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Monoclonal Antibodies That Detect Live Salmonellae

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Nine immunoglobulin G and nine immunoglobulin M murine monoclonal antibody-producing hybridomas reactive with live *Salmonella* bacteria were obtained from several fusions of immune spleen cells and Sp2/0 myeloma cells. The antibodies were selected by the magnetic immunoluminescence assay. The monoclonal antibodies were reactive with serogroups A, B, C_1, C_2, D, E, and K and *Salmonella choleraesuis* subsp. *diarizonae*. Each monoclonal antibody proved to be reactive with a distinct serotype. Clinical isolates belonging to these *Salmonella* serogroups could be detected. Reactivity with non-*Salmonella* bacteria proved to be minor.

Salmonella is a large genus of bacteria expressing over 2,000 serotypes. Although a large number of serotypes have been identified, the commonly encountered serotypes A, B, C, D, and E are responsible for >95% of isolates from humans (7). These gram-negative rods are the etiologic agents of food-borne salmonellosis and also the agents that cause typhoid and paratyphoid fever. Food products like meat, meat products, eggs, poultry, and chocolate (2, 8, 16, 24) are the most common sources of salmonellosis, but the presence of *Salmonella* spp. in recreational waters may also present a health hazard to humans (15).

The presence of salmonellae in food is of growing concern. It was demonstrated that fewer than 10 organisms per 100 g of chocolate are sufficient to cause symptomatic disease (9). Traditional methods for the isolation of *Salmonella* spp. involve the use of enrichment and selective media to allow the detection of small numbers of sometimes stressed bacteria in 4 to 5 days (7). The urgent need for faster methods has resulted in several assays based on immunochemical principles (3, 11, 16, 17, 21, 24). Recently, magnetic separation technology was introduced to make the assays faster and more specific (13, 25, 26). Those tests rely on the availability of specific monoclonal or polyclonal antibodies. However, the use of polyvalent antisera is limited by lack of specificity (1, 3). Several monoclonal antibodies reactive with the lipopolysaccharides of several *Salmonella* serogroups have been described (13, 14, 21–23).

In this article, we describe the production of serotype-specific monoclonal antibodies that bind to live or intact salmonellae belonging to serogroups A, B, C_1, C_2, D, E, and K and *Salmonella choleraesuis* subsp. *diarizonae*. These antibodies react with live salmonellae without the need for further processing of the bacteria to expose the epitopes (24). Such antibodies are mandatory for the use of either sensitive and more specific polymerase chain reaction tests (26) or immunosensors (5, 10) or simple test formats such as dipsticks, depending on the requirements of the test and the state of the sample.

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MATERIALS AND METHODS

Cultures. Bacterial isolates were obtained from specimens from patients at the University Hospital, Utrecht, and from food samples provided by LUMAC. A collection of serotyped strains was obtained from the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. O antigen information for these strains was deduced from published data (6, 11).

Bacteria were cultured on blood agar plates and, after collection, suspended in phosphate-buffered saline. The bacterial concentration was adjusted to 10^8 CFU/ml by comparing the optical density at 543 nm with calibrated readings.

Monoclonal antibodies. Female BALB/c mice, 6 to 8 weeks old, were immunized for several weeks with antibiotic-treated bacteria. Different isolates belonging to one serotype were used for immunization. Spleen cells obtained from mice expressing antibodies in their serum against all members of a given serogroup were fused with the Sp2/0 myeloma cell line as described before (20). Fusion was performed with an electrofusion apparatus (Braun Diessel Biotech, Melsungen, Germany). Ice-cold iso-osmolar buffers were used during the fusion process (18). The alignment frequency of 1.5 MHz before and after the fusion pulse lasted for 30 s. One fusion pulse of 3 kV/cm with a duration of 10 μs was given. After fusion, cells were seeded in microtiter plates and cultured for 1 day in phenol red-free medium (Gibco-BRL, Paisley, Renfrewshire, United Kingdom). All other procedures were as described before (19, 20).

