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Purification of a Surface-Specific Soluble Antigen from *Listeria monocytogenes*

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A complex antigenic preparation obtained from *Listeria monocytogenes* serovariant 4b by freeze-pressing, centrifugation, and gel filtration treatment was studied by crossed immunoelectrophoresis, with the aim of preparing an antigenic fraction that could be used to investigate the serological response to listeric infection. Of 17 immunoprecipitates revealed in the soluble extract, one of three major antigens (designated antigen 2) was shown to be a strong antigen in humans or rabbits infected with *L. monocytogenes* serovariant 4b. A monospecific anti-antigen 2 serum was obtained and used to prepare a serologically homogeneous antigen by immunoadsorption. Antigen 2, most probably located on the bacterial surface, is common to all serovariants of *L. monocytogenes* and to *Listeria grayi* and is not shared by the main bacterial species known to have common antigens with *L. monocytogenes*.

Antigens of *Listeria monocytogenes* have been extensively studied by agglutination, precipitation, hemagglutination, and other serological tests (8, 20). Such studies have characterized the somatic and flagellar antigenic factors used in serotyping *L. monocytogenes* (7, 14, 20). The finding that *L. monocytogenes* shares common antigen(s), including the Rantz antigen (14), with many gram-positive bacteria has complicated the antigenic scheme. Such an antigenic diversity may to some extent account for the problems encountered in interpretation of serological results (8, 20). In many instances, anti-*Listeria* antibodies have been observed in apparently normal subjects, and patients with confirmed listeric infection do not have elevated antibody levels (8). The identification and purification of specific antigenic fractions of *L. monocytogenes* appear, therefore, to be a desirable approach to the performance of a reliable serological test.

In the present study, antigen preparations from *L. monocytogenes* were analyzed by crossed (two-dimensional) immunoelectrophoresis and related techniques which have recently been shown to reveal the antigenic complexity of several microorganisms by increasing the resolution of the immunoprecipitate patterns (6, 9, 10, 12, 16, 18, 23-26). The analysis permitted the identification of a major soluble antigen, which was subsequently purified. The antigen, probably located at the cell surface, was found to be shared by all serovariants of *L. monocytogenes* and by *Listeria grayi*.

MATERIALS AND METHODS

Antigens. (i) Bacterial strains. *L. monocytogenes* serovariants (serovar.) 1/2a, 1/2b, 3a, 3b, 3c, 4ab, 4a, 4b, 4c, 4d, 4e, 4f, 4g, 5, 6, and 7 and *L. grayi* (collection of H. P. R. Seeliger) were kindly supplied by A. L. Courtieu (Unité d'Enseignement et de Recherche de Médecine, Nantes, France). *Streptococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Corynebacterium pseudodiphthericum* were isolated in this laboratory.

(ii) Bacterial cultures. Bacteria were inoculated from tryptose agar slant cultures into 1 liter of SP 2 medium (peptones, 22 g; monobasic potassium phosphate·$7H_2O$, 18 g; dibasic potassium phosphate, 2 g; yeast extract, 4 g; water, 1,000 ml; pH 7.3) and incubated at 37°C for 17 h. The 1-liter culture was used to seed a 20-liter Biolaffite fermentor (Gourdon, Poissy, France) containing 15 liters of SP2 medium. Incubation was at 37°C for 24 h with constant shaking (50 rpm). During the incubation period, the medium was aerated with 1 volume of air per volume of medium per min. The bacteria were collected by continuous centrifugation at 10,000 × *g* at a rate of 20 liters/h in a Westphalia centrifuge (Clarificatrice KDD 600) and stored at −20°C. By using this procedure, approximately 100 g (wet weight) of bacteria was obtained with *Listeria*, *Staphylococcus*, and *Bacillus*, and 50 g was obtained with *Streptococcus* and *Corynebacterium*.

(iii) Preparation of soluble antigens. Before treatment, bacteria were washed three times in 0.85% saline solution to eliminate traces of medium. The bacterial mass was passed five times through a freeze-press cell (X-Press, LKB-Biotech) at 28,000 lb/in^2 and was homogenized in a Virtis apparatus for 5 min at 2,000 rpm to obtain the crude extract (CE). The CE was centrifuged at 600 × *g* for 20 min to pellet the

cells. The supernatant, referred to as crude soluble extract (CSE), was tested for sterility before centrifugation at 40,000 × *g* for 1 h at 4°C in an SS-34 rotor (Sorval RC2-B centrifuge). At each step, samples of antigen-containing solution were dialyzed against distilled water for 16 h at 4°C and lyophilized for storage. About 200 mg (dry weight) of CSE was obtained from 20 g (wet weight) of bacteria.

