Monoclonal Antibody against *Leptospira interrogans* serovar *canicola*

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Nine cell lines producing monoclonal antibodies (MAbs) against *Leptospira interrogans* serovar *canicola* strain Moulton were established by the cell fusion technique. The immonological reactivity of these MAbs with various kinds of serogroups, serovars and strains were examined by microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). MAbs W1—W3 derived from mice, which were immunized with whole cells of the strain Moulton, reacted with the serogroups Canicola, Icterohaemorrhagiae and Pyrogenes. On the other hand, MAbs A1—A6 derived from mice immunized with the outer envelope (OE) fraction, which showed a potent protective activity and was extracted with ammonium hydroxide from the strain Moulton, reacted specifically with the serogroup Canicola alone in MAT. A difference in antigenic structure between subserogroup A (*canicola* subgroup) and subserogroups B (*schüeffneri* subgroup) of the serogroup Canicola was demonstrated by MAT using the MAbs. All the MAbs clearly agglutinated serovars of subserogroup A except for serovars *kamituga*, *jonsis* and *bindjei*, but did not react with any serovars of subserogroup B. These findings suggest that MAb highly specific to each serovar is readily available by OE immunization and is useful for the classification of *Leptospira*.

Keywords *Leptospira interrogans*; *Leptospira canicola*; outer envelope; monoclonal antibody; microscopic agglutination test; enzyme-linked immunosorbent assay

Introduction

Leptospira interrogans can be classified into about 190 serovars in 23 serogroups according to agglutination and cross-absorption tests with polyclonal antisera. However, the cross-absorption test is technically troublesome and interpretations of the results tend to be ambiguous. Recently, the classification of *Leptospira* using monoclonal antibodies (MAbs) has been examined by many investigators. We established a hybridoma producing MAbs against *L. interrogans* serovar *lai* strain 017.[1] Ono *et al.*[2] and Kobayashi *et al.*[3] established hybridomas producing MAbs against serovar-specific antigens of *Leptospira*. Adler and Faine[4] reported the establishment of a hybridoma cell line against a species-specific antigen, and Sakamoto *et al.*[5] also demonstrated that the hybridoma produced MAbs against leptospiral genus-specific protein antigen. However, there are few papers on comparative studies of the classification of *Leptospira* by MAbs against the whole cell, outer envelope (OE), or cellular fractions of *Leptospira*.

In the present study, in order to apply MAbs to the taxonomy of *Leptospira*, we established 3 and 6 hybridomas which were derived from mice immunized with whole cell and OE fractions of *L. interrogans* serovar *canicola* strain Moulton, respectively. The immunological specificity of the MAbs secreted by the hybridomas was examined by microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Bacterial Strains and Cultivation *Leptospira interrogans* serovar *canicola* strain Moulton and other leptospires (listed below in Tables III and IV) were grown in Korthof medium at 30 °C for 7 d.

Extraction of OE Cells of the strain Moulton were cultivated in Baseman–Cox medium[6] at 30 °C for 7 d and the OE fraction was extracted by the method described previously.[7,8] In brief, cultured cells were harvested and washed twice with physiological saline. One volume of leptospiral cells was extracted with 50 volumes of 1 N ammonium hydroxide at room temperature for 48 h. After centrifugation at 27000 × *g* for 30 min, the supernatant was neutralized with 4 N acetic acid and centrifugated at 18000 × *g* for 30 min. The supernatant was dialyzed against distilled water for 2 d and lyophilized as the OE fraction.

Immunization In the case of immunization with whole cells of the strain Moulton, the organisms were killed with formalin at a final concentration of 0.2% and were stored at 4 °C for 2 d. The cells were washed twice with physiological saline and were resuspended in saline at a concentration of 1×10^{10} cells/ml.

Seven- or 8-week-old BALB/c mice were injected intraperitoneally (i.p.) 3 times with 0.1 ml of the cell suspension at 3-day intervals. For booster immunization, the same volume of cell suspension was injected i.p. into the BALB/c mice 3 d before cell fusion.

In the case of the OE fraction, 5 μg of OE fraction was emulsified with 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich). Four- or 6-week-old BALB/c mice were injected subcutaneously (s.c.) with 0.1 ml of the emulsion. At the 3rd and 6th week later, 0.1 ml of the emulsion, which was prepared from 5 μg of OE fraction and 1 ml of Freund's incomplete adjuvant (Difco), was injected i.p. into the mice.

