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Evaluation and Standardization of an Agglutination Test for Human Listeriosis

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Human sera from patients with culturally confirmed listeriosis were tested for immunoglobulin M (IgM) and immunoglobulin G (IgG) agglutinating antibodies with trypsinized antigens of *Listeria monocytogenes*, *Streptococcus faecalis*, and *Staphylococcus aureus*. The response of humans to listeria infections is mainly IgM rather than IgG as found in animals. The antigens prepared from *L. monocytogenes* serotypes 1a, 1b, 2, 4b, and 4d were evaluated for specificity with normal sera, sera from patients with various other diseases, and sera from patients with listeriosis. The trypsinized antigens appeared to be specific for listeria antibodies with a cross-reaction rate of from 5.4 to 6%. Cross-reaction with *S. aureus* can be eliminated by absorption of the serum with *S. aureus*. This agglutination technique appears to be applicable for diagnostic testing, but, as with all serological procedures, both acute and convalescent sera should be tested.

Within the past decade and especially within the last 4 years, human listeriosis has been reported in the United States with increasing frequency (2). Although many listeria infections occur in infants and in patients with underlying disease, most of the recently reported cases of listeriosis have occurred in previously healthy urban residents in all age groups (4). The clinical syndrome of listeriosis in humans is varied and may be manifested by abortion, conjunctivitis, endocarditis, meningoencephalitis, pneumonitis, pyoderma, septicemia, or urethritis (3). To date, serological methods to support the clinical diagnosis of listeriosis have been inadequate (6, 10, 12). The *Listeria monocytogenes* antigen is serologically cross-reactive with various other bacteria, especially *Streptococcus faecalis* and *Staphylococcus aureus* (5, 8-11, 14, 16). Therefore, most tests, such as agglutination, complement fixation, indirect hemagglutination, and precipitation give false-positive reactions which are difficult to interpret (6, 10). In 1965, Osebold, Aalund, and Chrisp (7) in their study of the chemical and immunological composition of *L. monocytogenes* surface structures noted that treatment of the antigen with trypsin increased its sensitivity and eliminated some cross-reactions. In 1966, Aalund and his co-workers (1) studied the antibody response of sheep and cattle in experimental listeriosis and found that all of the sera examined from apparently normal sheep and cattle controls contained immunoglobulin M (IgM) antibodies to *L. monocytogenes*, whereas the sera of the experimentally infected animals contained both IgM and immunoglobulin G (IgG) antibodies. These investigators used 2-mercaptoethanol to inactivate the IgM agglutinating antibody and the trypsin-treated somatic antigen as a measure of biological activity. In 1968, Osebold and Aalund (6) continued their investigations with the trypsinized listeria antigen and serum reduction method with sera from uninfected humans and animals, sera from experimentally infected animals, and sera from naturally infected humans and animals. Again, they found the uninfected humans and animals to have reactive IgM antibodies and only 1 to 2% of the animals to have reactive IgG antibodies. None of the human subjects had listeria IgG antibodies. Of the experimentally infected animals all developed listeria IgG antibodies. Of the naturally infected humans and animals, only one human of the three tested was positive for IgG antibodies, whereas 26% of the animal sera tested had IgG-positive antibodies. In 1970, Seeliger and Emmerling (12) used non-trypsin-treated somatic and flagellar listeria antigens and 2-mercaptoethanol reduction and only occasionally found low titers of IgG antibodies in infected human sera; they found 2-mercaptoethanol-resistant antibodies in sera of both in-

fected and healthy animals. Unfortunately, the results of Seeliger and Emmerling (12) and those of Osebold and Aalund (6) are not in complete agreement. The following study is an evaluation of the method of Osebold, Aalund, and Chrisp (7) and Osebold and Aalund (6) as a diagnostic test for human listeriosis.