Detection. Supernatants from wells containing growing hybridomas were screened by the magnetic immunoluminescence assay (MILA) detection system. The MILA was performed as described before (20). Briefly, Magnisort M magnetic beads (E. I. du Pont de Nemours, Wilmington, Del.) were incubated with hybridoma supernatant for 5 min at room temperature. The fluid phase was removed, and a bacterial suspension was added. Unbound bacteria were removed by washing. Bound bacteria were lysed, and the liberated ATP was measured by the ATP-dependent luciferin-luciferase enzyme system. Bioluminescence reagents and bacterial lysis buffer were from LUMAC B.V. (Landgraaf, The Netherlands). Bioluminescence was measured in a Biocounter M-2500 (LUMAC). The MILA value

TABLE 1. Reactivity of monoclonal antibodies with serotyped *Salmonella* strains

Strain	Serogroup	Reactivity[a] with monoclonal antibody:																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Durazzo	A	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
S. paratyphi A	A	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. paratyphi B	B	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
S. typhimurium	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
S. typhimurium S2	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Heidelberg	B	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
S. abortusequi	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Derby	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Reading	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Schwarzengrund	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
S. abortusbovis	B	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Stanley	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Brandenburg	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Indiana	B	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Agona	B	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Nigeria	C_1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Virchov	C_1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Amersfoort	C_1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Eimsbuettel	C_1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Omderman	C_1	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−
Oranienburg	C_1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
S. infantis	C_1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Bareilly	C_1	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Glostrup	C_2	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Takoradi	C_2	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Newport	C_2	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Virginia	C_2	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Amherstiana	C_2	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Kentucky	C_2	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Emek	C_2	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Hadar	C_2	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
Dublin	D	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
Panama	D	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
S. typhi	D	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
S. enteritidis	D	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
Eastborne	D	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
S. meleagridis	E	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
S. anatum	E	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Newbrunswick	E	−	−	−	−	+	−	−	−	−	−	−	+	+	+	−	+	+	+
Goerlitz	E	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Canoga	E	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Minneapolis	E	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Taksony	E	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Senftenberg	E	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Niloese	E	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Cerro	K	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
Fluntern	K	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
S. choleraesuis subsp. *diarizonae*	C_1	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
	Y	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+

[a] +, MILA value of >3,000 RLU and MILA ratio of ≥3 (see text).

was expressed as relative light units (RLU). Control incubations with an irrelevant monoclonal antibody served as a negative control. A result was regarded positive if the ratio of the MILA value obtained with a monoclonal antibody and the MILA value obtained with an irrelevant monoclonal antibody was >3 and the absolute MILA value was above 3,000 RLU.

Dot spot analysis. Monoclonal antibodies were purified by the fast protein liquid chromatography method on a Mono Q column (Pharmacia-LKB, Uppsala, Sweden). The binding and elution buffers were those described before (4). Purified monoclonal antibody was spotted on a nitrocellulose membrane, and residual binding places were blocked with bovine serum albumin (Organon Teknika, Turnhout, Belgium). The membranes were incubated with live salmonellae (10^6 CFU/ml) for 20 min and subsequently with the same monoclonal antibody but now coupled to alkaline phosphatase. Alkaline phosphatase (Sigma, St. Louis, Mo.) was conjugated to the monoclonal antibody with glutaraldehyde by standard techniques.

Nomenclature. All *Salmonella* serotypes used belong to the species *S. choleraesuis*. The serotypes belong to the two subspecies *S. choleraesuis* subsp. *choleraesuis* and *S. choleraesuis* subsp. *diarizonae*. The antigenic formulas of *S. choleraesuis* subsp. *diarizonae* are 6,7:l,v:z53 and 48:i:z57:(z53).