Hyperimmune sera. Hyperimmune sera were produced in male New Zealand white rabbits (Janvier, le Genest, France) weighing about 3.5 kg. All animals were controlled for absence of precipitating and agglutinating anti-*Listeria* antibodies.

Some rabbits were injected intradermally with 10^9 heat-killed bacteria in 1 ml of 0.85% saline solution mixed with 1 ml of incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.) to obtain the following immune sera: anti-*L. monocytogenes* serovar 4b; anti-*L. monocytogenes* serovar 1/2a; anti-*S. faecalis*; anti-*S. aureus*; anti-*S. epidermidis*; anti-*B. subtilis*; anti-*C. pseudodiphthericum*.

Other rabbits received intradermal injections containing 2 mg of the CE from *L. monocytogenes* serovar 4b (anti-CE 4).

Injections by the same route were repeated after 3 weeks. Beginning 1 week after the final injection, the rabbits were bled at monthly intervals, and sera were stored at −20°C.

Monospecific antiserum preparation. The preparation of monospecific antiserum was based on the finding of Shivers and James (22) that individual precipitin bands formed in agar gel diffusion plates may be used for the production of specific antisera against a single antigen in a complex mixture. The procedure used was basically that described by Vestergaard (27). The antigen mixture was submitted to crossed immunoelectrophoresis against polyvalent antiserum. The gel was exhaustively washed in 0.85% saline solution. The upper one-quarter of a selected peak was cut from 10 gels and placed in 3 volumes of saline per volume of gel. The gel suspension was sonicated, mixed with adjuvant, and intradermally injected into rabbits. Injections were repeated after 3 weeks, and the rabbit(s) were bled 2 weeks after the last inoculation. The antiserum obtained was absorbed against agarose before use.

Human sera. Human sera were separated into potential positive and negative sera. Positive sera were collected from patients with confirmed listeriosis between 15 to 30 days after positivity of blood cultures. Negative sera were obtained from healthy individuals and patients with other diseases.

Immunological methods. (i) Double-diffusion assay. For the double-diffusion assay, a microscope slide assay using 1% barbital-buffered agarose was used, as described by Ouchterlony (15). Wells were filled with antiserum or antigen solutions and incubated at 4°C for 48 h. Slides were treated with 5% citrate for 1 h, washed in saline, dried, and stained with amido black 10B.

(ii) Immunoelectrophoresis. Immunoelectrophoresis was carried out according to Scheidegger (19). Electrophoresis was performed at 10 V/cm for 1 h in a 1% agarose gel buffered with barbital, pH 8.6. Antigen-containing fractions were detected with unconcentrated or five-times-concentrated sera. Slides were washed and stained as indicated above.

(iii) Crossed immunoelectrophoresis. An adaptation of the procedure described by Weeke (29) was employed.

(a) First dimension. Samples (5 to 20 μl) were applied in wells punched in an agarose gel (four wells on a 90- by 110- by 1.5-mm glass plate, corresponding to 15 ml of 1% agarose). Electrophoresis was carried out at 10 V/cm for 60 min in an agarose gel buffered with 0.02 M barbital, pH 8.6. The electrophoresis tank was cooled to 15°C by tap water circulation. After the first-dimension electrophoresis, the gel was divided into four slabs, each corresponding to one well.

(b) Second dimension. Each of the four first-dimension gel slabs was then transferred to the edge of a glass plate, and the remaining part of the plate was filled with antibody-containing agarose (500 to 750 μl of antiserum mixed with 13 ml of 1% agarose solution maintained in a 52°C water bath). After electrophoresis for 16 h (overnight) at 3 V/cm and 15°C, the gel was washed in saline, pressed, dried, stained with Coomassie brilliant blue R-250 (0.5% in 50% ethanol–10% acetic acid), and destained in 10% acetic acid–50% ethanol.

(iv) Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed by the method of Axelsen (2). Electrophoresis was carried out in an agarose gel buffered with barbital (pH 8.6) and containing monospecific antiserum. The samples were added to the wells with the current on (2 V/cm). After application of all samples, the electrophoresis was continued for 16 h. The gel was then treated as indicated above for crossed immunoelectrophoresis. The quantitation of antigen was based upon the height of the antigen-antibody precipitin peaks.