MATERIALS AND METHODS

Cultures. The *L. monocytogenes* strains of serotypes 1a (7973) and 2 (5348) were originally obtained from H. P. R. Seeliger. Strains of serotypes 4b (F4) and 4d (21) were obtained from J. Donker-Voet. The strain of serotype 1b was isolated from a suspected case of human listeriosis and serotyped in this laboratory. Cultures of *S. faecalis* and *S. aureus* were obtained from the Clinical Bacteriology Unit at the Center for Disease Control (CDC).

Antisera. Normal human antisera for controls were obtained from the CDC serum bank and from sera submitted to this laboratory for estimation of tetanus and diphtheria antitoxin levels. These sera were taken from apparently healthy individuals of all ages throughout the United States (Table 1). Human sera thought to contain antibodies to *L. monocytogenes* were submitted by private physicians from patients with culturally confirmed cases of listeriosis (Table 2). Sera from patients with melioidosis, tularemia, typhoid and paratyphoid, mononucleosis, brucellosis, rat bite fever, rickettsial infections, leptospirosis, and group A streptococcal infections were submitted to this laboratory for specific serological tests through the state health departments. Human sera containing antibodies to cholera were supplied by Wallis DeWitt of the CDC Epidemiology Program. Control rabbit antisera for listeria factors 1a, 4b, and 4d were produced in this laboratory by Geraldine Wiggins.

Antigen preparation. The antigens of *L. monocytogenes*, *S. aureus*, and *S. faecalis* were prepared by the method of Osebold, Aalund, and Chrisp (7). The cultures were incubated for 24 hr at 37 C in 5 ml of tryptose broth (Difco); these broths were then used to seed 32-oz (ca. 1-liter) prescription bottles containing 250 ml of tryptose agar (tryptose broth plus 1.5% agar). The bottles were incubated for 48 hr at 37 C. Cells were harvested from the agar surface in a minimal amount of saline (0.85% NaCl) and steamed at 100 C for 1 hr. The suspensions were then washed twice in Sorensen's phosphate-buffered saline, *p*H 7.3. A concentrated suspension of cells in buffer was prepared; when diluted 1:20 it read 50 to 53% optical transmission at 430 nm on a Coleman Jr. spectrophotometer. This concentrated suspension was treated with 0.1% crude trypsin (Difco 1-300) by adding one part of a 1% trypsin solution to nine parts of cell suspension. The cells were trypsinized for 15 min at 37 C, washed twice in saline, and then resuspended to a concentration that would read 50 to 53% optical transmission when diluted 1:20. Antigens were preserved with Merthiolate at a final concentration of 1:10,000.

Serum reduction. Each serum sample was tested as whole serum and 2-mercaptoethanol-reduced serum. Serum was reduced by the method of Osebold and Aalund (6). The serum was diluted 1:6.25 with saline. The 2-mercaptoethanol was diluted 1:4 with distilled water, and one drop was added to 1 ml of the diluted serum. Twenty-eight drops per ml were formed at the aperture of the dropper to approximate 0.1 M 2-mercaptoethanol in the serum solution. Tubes were stoppered and incubated at 37 C for 30 min.

Test procedure. Twofold serial dilutions of both reduced and untreated sera were prepared in 0.85% saline in 0.25-ml volumes. Then an equal volume of antigen diluted 1:20 in saline was added. Therefore the final serum dilution in the first tube was 1:12.5. We found volumes of 0.25 ml for diluted serum, diluent, and antigen to be satisfactory in tubes 12 by 75 mm. The standard incubation conditions were 50 C

TABLE 1. *Geographical location, age, and sex of sources of "normal" human sera*

U.S. geographical area	1-9[a]		10-19		20-29		30-39		40-49		50-59		60-69		Over 70		Total/area	
	M[b]	F[b]	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
North East		1		1	2					1		1			1		3	4
Middle Atlantic				1	1	1	1	1	2			2	1	1	1		6	6
East North Central						4		3	5	5		1					5	13
West North Central	2		1	1		1	2	1	1	2	5			1			11	6
South Atlantic		7	11	1			1					5	4	2	1		17	15
East South Central	1			3			1			1	2	1		1		2	4	8
West South Central	3		3	10	7	3	10	4					2	4			25	21
Mountain					1	2		1			1	1	1	1	1	1	4	6
Pacific	3			1		1					3		2	1	2		10	3
Puerto Rico								1										1
Total/age group	9		15	18	11	12	15	11	8	9	11	11	10	11	6	3		
Total M and F																	85	83

[a] Age group in years.
[b] Sex.