TABLE 2. Reactivity of monoclonal antibodies with clinical *Salmonella* isolates

Serogroup	Isolate	MILA value (RLU) with monoclonal antibody[a]:		
		1	2	Control
A	14A	16,716	4,852	67
	BK	7,168	9,851	73
	MH	12,129	4,280	50
	211188	11,246	3,186	119
	6488	5,509	1,675	38
	Gouda	56,175	2,188	87
	Den Haag	43,234	520	83
	K01	60,172	228	87
	K02	39,837	19,983	722
	Leiden	61,369	728	86
	Kiel	39,305	97	49
B	Heidelberg	13,002	11,321	372
	14K	14,209	14,231	361
	Essen	26,921	25,341	663
	10.2	32,824	ND[b]	176
	SaintPaul	981	5,845	767
	10.4	5,975	ND	629
	10.5	6,488	ND	72
	Derby	2,423	16,002	1,119
	10.12	17,633	ND	550
	10.50	17,950	ND	276
C_1	8562	10,085	12,209	446
	6488	10,134	13,795	545
C_2	Newport	252	374	626
	S. bovismorbificans	182	285	114
D	Dublin	31,158	26,142	135
	S. enteritidis	20,240	27,887	757
	S. typhi	40,187	6,677	310
	S. typhi	4,234	3,412	147
	Panama	24,237	22,632	901
	S. typhi	520	581	121
	Gnos	15,786	14,609	646
	S. typhi	34,887	40,443	217
	Vis	17,671	22,836	961
	Balt	18,251	21,632	1,124
	Veed	25,815	22,771	702
E	14E	38,475		2,903
	14O	20,744		1,241
	14P	17,907		295
	14R	17,534		66
	14T	18,418		2,612

[a] Pairs of monoclonal antibodies (1 and 2) used for each serogroup: A, 1 and 2; B, 3 and 4; C_1 and C_2, 7 and 8; and D, 11 and 12. Only one monoclonal antibody, 14, was used for serogroup E. The controls consisted of irrelevant monoclonal antibodies.
[b] ND, not done.

RESULTS AND DISCUSSION

After immunization with isolates belonging to one serogroup, an immune response to all isolates in that serogroup was detected. Subsequently, several fusions were performed, yielding hundreds of specific hybridomas. Initial screening of the hybridoma supernatants was only possible against one member of a given serogroup because of the limited availability of supernatant at this stage. Positive hybridomas were cloned, and sufficient supernatant was collected.

The screening of these monoclonal antibodies against the whole panel of *Salmonella* spp. yielded 18 monoclonal antibodies. The reactivity of these monoclonal antibodies is shown in Table 1. Some monoclonal antibodies showed a limited reactivity within one serogroup. The monoclonal antibodies against *Salmonella* group A were screened with a clinical isolate. Later on, it appeared that monoclonal antibody 2 showed no reactivity towards the *Salmonella* serotype A strains. However, it was reactive with several clinical isolates (Table 2). Such a reaction pattern was also observed for other group-specific monoclonal antibodies. This limited reactivity within a serogroup is not easily explained. The monoclonal antibodies against serogroup C_1 failed to detect strain Eimsbuettel and partially failed to detect strain Omderman. These strains were formerly classified as serogroup C_4 (12). It is remarkable that both strains are also reactive with the serogroup K-specific monoclonal antibodies.

Strain Newbrunswick showed peculiar binding behavior. Besides strong reactivity with serogroup E-specific monoclonal antibodies, reactivity with antibodies specific for several other serogroups was also seen.

The serogroup E-specific monoclonal antibodies showed a differential binding pattern. Monoclonal antibody 13 was reactive with all strains, indicating that it is reactive with the O:3 antigen. On the other hand, monoclonal antibody 14 was reactive with serogroup E strains lacking the O:34 antigen. Expression of this O antigen type seemed to prevent the binding of this monoclonal antibody.

Monoclonal antibodies 3 and 4, directed towards serogroup B, did not recognize *S. abortusbovis*. Therefore, new monoclonal antibodies were raised with this strain as the screening organism. Most of the monoclonal antibodies isolated showed reactivity only with this strain. Monoclonal antibody 6 showed reactivity not only with *S. abortusbovis* but also with the other serogroup B isolates (Table 1).

