mip proteins, MAbs were used to determine the reactivity with 34 *Legionella* species by immunoblot analysis. Based on their reactivities, the *Legionella* species fell into at least six antigenically distinct groups (Table 3). For MAbs 2D8 and 18/1 as well as for MAbs 29/2 and 20/5, the same reaction patterns were observed. As demonstrated for *L. pneumophila* and other species (*L. bozemanii*, *L. feeleii*, *L. hackeliae*, *L. longbeacheae*, and *L. sainthelensi*), various serogroups of the same species exhibited identical MAb reaction patterns.

The molecular masses of Mip proteins detected by our MAbs were in the range from 24 to 31 kDa. According to Cianciotto et al. (3), the highest molecular masses were found for *L. hackeliae*, *L. micdadei*, *L. jordanis*, and *L. jamestowni-*

TABLE 3. Reaction types of MAbs against *Legionella* Mip proteins demonstrated by immunoblot analysis

Type	Reactivity of MAb[a]						*Legionella* spp.[b]
	22/1	23/1	2D8, 18/1	23/3	20/5, 29/2	20/2	
A	+	+	+	0	+	+	*L. pneumophila*[c]
B	+	+	+	0	+	0	*L. cincinnatiensis*, *L. dumoffi*, *L. fairfieldensis*, *L. gormanii*, *L. moravica*, *L. rubrilucens*
C	+	+	+	0	0	0	*L. bozemanii*,* *L. longbeachae*,* *L. parisiensis*, *L. sainthelensi*,* *L. santicrucis*, *L. shakespearei**, *L. steigerwaltii*
D	+	+	0	+	0	0	*L. cherrii*, *L. feeleii*,* *L. maceachernii*, *L. micdadei*, *L. spiritensis*
E	+	+	0	0	0	0	*L. anisa*, *L. erythra*, *L. israelensis*, *L. jamestowniensis*, *L. jordanis*, *L. oakridgensis*, *L. wadsworthii*
F	+	0	0	0	0	0	*L. adelaidensis*, *L. birminghamensis*, *L. brunensis*, *L. gratiana*, *L. hackeliae*,* *L. lansingensis*, *L. quinlivanii*, *L. tucsonensis*

[a] MAbs 2D8 and 18/1 and MAbs 20/5 and 29/2 exhibited the same reaction patterns. +, positive reaction; 0, no reaction.
[b] *, serogroups 1 and 2 tested.
[c] Forty-three strains representing the 15 serogroups tested.

ensis. Contrary to another study, which reported the reaction of a 40-kDa protein in *L. rubrilucens* to polyclonal Mip antiserum (3), our study revealed the presence of only a 29-kDa protein reacting against MAbs raised against Mip (see Fig. 1A).

Characterization of MAbs by ELISA. In order to examine the epitope identification on non-lysed bacterial cells, an ELISA was performed with MAbs specific for Mip proteins. For this, polystyrene plates were coated with living *Legionella* cells. Heat or Formalin killing of bacteria prior to coating resulted in a significantly lower OD for MAbs 18/1, 20/2, 20/5, and 23/1 (data not shown). In comparison to immunoblot analysis, which was used as a reference method for epitope recognition by the above-described MAbs (Table 3), the differences obtained in an ELISA with living cells in a few cases depended on both the MAbs and strains tested (see Table 4). As mentioned above, the genus-specific MAb 22/1 was shown to exhibit only weak reactivity by ELISA for six *Legionella* species, indicating differences in the accessibility of this epitope on the intact bacteria.

For MAb 23/1, showing positive results for 27 *Legionella* species by immunoblot analysis, no corresponding epitope was detected in seven species (*L. anisa*, *L. cherrii*, *L. feelii*, *L. gormanii*, *L. maceachernii*, *L. oakridgensis*, and *L. wadsworthii*) by ELISA. Considering MAbs 2D8 and 18/1, epitope recognition documented by immunoblot analysis was not confirmed by ELISA in the case of *L. gormanii*. Furthermore, the reactivity of MAb 20/5 in the ELISA was shown to be weak for all species, whereas only seven of them were detectable by immunoblot analysis. On the other hand, no differences were seen for MAbs 29/2 and 23/3 in their ability to detect *Legionella* species by immunoblot analysis or ELISA.