TABLE 2. *Geographical location, age, and sex of sources of possible positive human sera*

U.S. geographical area	Neonates	1-19[a]		20-29		30-39		40-59		60-69		Over 70		Unknown		Total/area		
		M[b]	F[b]	M	F	M	F	M	F	M	F	M	F	M	F	M	F	Neonates
North East	1			1			1					1		1	2	3	3	1
Middle Atlantic	9		1	1	1	1		4		1		1		3	1	11	3	9
East North Central	4				2		2	3						1	2	4	6	4
West North Central														1		1		
South Atlantic						1				1				1	4	3	4	
East South Central	1				1										3		4	1
West South Central	1			1	2	1	2		1		1	1		1	2	4	8	1
Mountain	1					1				1						2		1
Pacific		1	1		2									4	1	5	4	
Puerto Rico		1														1		
Total/age group	17	2	2	3	8	4	5	7	1	3	1	3		12	15			
Total M, F, and neonates																34	32	17

[a] Age group in years.
[b] Sex.

for 2 hr followed by 4 C for 24 hr. Tests were read by tapping the tubes and then holding them against a black background near the hood of a fluorescent lamp for viewing. Titers were recorded as the highest dilution with 2+ or stronger agglutination. All agglutination reactions were definite, and therefore centrifugation was not necessary to facilitate reading.

Serum absorption. Whole sera that cross-reacted with the trypsinized *S. aureus* antigen were absorbed as follows. A 2-ml amount of the trypsinized *S. aureus* diluted 1:20 was centrifuged, and the supernatant fluid was drawn off. The *S. aureus* pellet was mixed with 2 ml of the serum diluted 1:6.25. This mixture was incubated at 50 C for 2 hr and then refrigerated overnight at 4 C. The next day the serum was centrifuged, separated from the agglutinated pellet, and then retested with the *L. monocytogenes* and *S. aureus* antigens. After the tubes were incubated at 50 C and held at 4 C overnight, they were read, centrifuged for 10 min at 2,000 rev/min, and reread, and titers were recorded.

Immunoelectrophoresis. Selected human sera were subjected to electrophoresis in 0.05 M sodium barbital buffer, pH 8.6, on an LKB (LKB-Produkter AB, Stockholm, Sweden) apparatus. Glass slides were layered with 1.5% Noble agar in 75 ml of the sodium barbital buffer and 25 ml of distilled water. Electrophoresis was carried out at room temperature at 250 v for 50 min. After electrophoresis, sera were diffused against concentrated listeria antigen and goat anti-human IgM. Slides were incubated at 25 C overnight.

RESULTS

Although smooth trypsinized antigens could be produced from all five serotypes of *L. monocytogenes*, strains 1b and 4b consistently produced heavier growth than did strains 1a, 2, and 4d. Therefore, antigens 1b and 4b were used as the primary screening antigens. Before being tested with human sera, antigens were checked for specificity and titer with whole and 2-mercaptoethanol-reduced rabbit antisera to factors 1a, 4b, and 4d (Table 3). Antigens 1a, 1b, 2, and 4d appeared to be specific for the corresponding 1a and 4d whole antisera. The reduction of these antisera had no apparent effect on the titer, which indicated that these antibodies were IgG. The 4b antigen reacted with all three whole antisera, but it reacted with only the 4b and 4d reduced antisera. Apparently there were some IgM antibodies to a 4b factor in the 1a antiserum as well as IgG antibodies to factors 1a, 1b, and 2.