TABLE 3. Immunoglobulin subclass and cross-reactivity of monoclonal antibodies with non-*Salmonella* bacteria[a]

Monoclonal antibody no.	Subclass	No. of strains positive/no. tested
1	IgG1	0/100
2	IgM	0/100
3	IgM	0/40
4	IgM	0/40
5	IgG1	0/40
6	IgG3	0/40
7	IgG2b	0/40
8	IgM	0/40
9	IgM	3/140
10	IgG1	2/140
11	IgG1	0/140
12	IgM	3/140
13	IgG3	1/140
14	IgM	0/40
15	IgM	4/140
16	IgG3	2/140
17	IgG1	0/140
18	IgM	2/140

[a] The 100 clinical isolates comprised 25 *E. coli*, 26 *Klebsiella* spp., 15 *Enterobacter* spp., 13 *Pseudomonas* spp., 10 *Citrobacter* spp., 4 *Morganella* spp., 3 *Serratia* spp., 3 *Acinetobacter* spp., and 1 *Flavobacterium* sp. The 40 isolates from food included one *Aeromonas* sp., five *Bacillus* spp., one *Citrobacter* sp., two *Enterobacter* sp., seven *E. coli* strains, one *Hafnia* sp., two *Klebsiella* spp., three *Lactobacillus* spp., two *Proteus* spp., three *Pseudomonas* spp., three *Serratia* spp., five *Shigella* spp., two *Streptococcus* spp., and three *Yersinia* spp.

TABLE 4. Sensitivity of the MILA for the 18 monoclonal antibodies[a]

Monoclonal antibody no.	Isolate (serogroup)	MILA ratio at a bacterial concn (CFU/ml) of:				
		10^8	10^7	10^6	10^5	10^4
1	K02 (A)	53	114	27	7	2
2	K02 (A)	6	8	3	1	1
3	Brandenburg (B)	10	9	2	1	1
4	Brandenburg (B)	14	14	2	1	1
5	Brandenburg (B)	14	19	4	3	2
6	Brandenburg (B)	20	26	5	3	2
7	*S. infantis* (C_1)	12	12	9	4	
8	*S. infantis* (C_1)	12	15	13	6	
9	Newport (C_2)	21	33	32	8	2
10	Newport (C_2)	26	34	59	10	2
11	*S. enteritidis* (D)	17	39	13	5	1
12	*S. enteritidis* (D)	14	23	11	3	3
13	Senftenberg (E)	22	42	8	3	1
14	Senftenberg (E)	13	17	6	2	1
15	Cerro (K)	8	25	15	6	2
16	Cerro (K)	13	23	10	5	2
17	*S. choleraesuis* subsp. *diarizonae* (Y)	169	76	32	7	3
18	*S. choleraesuis* subsp. *diarizonae* (Y)	204	68	25	5	3

[a] The reactivity of the monoclonal antibodies was tested with the indicated isolates as the antigen. Background reaction was determined with an irrelevant monoclonal antibody. Results are expressed as the MILA ratio (see text). Both MILA values used for this ratio were obtained at the same bacterial concentration. A ratio of ≥3 was considered positive.

Monoclonal antibody 7 was also reactive with *S. choleraesuis* subsp. *diarizonae*. This strain has the same O antigen as serotype C_1.

The reactivity of the specific monoclonal antibodies with clinical *Salmonella* isolates is shown in Table 2. Selected monoclonal antibodies were tested against a panel of 100 clinical isolates of members of the family the *Enterobacteriaceae*. In addition, reactivity with 40 different isolates from food products was tested. The reactivity is shown in Table 3. In less than 1% of cases, a positive reaction with non-*Salmonella* bacteria was seen when the same criteria were used as for the *Salmonella* isolates. No pattern to this cross-reactivity was observed. The 17 cross-reactions (Table 3) were found with five different *Escherichia coli* strains, two different *Citrobacter freundii* strains, and one strain each of *Enterobacter agglomerans*, *Pseudomonas aeruginosa*, *Serratia liquefaciens*, *Proteus* sp., and *Morganella morganii*. The *Serratia* isolate reacted with monoclonal antibodies 10, 12, 13, and 15, one *Citrobacter* isolate reacted with monoclonal antibodies 9 and 12, and the *Morganella* isolate reacted with monoclonal antibodies 15 and 16. All other cross-reactivity occurred with different isolates.