(v) Indirect immunofluorescence. Suspensions (optical density at 650 nm, 10) of *L. monocytogenes*, fixed on slides with absolute alcohol, were incubated at 37°C for 30 min with a 1:200 dilution of anti-antigen 2 serum. The preparations were washed with phosphate buffer, pH 7.6, and incubated at 37°C for 30 min with a 1:5 dilution of sheep anti-rabbit immunoglobulin fluorescent conjugate. Controls consisted of preimmunization rabbit serum and fluorescent serum in the presence and absence of anti-antigen 2 serum.

Biochemical methods. (i) Gel filtration chromatography on acrylamide-agarose. A glass column containing acrylamide-agarose ACA/44 was equilibrated with 500 ml of 0.01 M tris(hydroxymethyl)aminomethane–0.1 M NaCl buffer, pH 8.2. The flow rate was maintained at 8 ml/h. Molecular weight calibration of the column was done by using a Combitek kit (size 1). A 1-ml amount of buffer containing 30 mg of CSE was applied to the column with the same flow rate. The eluate was analyzed by absorbance at 280 nm. Fractions were pooled and concentrated by filtration on a UM-10 membrane in an Amicon cell (Amicon SARL, Ivry-sur-Seine, France).

(ii) Immunosorbent. Immunoglobulins of monospecific antiserum were precipitated in 33% ammonium sulfate. A 40-mg amount of immunoglobulins was coupled to 1 g of swollen cyanogen bromide-activated Sepharose 4B by the method of Axen et al. (4). A 4-mg amount of antigen extract was then allowed to

react with the immunosorbent for 1 h at room temperature. After washing, the column was eluted with 0.2 M glycine-hydrochloride buffer, pH 2.8. The eluate was dialyzed overnight against phosphate buffer, pH 7.2, and lyophilized.

Chemicals and reagents. Agarose (Indubiose; batch C 321) and acrylamide-agarose ACA/44 were obtained from l'Industrie Biologique Française, Clichy, France; peptones, yeast extract, and culture media were purchased from Bio-Service Institut Pasteur Lille, Lille, France; sheep anti-rabbit immunoglobulin fluorescent conjugate was obtained from Institut Pasteur Production, Paris, France; Combitek kit (size 1) was purchased from Boehringer, Mannheim, West Germany; and Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden.

RESULTS

Antigenic analysis of *L. monocytogenes* serovar. 4b CSE. *L. monocytogenes* serovar 4b was chosen for the study as almost all listeric infections diagnosed in the laboratory were caused by this serovariant. Before any investigation, it was necessary to search for human precipitating antibodies against the *L. monocytogenes* serovar 4b CSE. This was achieved by testing 50 suspected positive and 40 suspected negative human sera. All positive sera gave a strong precipitin line by double-diffusion tests against the CSE. In contrast, no precipitin line appeared with all negative sera. As strongly reactive positive human sera were not often available, five anti-whole bacteria and three anti-CE rabbit sera were prepared and used to perform CSE antigen analysis by crossed immunoelectrophoresis. Nine peaks were observed with the anti-whole bacteria serum (Fig. 1). Three, designated A, B, and C, were easily recognizable. This pattern differed slightly from one serum or extract to another, but peak B was always markedly evident. Seventeen peaks were observed with anti-CE serum, with three major peaks, designated 1, 2, and 3, present (Fig. 2). This pattern varied somewhat from extract to extract with a major peak always appearing in the peak 2 migration region. Cross-reactivity between the anti-whole bacteria serum and the anti-CE serum with CSE as antigen was studied by using crossed immunoelectrophoresis with an intermediate gel. The two antisera were compared by running two plates which were identical except for the absence of antiserum in the intermediate gel of the control plate. Figure 3 shows the plate with the anti-whole bacteria serum in the intermediate gel and the anti-CE serum in the revealing gel. The three major precipitates in the intermediate gel correspond to peaks A, B, and C in Fig. 1, whereas precipitates 1 and 2 (Fig. 2) are absent from the reference gel. This pattern demonstrates a reaction of identity between precipitates A and 1 and between B and 2.