Fusion Protocol The fusion protocol was as described previously.[1] Spleen cells of BALB/c mice immunized with the whole cells or OE fraction were fused with mouse myeloma cells, P3-X63-Ag8-U1 (P3-U1), using a 45% solution of polyethylene glycol 4000 (Merck, Darmstadt).[9] Two or 3 weeks after the fusion, the culture supernatant was tested for the production of antibody specific for the strain Moulton by ELISA.[1]

The hybridoma cells secreting MAbs against the strain Moulton were cloned at least twice by a limiting dilution procedure employing spleen cells of BALB/c mice as feeder layers.

MAb Production *in Vitro* and *in Vivo* Hybridoma cells producing MAbs were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS; GIBCO Laboratories, Grand Island, NY) and the culture supernatant was used as MAbs.

To produce a large amount of MAbs, the hybridoma cells (1×10^6 cells/mouse) were inoculated i.p. into pristane-primed BALB/c mice. Two to 3 weeks later, ascites fluid of the mice was harvested and used as MAbs.

Determination of Immunoglobulin Class and Subclass of MAbs The immunoglobulin class and subclass of the MAbs were determined by the micro-Ouchterlony method.[10] Goat anti-mouse IgG1, goat anti-mouse IgG2a, goat anti-mouse IgG2b and goat anti-mouse IgM (Meloy, Laboratories, Inc. Springfield, Va) were employed as antiserum.

MAT The MAT was carried out according to the method described in the guidelines of the World Health Organization.[11] Culture fluid (25 μl) of *Leptospira* and the same volume of 2-fold serial dilutions of the culture supernatant of each hybridoma were mixed and incubated for 2 h at 30 °C. The MAT titer was designated as the reciprocal of the highest dilution in which 50% of the leptospires was agglutinated.

Results

Establishment of Antibody-Producing Hybridoma Spleen cells from BALB/c mice immunized with whole cells or OE fraction of the strain Moulton were fused with P3-U1 myeloma cells and seeded into 576 wells (6 trays). After selection by HAT medium and cloning, hybridomas fused with splenocytes of mice immunized with whole cells and OE fraction were grown in 76 wells (13.2%) and 59 wells (12.0%), respectively.

When the MAb production of these hybridomas was tested by ELISA, 6 hybridomas (1.0%, whole cell immunization) and 25 hybridomas (4.3%, OE fraction immunization) were positive, respectively. Finally, 3 MAb-producing hydridoma clones derived from mice immunized with whole cells of Moulton and 6 hybridoma clones derived from mice immunized with OE of Moulton were established, respectively.

TABLE I. Immunoglobulin Class and Subclass of Monoclonal Antibodies and Their Agglutination Titers

Immunogen	Monoclonal antibodies	Immunoglobulin class (subclass)	Agglutination titers	
			Culture supernatant	Ascites fluid
Whole cells	W1	IgG (IgG1)	2^7	2^{15}
	W2	IgG (IgG2a)	2^9	2^{17}
	W3	IgG (IgG1)	2^7	2^{16}
Outer envelope fraction	A1	IgG NT[a)]	2^9	NT
	A2	IgG (IgG2a)	2^9	2^{17}
	A3	IgG (IgG2b)	2^{11}	2^{14}
	A4	IgG (IgG2a)	2^9	2^{14}
	A5	IgG (IgG2a)	2^{13}	2^{21}
	A6	IgM	2^7	NT[a)]

a) Not tested.

Immunoglobulin Class, Subclass, and Agglutination Titer of MAbs As shown in Table I, the classes and subclasses were classified at two IgG1 clones (W1 and W3), four IgG2a clones (W2, A2, A4, and A5), one IgG2a clone (A3), and one IgM clone (A6). These MAbs strongly agglutinated the strain Moulton. The agglutination titers (2^{14} to 2^{21}) of the ascites fluids were higher than those (2^7 to 2^{13}) of the culture supernatants. In particular, MAb A5 exhibited an extraordinarily high agglutination titer (2^{21}).

Reactivity of MAbs with Various Serovars in ELISA The immunological specificity of the MAbs was examined by ELISA using 15 serovars (Table II). All the MAbs produced by the hybridomas reacted strongly with both homologous serovars, the *canicola* strain Moulton and strain Hond Utrecht IV (type strain of the serovar *canicola*), to similar degrees.

The MAbs, W1 and W3, revealed weak cross-reaction with various kinds of serovars such as *patoc*, *bataviae*, *hebdomadis*, *pyrogenes*, *etc.*, in addition to the serovar *canicola*. On the other hand, the reactivity of the A series of MAbs, other than A5, was broadly specific for the serovars *canicola* and *hebdomadis*. MAb A5 exhibited a high specificity to the strains Moulton and Hond Utrecht IV of the serovar *canicola* only.