Failure of cross-reactions of MAbs and non-*Legionella* strains. For cross-reactivity studies of the Mip-specific MAbs, 18 non-*Legionella* species were included (Table 1). As demonstrated by both immunoblot analysis and ELISA, our MAbs did not exhibit any reactions with gram-negative rods (*Citrobacter diversus*, *Enterobacter aerogenes*, *E. coli*, *Erwinia* spp., *Hafnia alvei*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Shigella* spp., *Serratia* spp., and *Yersinia pseudotuberculosis*). In addition, no reactivity was found with species which are known to carry genes with homology to the *mip* gene of *Legionella* species (*Chlamydia trachomatis*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*) (12, 19).

TABLE 4. ELISA studies with MAbs raised against *Legionella* Mip proteins

MAb	No. of *Legionella* species recognized/34 tested	Reactivity in ELISA[a] (OD_{492})	Divergence from reactivity observed by immunoblot analysis[b]
22/1	34 (genus specific)	0.39–2.2[c]	None
20/2	1 (*L. pneumophila* specific)	0.35–0.85[d]	None
23/1	20	0.30–0.65	Negative for *L. anisa*, *L. cherrii*, *L. feeleii*, *L. gormanii*, *L. maceachernii*, *L. oakridgensis*, and *L. wadsworthii*
2/D8	13	1.3–1.8	Negative for *L. gormanii*
18/1	13	1.0–1.8	Negative for *L. gormanii*
23/3	5	0.44–0.98	None
20/5	34	0.25–0.62	Positive for all species tested
29/2	7	0.32–0.68	None

[a] An OD_{492} of $\geq$0.15 was judged to be a positive result. The OD_{492} of blanks did not exceed 0.075.
[b] For comparison, see Table 3.
[c] With the exception of *L. cherrii*, *L. gormanii*, *L. hackeliae*, *L. quinlivanii*, *L. wadsworthii, and L. tucsonensis*, which had an OD_{492} in the range from 0.39 to 0.60, an OD_{492} of $\geq$1.0 was obtained for all *Legionella* spp.
[d] Forty-three *L. pneumophila* strains were tested.

DISCUSSION

In this paper we report the production and characterization of eight different MAbs recognizing *Legionella* Mip proteins. In previous studies, Mip proteins were discovered in 29 *Legionella* species by using a polyclonal antiserum prepared by immunization with Mip protein derived from *L. pneumophila* (3). In order to determine the antigenic diversity of the Mip proteins as well as to detect common epitopes, the eight MAbs introduced in this study were tested against 82 *Legionella* strains representing 34 species. As shown in Fig. 1A, the epitope recognized by MAb 22/1 was discovered on all *Legionella* strains, including *L. brunensis*, for which Cianciotto et al. (3) failed to detect the Mip protein. Eighteen organisms, including *Chlamydia trachomatis*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*, which are known to carry *mip* homolog genes (12, 19) showed no cross-reactivity with MAb 22/1. Therefore, we demonstrated that the *Legionella* Mip proteins possess a genus-specific epitope. To our knowledge, the Mip epitope recognized by MAb 22/1 is the second *Legionella* genus-specific epitope reported. Evidence of a genus-specific epitope located on *Legionella* proteins was found only for the 60-kDa heat shock protein (24).

In contrast to the genus-specific epitope recognized by MAb 22/1, the epitope specific for MAb 20/2 is expressed only on *L. pneumophila* and therefore has been proven to be species specific (Fig. 1B). Up to now, species-specific protein epitopes of *L. pneumophila* were detected on the 28-kDa major outer membrane protein (20) and a 135-kDa protein of the outer membrane (17). In total, 43 *L. pneumophila* strains representing all serogroups, as well as strains with differences in their ability to grow intracellularly or to be infective for guinea pigs, were tested. All these strains expressed the species-specific Mip epitope. In addition, the immunoblot analysis demonstrates that all *L. pneumophila* strains tested possess epitopes recognized by the other MAbs except MAb 23/3 (see Table 3). These data are in agreement with genetic studies. DNA-DNA hybridizations with two *mip*-specific gene probes under high-stringency conditions indicate that the *mip* genes are conserved in different *L. pneumophila* isolates (3). A comparison of the amino acid sequences of five *L. pneumophila*-specific Mip proteins on the basis of their nucleotide sequences showed only one amino acid exchange which does not influence the properties of the protein (18). It therefore seems that Mip proteins are housekeeping proteins produced by all known *Legionella* species and that the Mip molecules of *L. pneumophila* are very similar if not identical to each other.