The degree of cross-reactivity of the trypsinized listeria antigens 1b and 4b was determined with normal human sera, both whole and reduced with 2-mercaptoethanol. When the 1b antigen was reacted with whole normal sera, it gave titers ranging from <12.5 to 100, with a geometric mean titer (GMT) of 25. Of the 168 untreated sera tested, 95.6% had a titer of 50 or less with the 1b antigen. Nine sera had titers of 100 with the 1b antigen for a rate of cross-reaction of 5.4% (We considered a titer of 100 or greater positive for listeriosis as suggested by Seeliger and Potel [13].) The subjects from which these nine sera were drawn appeared to be unrelated according to age, sex, or geographical location. When the untreated normal sera were reacted with the 4b antigen, the GMT was 14, with a range of titers of <12.5 to 50. When the normal sera were reduced with 2-mercaptoethanol, 167 of the 168 sera had titers <12.5. The one serum that con-

tained mercaptoethanol-resistant antibodies (IgG) for the 1b antigen had been positive with the 1b antigen in the whole state. Unfortunately, a history on this subject was not available.

The specificities of the trypsinized listeria antigens 1a, 1b, 4b, and 4d were determined with human sera from diseases other than listeriosis (Table 4). The GMT for the 46 whole sera tested was 16 for antigen 1a, 18 for 1b, 10 for 4b, and 8 for 4d. Three of the 46 sera cross-reacted at a dilution of 1:100; the rate of cross-reaction was 6.5%. Two of these sera were from cases of melioidosis and the other from a case of brucellosis. All three cross-reactions were with either the 1a or 1b antigens, or both, but not with the 4b or 4d antigens. When the 46 sera were reduced, no titers remained to the listeria antigens.

Antigens 1a, 1b, 2, 4b, and 4d were tested with the 83 sera submitted from culture-positive cases of listeriosis (Table 2). Of the 83 untreated sera tested, 50 had titers of 100 or greater. Thirty sera were positive only with antigens 1a, 1b, and 2; fifteen sera were positive only with antigens 4b and 4d; and five sera were positive with all five antigens. The GMT of the positive sera with each of the antigens is listed in Table 5. Thirty-three of the sera failed to react with any of the antigens. Of these 33 sera, 17 were from neonates with an average age of 15 days, nine were from patients with underlying malignant diseases, and three were acute sera from patients with listeria infections. The convalescent sera from these three patients were positive with the listeria antigens. Unfortunately, histories were unavailable on the other four patients whose sera failed to react. The 50 sera that did give

TABLE 3. *Effect of 2-mercaptoethanol reduction on rabbit antisera*

Antigen	Antiserum	GMT[a] with whole antiserum	GMT[a] with reduced antiserum
1a	1a	400[a, b]	400
	4b	0[c]	0
	4d	0	0
1b	1a	200	200
	4b	0	0
	4d	0	0
2	1a	200	200
	4b	0	0
	4d	0	0
4b	1a	100	0
	4b	200	400
	4d	200	100
4d	1a	25	25
	4b	50	50
	4d	200	100

[a] Geometric mean titer (GMT) where titer is the dilution factor of the end-point dilution.

[b] For calculations, log of 6.25 used for titers less than 12.5.

[c] Zero titer equals less than 2+ at a serum dilution of 1:12.5.

TABLE 4. *Specificity of listeria antigens, serotypes 1a, 1b, 4b, and 4d, with human sera from diseases other than listeriosis*