The beads used here are coated with immunoglobulin isolated from immunized goats. No affinity purification to obtain pure goat anti-mouse immunoglobulin was undertaken by the manufacturer. Indeed, this unpurified goat immunoglobulin reacted with some *Pseudomonas* isolates. Beads not coated with immunoglobulins did not show those reactivities, although certain isolates showed reactivity towards the magnetic bead.

Some other bacterial strains showed nonspecific binding to either the magnetic particle or the goat anti-mouse immunoglobulin coated to the magnetic bead. This nonspecific binding was independent of the monoclonal antibody used. By choosing an MILA ratio of >3 as the cutoff for a positive reaction, in most cases this nonspecific reactivity gave a negative result.

To assess the usefulness of these monoclonal antibodies for the dipstick technique, monoclonal antibody 5 and three irrelevant monoclonal antibodies were used to coat a nitrocellulose membrane. As indicated in Fig. 1, only the monoclonal antibody directed towards serogroup B showed a positive reaction when the stick was incubated with salmonellae from serogroup B. No reaction was observed when a monoclonal antibody against serogroup C_1 or irrelevant monoclonal antibodies were used to coat the nitrocellulose and incubated with salmonellae from serogroup B. No reaction was observed when the stick was incubated with *E. coli*.

The general applicability of this dipstick technique was deduced from experiments in which the immunoglobulin M (IgM) monoclonal antibodies were spotted onto nitrocellulose, incubated with different bacterial isolates, and subsequently incubated with the IgG monoclonal antibodies. A clear positive reaction was seen in the proper combinations after bound IgG was visualized with peroxidase-labeled IgG class-specific goat anti-mouse immunoglobulins. More background reactivity was seen in these experiments, which is due to the cross-reactivity of the IgG class-specific antibodies.

The MILA is a very rapid method for detecting the reactivity of monoclonal antibodies with live salmonellae. The sensitivity of the luminescence assay as used here is approximately 10^4 to 10^7 CFU/ml (Table 4). This sensitivity is comparable to that of other enzyme immunoassays (11, 13, 24, 25).

The specificity of detection of salmonellae can be increased further by using polymerase chain reaction methods in concert with the monoclonal antibodies (26).

For direct detection of salmonellae in enrichment cultures, other test formats than that used here can also be used. A simple test format is a dipstick, in which monoclonal antibodies are used to coat a nitrocellulose membrane fitted on a plastic strip. The stick is subsequently immersed in a test sample and developed with a second antibody labeled with an enzyme. The feasibility of such a test format is clearly shown.

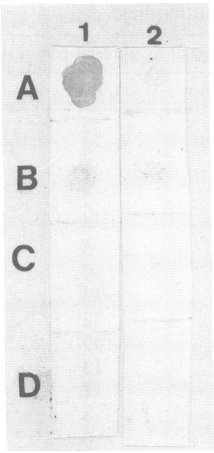

FIG. 1. Reactivity of monoclonal antibody 5 (anti-*Salmonella* serogroup B) with salmonellae and irrelevant bacteria. Nitrocellulose membranes were coated with: row A, monoclonal antibody 5; row B, monoclonal antibody 8 (anti-*Salmonella* serogroup C_1); row C, monoclonal antibody to *Enterobacter* spp.; row D, monoclonal antibody to *Pseudomonas* spp. Lane 1, *Salmonella* sp. strain Brandenburg; lane 2, *E. coli* K1. Possible bound bacteria were detected with monoclonal antibody 5 coupled to alkaline phosphatase.

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