On the basis of their reaction pattern to Mip-specific MAbs, as demonstrated by immunoblot analysis, the *Legionella* strains included in this study can be divided into six groups, A to F (see Table 3). Bangsborg et al. (1) divided the *mip* gene family of *Legionella* spp. into three groups based on the degree of homology between *mip*-specific sequences of various species and the *mip* genes of *L. pneumophila* and *L. micdadei*. The *mip* loci of the two species have 71% homology. The three homology groups include (i) *mip* of *L. pneumophila*, (ii) *mip*-like genes of *L. micdadei*, *L. feeleii*, *L. jamestowniensis*, *L. oakridgensis*, *L. quinlivanii*, *L. sainthelensi*, *L. spiritiensis*, and *L. wadsworthii*, which have moderate homology to *L. pneumophila mip* and extensive homology to *L. micdadei mip*, and (iii) *mip*-like genes which have moderate homology to *mip* of both *L. pneumophila* and *L. micdadei*. Of course, the Mip proteins of *L. pneumophila*, could be distinguished from those of all other species by having a species-specific epitope recognized by our MAb 20/2. Species belonging to *mip* homology group ii of Bangsborg et al. (1) possess our Mip types C, D, and E and have two or three common epitopes, whereas the species of group iii possess our types B to F, having one to four common epitopes. Including MAb 12F4 raised against Mip proteins, which has been introduced by Cianciotto et al. (3, 5), a seventh Mip type is given. The reaction pattern of MAb 12F4 is similar to those of our MAbs 2D8 and 18/1, but no reactivity was found for *L. dumoffii*, *L. parisiensis*, and *L. rubrilucens*, whereas MAb 12F4 recognized *L. cherrii*. Concerning the epitope expression of the Mip proteins, Table 3 shows that *L. cincinnatiensis*, *L. dumoffii*, *L. fairfieldensis*, *L. gormanii*, *L. moravica*, and *L. rubrilucens* have a high degree of relationship to *L. pneumophila*. On the other hand, *L. adelaidensis*, *L. birminghamsis*, *L. brunensis*, *L. gratiana*, *L. hackeliae*, *L. lansingensis*, and *L. tucsonensis* have only one Mip epitope in common with *L. pneumophila*.

The eight MAbs against the Mip proteins resulted from fusion experiments with native *Legionella* cells or native Mip protein used for immunization of mice. These immunogens were selected to get MAbs recognizing predominantly native epitopes expressed on the surface of bacteria. Although the screening of seeded wells was done by SDS-immunoblot analysis, all of the eight hybridomas collected in this study recognized Mip epitopes on the intact bacterial surface of *Legionella* species and were accessible to antibodies, as demonstrated by ELISA. It is therefore suggested that the MAbs can be used in routine work to detect Mip proteins on the surface of viable bacteria.

Besides the implications for organism identification, the knowledge about the immunological specificity of the Mip proteins is usable in serologic diagnosis. Traditionally, the detection of antibodies in patient sera was based on the use of intact *Legionella* cells carrying the lipopolysaccharide as the major antigenic component. The lipopolysaccharide of *Legionella* spp. is known to have pronounced antigenic diversity, without genus-wide epitopes. Therefore, the use of a large number of antigens is necessary for a valid diagnosis. On the other hand, cross-reactions between *Legionella* spp. and other gram-negative rods have been observed. In the present paper, we have demonstrated the existence of a genus-wide epitope on the *Legionella* Mip proteins that is also located on the recombinant Mip protein cloned from *L. pneumophila* (Table 2). The detection of *Legionella*-specific antibodies in patient sera by ELISA with the recombinant antigen or a synthetic peptide typifying the genus-specific epitope might be a suitable alternative to the established serological methods.

Taken together, the data presented in this study substantiate that recognition of Mip proteins by MAbs is an effective tool for detecting *Legionella* spp. (i) on the genus level, (ii) on the basis of Mip types, or even (iii) on the species level, as demonstrated for *L. pneumophila*. Additionally, the knowledge about the epitope patterns of the Mip proteins revealed structural differences that probably reflect differences in the functions of these proteins.

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