Disease	No. of sera tested	GMT[a] original disease	Range	GMT for listeria antigen (range)			
				1a	1b	4b	4d
Brucellosis	6	113	20–2,560	18 (0–50)	37 (0–100)	9 (0–25)	7 (0–12.5)
Cholera	4	1,618	1,280–20,480	7 (0–12.5)	8 (0–25)	0 (0)	0 (0)
Leptosporosis	2	5,790	3,200–10,240	13 (0–25)	18 (0–50)	0 (0)	0 (0)
Melioidosis	16	79	20–5,120	26 (0–100)	26 (0–100)	13 (0–50)	9 (0–50)
Mononucleosis	3	Unabsorbed 508	160–2,560	10 (0–25)	10 (0–25)	8 (0–12.5)	0 (0)
Paratyphoid	1	Ha 80 Hb 20		0 (0)	0 (0)	0 (0)	0 (0)
Rat Bite Fever	1	5,120		25 (25)	25 (25)	0 (0)	0 (0)
Rickettsial infections	5	OX_{19} 61 OX_2 53 OX_K 12	0–640 20–80 0–20	11 (0–12.5)	11 (0–12.5)	8 (0–12.5)	8 (0–12.5)
Streptococcal infections	2	ASO 680	680	9 (0–12.5)	9 (0–12.5)	0 (0)	0 (0)
Tularemia	3	80	10–320	13 (12.5–25)	13 (12.5–25)	10 (0–25)	10 (0–25)
Typhoid	1	Ho 1,280		25 (25)	25 (25)	0 (0)	0 (0)
Typhoid	2	Vi 452	160–12,80	0 (0)	0 (0)	0 (0)	0 (0)

[a] See Table 3 for footnotes.

positive reactions with the listeria antigens were reduced with 2-mercaptoethanol and then reacted with antigens 1b and 4b. Only three reduced sera had persistent IgG titers. Two of these three sera were positive with the 4b and 4d antigens before reduction and retained a titer of 25 after reduction. Before reduction, the third serum was positive with all five antigens, and, after reduction, it retained a titer of 50 with the 4b antigen.

Since no sera were available from patients with *S. faecalis* or *S. aureus* infection, 50 untreated normal sera and 50 listeria-positive untreated sera were tested against antigens of *S. faecalis* and *S. aureus* that were prepared in the same way as the listeria antigens. When reacted with the *S. faecalis* antigen, the normal sera had a GMT of 13; the range of titers was from <12.5 to 25. The listeria-positive sera, when reacted with the *S. faecalis* antigen, had a GMT of 15; the range of titers was from <12.5 to 25. On the other hand, when reacted with the *S. aureus* antigen, the normal sera had a GMT of 18 and a range of from <12.5 to 100. Three normal sera had titers of 100 with the *S. aureus* antigen for a cross-reaction rate of 6%. The listeria-positive sera, when reacted with the *S. aureus* antigen, had a GMT of 25 with a range in titers from <12.5 to 100. Five of the listeria-positive sera had titers of 100 for a cross-reaction rate of 10%. To alleviate this large percentage of cross-reactions, we absorbed sera with the *S. aureus* antigen as previously described. After absorption and retesting with both the *S. aureus* and *L. monocytogenes* antigens, tubes were read, and the titers were recorded before and after centrifugation (Table 6). The three normal sera that had titers of 100 before absorption had titers of <12.5 after absorption and centrifugation. The five listeria-positive sera which were also positive with the *S. aureus* antigen remained positive for listeria after absorption, while their titers for *S. aureus* were reduced to <12.5 to 50. In general, the end-point dilutions of the listeria-positive sera with the corresponding listeria antigen were one to two dilutions higher after absorption and centrifugation than before.

TABLE 5. *Geometric mean titers (GMT) of positive listeria sera with specific antigens*

No. positive sera	Antigen	GMT	Range of titers
30	1a	171	100–400
30	1b	155	100–400
30	2	171	100–400
15	4b	210	100–800
15	4d	183	100–400
	1a	115	100–200
	1b	115	100–200
5[a]	2	115	100–200
	4b	264	100–800
	4d	229	100–800

[a] Positive with all five antigens.

The immunoelectrophoresis of selected positive and negative untreated normal sera and sera from confirmed cases of listeriosis revealed two facts. First, the sera of neonates of mothers with confirmed listeriosis had little if any precipitable IgM antibodies when reacted with the anti-human IgM antiserum. And second, all the human sera failed to produce precipitin bands with the trypsinized listeria antigens, which indicates that the agglutination observed was probably an IgM reaction.