Reactivity of MAbs with Various Serovars in MAT We next tested the agglutination reactivity of 9 MAbs against 24 serovars of 13 serogroups by MAT. As shown in Table III, 2 of the MAbs (W1 and W3) agglutinated the serovars *pyrogenes*, *copenhageni* and *icterohaemorragiae* strain CF 1, in addition to the serogroup Canicola, but did not react with the other serovars. However, the remaining 7 MAbs (W2 and A1—A6) displayed no reactivity to all the serovars tested except for the serogroup *Canicola*.

The serogroup Canicola can be classified into two sub-

TABLE II. Cross-Reaction of Monoclonal Antibodies to Various Serovars of *Leptospira*

Antigens		Monoclonal antibodies								
Serovar	Strain	W1	W2	W3	A1	A2	A3	A4	A5	A6
Leptospira										
Species *biflexa*										
andamana	CH-11	−	−	−	−	−	−	−	−	−
?	Urawa	−	−	−	−	−	−	−	−	−
patoc	Patoc I	+	−	+	−	−	−	−	−	−
Species *interrogans*										
australis	Akiyami C	−	−	−	−	−	−	−	−	−
bataviae	Van tienen	+	+	+	−	−	−	−	−	−
benjamin	Benjamin	−	+	−	−	−	−	−	−	−
canicola	Cong 13-127	+	+	+	−	−	−	+	−	+
	Hond Utrecht IV	++	+++	++	+++	+++	+++	+++	++	+++
	Kahendo	+	+	+	+	+	+	+	−	+
	Moulton	+++	+++	+++	+++	+++	+++	+++	+++	+++
hebdomadis	Hebdomadis	+	−	+	+	+	+	+	−	+
icterhaemorrhagiae	CF 1	+	−	+	−	−	−	−	−	−
	RGA	+	−	−	−	−	−	−	−	−
javanica	Veldrat Batavia 46	+	−	−	−	−	−	−	−	−
pomona	Pomona	+	−	+	−	−	−	+	−	−
pyrogenes	Salinem	++	−	+	−	−	−	+	−	−
tarassovi	Mitis-Johnson	+	−	−	−	−	−	−	−	−
copenhageni	Shibaura	++	+	+	−	−	+	+	−	+
Leptonema										
Species *illini*										
illini	3055	+	+	−	−	−	−	−	−	−

The OD values at 500 nm determined by ELISA are expressed as follows: +++, 9—10; ++, 6—8; +, 3—5; −, 0—1.

TABLE III. Microscopic Agglutination Titers of Monoclonal Antibodies Secreted in Culture Supernatants of Hybridomas Established from Mice Immunized with *L. canicola* Moulton

Antigens		Monoclonal antibodies								
Serogroup	Serovar	W1	W2	W3	A1	A2	A3	A4	A5	A6
Species *biflexa*										
Andamana	*andamana*	—	—	—	—	—	—	—	—	—
Semaranga	*patoc*	—	—	—	—	—	—	—	—	—
Species *interrogans*										
Australis	*australis*	—	—	—	—	—	—	—	—	—
Bataviae	*bataviae*	—	—	—	—	—	—	—	—	—
Hebdomadis	*hebdomadis*	—	—	—	—	—	—	—	—	—
Javanica	*javanica*	—	—	—	—	—	—	—	—	—
Pomona	*pomona*	—	—	—	—	—	—	—	—	—
Sejroe	*hardjo*	—	—	—	—	—	—	—	—	—
Tarassovi	*tarassovi*	—	—	—	—	—	—	—	—	—
Canicola										
(A) *canicola*	*canicola*	64	512	128	512	512	512	128	512	128
	broomi	8	64	4	4	16	32	4	16	16
	sumneri	8	16	4	32	16	16	64	32	32
	galtoni	4	8	4	4	4	4	16	4	4
	bafani	4	16	8	—	—	—	—	—	—
	kamituga	—	—	—	—	—	—	—	—	—
	jonsis	—	—	—	—	—	—	—	—	—
	bindjei	—	—	—	—	—	—	—	—	—
(B) *schüeffneri*	*schüeffneri*	—	—	—	—	—	—	—	—	—
	benjamin	—	—	—	—	—	—	—	—	—
	malaya	—	—	—	—	—	—	—	—	—
Icterohaemorrhagiae	*icterohaemorrhagiae*	8	—	8	—	—	—	—	—	—
	copenhageni	16	—	8	—	—	—	—	—	—
Pyrogenes	*pyrogenes*	32	—	16	—	—	—	—	—	—
Species *illini*										
Illini	*illini*	—	—	—	—	—	—	—	—	—

W1—W3: clones derived from mice immunized with whole cells of the strain Moulton. A1—A6: clones derived from mice immunized with outer envelope fraction of the strain Moulton.