DISCUSSION

Unlike Seeliger and Emmerling (12), we found only 5.4% of the untreated normal sera to yield titers of 100 to the listeria 1b antigen, and no serum gave a titer greater than 50 with the 4b antigen. Perhaps the differences in the antigens used account for the disparity in results. In a preliminary study in which we compared nontrypsinized antigens with trypsinized antigens, we confirmed the finding of

TABLE 6. *Effect of absorption and centrifugation on titer*

Serum	Antigen	GMT before absorption (range)	GMT after absorption (range)	GMT after absorption and centrifugation (range)
Normal	*S. aureus*	18 (0–100)	7 (0–25)	11 (0–50)
Listeria-positive	*S. aureus*	25 (0–100)	9 (0–50)	17 (0–50)
Normal	Listeria 1b	25 (0–100)	8 (0–50)	14 (0–100)
Listeria-positive	Listeria 1b	155 (100–400)	105 (100–400)	210 (100–800)
Normal	Listeria 4b	14 (0–50)	7 (0–25)	10 (0–25)
Listeria-positive	Listeria 4b	210 (100–800)	121 (100–400)	332 (100–800)

Osebold, Aalund, and Chrisp (7) that trypsinization of the listeria antigen increases its specificity. With untreated human sera from diseases other than listeriosis, the antigens still appeared to be specific. In general, the antigens of serotypes 4b and 4d were less cross-reactive than those of serotypes 1a, 1b, and 2. However, because of the formulation of the O factors of the listeria cells, it is necessary to use both 1b and 4b antigens to detect listeria antibodies.

Seeliger and Potel (13) found that the O titer values of serum in cases of clinical illness often lie in the range of 100 to 160 and higher. We found 60% of the sera from confirmed cases of listeriosis to give titers of 100 or greater. Of the untreated sera that did not react with the listeria antigens, 51% were from neonates. Seeliger (10) and Seeliger and Potel (13) stated that no titers are generally detectable in neonates. We were unable to detect a precipitable amount of IgM in the sera of neonates by immunoelectrophoresis or immunodiffusion with anti-human IgM. This lack of IgM along with early antibiotic treatment probably explains the failure of these sera to agglutinate. Twenty-seven percent of the sera that failed to react were from patients with underlying malignant diseases. Tripathy and Mackaness (15) found that the administration of cancer chemotherapeutic agents suppressed the immune response of mice infected with *L. monocytogenes*. These investigators inferred that this suppression of the immune response also occurs in human chemotherapeutically treated cancer patients. Therefore, antibodies to concurrent *L. monocytogenes* infections might not be expected in the sera of these patients.

Reduction of human sera with 2-mercaptoethanol did not resolve the cross-reactive problem as it did with animal sera (1, 6). Only three positive whole human sera had titers remaining after reduction. Histories of the three patients listed no previous infections with *L. monocytogenes*, and only one patient had prior contact with animals or raw dairy products. The IgG response in these patients therefore remains unexplained.

The absorption of cross-reacting whole human sera with *S. aureus* antigen eliminated the cross-reactions in this agglutination test. Seeliger and Potel (13) suggested neutralizing sera with the cross-reactive antigen before using the complement fixation test for the detection of listeriosis, but apparently they did not extend this absorption procedure to the agglutination test.

The listeria antigen prepared as described by Osebold, Aalund, and Chrisp (7) and modified here can be used in a diagnostic test for human listeriosis if certain conditions are met. (i) All sera must be tested against an antigen with the O formulation of either serotype 1a, 1b, or 2 and with an antigen with the O formulation of either serotype 4a, 4b, 4c, 4d, or 4e. (ii) All sera must be tested with an antigen prepared from *S. aureus*, and, if cross-reactions appear, the sera should be absorbed and the test repeated. (iii) As in all serological procedures, both acute and convalescent sera should be tested, because the precise level of a "diagnostic" titer cannot be defined.

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