TABLE IV. Microscopic Agglutination Titers of Monoclonal Antibodies Secreted in Culture Supernatant of Hybridomas Established from Mice Immunized with *L. interrogans* Serovar *canicola* Strain Moulton

Antigens		Monoclonal antibodies								
Serovar	Strain	W1	W2	W3	A1	A2	A3	A4	A5	A6
canicola	Moulton	128	512	128	512	512	2048	512	4096	64
	Hond Utrecht IV	32	1024	32	512	512	1024	128	512	32
	Kodaira	128	128	512	1024	1024	64	32	64	128
	HD 543	32	512	8	128	128	64	64	128	32
	Ooyama	16	16	4	512	128	64	16	32	64
	Congo 13-127	—	32	—	16	64	—	8	16	—
	Kahendo	—	32	—	64	16	—	32	32	—

W1—W3: clones derived from mice immunized with whole cells of the strain Moulton. A1—A6: clones derived from mice immunized with OE fraction of the strain Moulton.

serogroups, A (*canicola* subgroup) and B (*schüeffneri* subgroup). All of the MAbs reacted with the serovar *canicola* at high titers, but agglutinated with the serovars *broomi*, *sumneri* and *galtoni* at low titers. The serovar *bafani* was merely agglutinated by 3 of the MAbs (W1—W3). None of the MAbs reacted with the *schüeffneri* group or with the serovars *kamituga*, *jonsis* and *bindjei* which belong to subserogroup A (*canicola* subgroup).

As shown in Table IV, all of the MAbs agglutinated various strains of the serovar *canicola*, such as Moulton, Hond Utrecht IV, Kodaira, HD 543 and Ooyama. In particular, the strains Moulton, Hond Utrecht IV and Kodaira showed higher titers than those of HD 543 and Ooyama. However, the strains Congo 13-127 and Kahendo were not agglutinated appreciably by these MAbs.

Discussion

Extermination of leptospirosis is thought to be impossible, since this disease as a zoonosis is spread worldwide and carriers of the pathogens are wild animals, especially rodents. At present, vaccination with inactivated leptospiral cells appears to be the most effective means of protecting against the infection. Auran *et al.*[12] showed that a potent protective antigen against leptospiral infection was present in the OE of the organisms in their investigations using hamsters.

In the present study, we employed the used virulent strain, Moulton, instead of the strain Hond Utrecht IV (standard strain of the serovar *canicola*) in order to examine the passive protective activity of MAbs against *canicola* infection. We established 9 cell lines producing

MAbs against the serovar *canicola* strain Moulton by the cell fusion technique. The MAbs, W1—W3, were derived from mice immunized with whole cells while the MAbs, A1—A6, were derived from mice immunized with OE fraction extracted from the strain Moulton. The different antigens (whole cell or OE fraction) and procedures of immunization were not related to the production of immunoglobulin classes and subclasses of MAbs in this experiment (Table I).

The results obtained suggested that the immunological specificity of MAbs derived from mice immunized with OE fraction was higher than that of MAbs derived from mice immunized with whole cells. It appeared that MAbs which specifically reacted only with a homologous serovar or strain were readily obtained by immunization with OE. Hamsters pre-immunized with OE fraction (10 μg/hamster) were effectively protected from infection by the virulent strain, Moulton (data not shown). It seems likely that the OE fraction contains more efficient antigens than the whole cells in the production of specific antibody. Johnson *et al.*[12,13,14] reported that OE of leptospiral cells possessed the most effective antigen for protective immunity. However, the chemical nature of the protective antigen in OE is unclear.

The MAbs, A1—A6, reacted specifically with the serogroup Canicola alone and not with the other serogroups by MAT. On the other hand, the MAbs, W1—W3, reacted not only with serovars of the serogroup Canicola at high titers but also with the serogroups Icterohaemorrhagiae and Pyrogenes at low titers. Previously, Auran *et al.*[15] demonstrated that polyclonal antiserum against the strain Hond Utrecht IV reacted with serovars of the serogroup Icterohaemorrhagiae at low titers and some serovars of the serogroup Pyrogenes at high titers. Furthermore, the present authors[1] and Kobayashi *et al.*[16] found that MAbs against the serogroup Icterohaemorrhagiae exhibited a cross-reaction to the serogroup Canicola. The above findings suggest that similar antigenic determinants may exist on the cell surface of the serogroups Canicola, Icterohaemorrhagiae and Pyrogenes.

The MAbs, A1—A6, did not agglutinate any of the serovars belonging to subserogroup B of the serogroup Canicola or 4 of the serovars (*bafani*, *kamituga*, *jonsis* and *bindjei*) belonging to the subserogroup A, but reacted merely with the serovars *canicola*, *sumneri*, *broomi* and *galtoni* belonging to subserogroup A of the serogroup Canicola specifically. The immunological specificity of the MAbs, W1—W3, against various serovars of the serogroup Canicola (except the *bafani* serovar) was the same as that of the MAbs, A1—A6. However, the agglutination titer of the MAbs against the serovar *canicola* was higher than that of the MAbs against the 10 other serovars of the serogroup Canicola.

Ono *et al.*[2] indicated the possibility that the antigenic determinant of the *canicola* Type-specific main (TM) antigen had at least 3 types based on results for MAT using the CT series MAbs against the TM antigen. However, it seems that the antigenic determinant recognized by the MAbs established in the present study is different from that of the CT series MAbs. The W series MAbs and the A series MAbs may recognize similar antigenic determinants present in the serovars *canicola*, *broomi*, *samneri*, *galtoni* and/or *bafani* (Table III).

We examined the reactivity of MAbs against various strains belonging to the serovar *canicola* by MAT. The MAbs reacted significantly with both the strains Moulton and Hond Utrecht IV, and showed a weak cross-reactivity to the other strains belonging to the serovar *canicola*. Exceptionally, the MAbs W1, W3, and A6 revealed low or no agglutination titers to the strains Congo 13-127 and Kahendo. MAb A5 among the MAbs tested displayed the highest agglutination titer and the highest specificity against the strain Moulton. These results indicate that application of MAbs is indispensable for the classification of *Leptospira*, since polyclonal antibody shows a cross-reaction with various serovars of *Leptospira* and necessitates a cross-absorption procedure in the classification process. Standard MAbs specifically reacting with a serovar or serogroup are necessary for the classification of *Leptospira* in place of the classification using polyclonal antiserum. It is expected that the classification of *Leptospira* employing standard MAbs will be generally adopted in the near future.

To investigate the passive immunity of MAbs in hamsters and the chemical nature of the antigenic determinants of the strain Moulton, purification of the MAbs on a protein A–Sepharose affinity column is currently in progress. The results will be published elsewhere.

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References

1) T. Masuzawa, M. Kumagai, T. Shimizu, and Y. Yanagihara, *J. Clin. Microbiol.*, **26**, 2332 (1988).
2) E. Ono, M. Naiki, and R. Yanagawa, *Zbl. Bakt. Hyg. A.*, **252**, 414 (1982).
3) Y. Kobayashi, T. Tamai, T. Oyama, H. Hasegawa, E. Sada, T. Kusaba, and M. Hamaji, *Microbiol. Immunol.*, **28**, 359 (1984).
4) B. Adler and S. Faine, *Zbl. Bakt. Hyg. A.*, **255**, 317 (1983).
5) N. Sakamoto, E. Ono, H. Kida, and R. Yanagawa, *Zbl. Bakt. Hyg. A.*, **259**, 557 (1985).
6) J. B. Baseman and C. D. Cox, *J. Bacteriol.*, **97**, 992 (1969).
7) T. Taniyama, Y. Yanagihara, I. Mifuchi, I. Azuma, and Y. Yamamura, *Infect. Immun.*, **6**, 414 (1972).
8) Y. Yanagihara, K. Kamisango, S. Yasuda, S. Kobayashi, I. Mifuchi, I. Azuma, Y. Yamamura, and R. C. Johnson, *Microbiol. Immunol.*, **28**, 535 (1984).
9) G. Köhler and C. Milstein, *Nature* (London), **256**, 495 (1975).
10) O. Ouchterlony, *Allergy*, **5**, 1 (1958).
11) World Health Organization, Guidelines for the Control of Leptospirosis, W. H. O. Offset Publication, No. 67.
12) N. E. Auran, R. C. Johnson, and M. D. Ritzi, *Infect. Immun.*, **5**, 968 (1972).
13) R. F. Bey, N. E. Auran, and R. C. Johnson, *Infect. Immun.*, **10**, 1051 (1974).
14) J. E. Glosser, R. C. Johnson, C. R. Sulzer, and N. E. Auran, *Am. J. Vet. Res.*, **35**, 681 (1973).
15) N. E. Auran, R. C. Johnson, and A. D. Alexander, in the Symposium on Leptospirosis, *Leptospira* and Other Spirochetes, Bucharest, 1975, p. 275.
16) Y. Kobayashi, T. Tamai, and E. Sada, *Microbiol. Immunol.*, **29**, 1229 (1985).