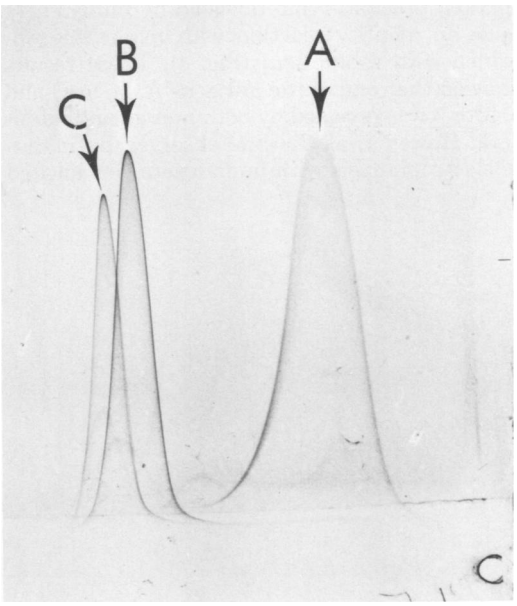

FIG. 1. *Crossed immunoelectrophoresis of L. monocytogenes 4b CSE against anti-whole L. monocytogenes 4 serum. Antigen, 10 μl of a 20-mg/ml solution; antiserum, 6 μl/cm². Nine precipitates can be seen, with the main antigens designated A, B, and C.*

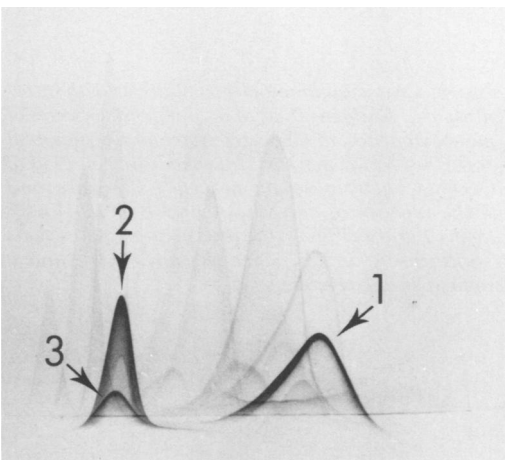

FIG. 2. *Crossed immunoelectrophoresis of L. monocytogenes 4b CSE against anti-L. monocytogenes 4 CE serum. Antigen, 10 μl of a 20-mg/ml solution; antiserum, 9 μl/cm². Seventeen precipitates can be seen, with the main antigens designated 1, 2, and 3.*

Attempts to detect the antigen fraction which precipitated with human antibodies were carried out. Double-diffusion tests with rabbit antisera (anti-whole bacteria and anti-CE) and positive human sera against CSE showed that the

marked precipitin line revealed by human sera gave an identity reaction with major line obtained with rabbit sera (Fig. 4). These results showed that one of the antigens A, B, or C and 1, 2, or 3 was revealed by both human and rabbit sera. However, as the line observed by immunoelectrophoresis with human sera was located in the same region as peaks 1 and 2 and peak B (data not shown), it appeared plausible that the precipitate corresponded to peak B or 2.

These data suggest that the *L. monocytogenes* serovar 4b CE contains a fraction, corresponding to peak B or 2, which is present in large amounts in the bacteria or is highly antigenic in both humans and rabbits.

Preparation of anti-antigen 2 monospecific serum. Since antigen 2, corresponding to peak 2, seemed to be the most interesting fraction to study, a monospecific anti-antigen 2 serum was prepared for easy detection of the antigen during purification. *L. monocytogenes* serovar 4b CSE (0.2 mg [dry weight] per plate) was submitted to crossed immunoelectrophoresis against the anti-whole bacteria serum, and the immunoprecipitate B (Fig. 1) was removed from the gels and utilized for immunization of rabbits as described above. The antiserum was tested against *L. monocytogenes* CSE by crossed immunoelectrophoresis. Figure 5 shows that the antiserum contained a high concentration of antibodies exclusively directed against antigen B or 2. Double-diffusion tests and crossed immunoelectrophoresis carried out with the antiserum against CSEs from *S. faecalis*, *S. aureus*, *S. epidermidis*, *B. subtilis*, and *C. pseudodiphtheriticum* showed no precipitate.

Purification of antigen 2. Before purification, it was necessary to demonstrate that antigen 2 was present in the supernatant after centrifugation of CSE at 40,000 × *g* for 1 h.

(i) Acrylamide-agarose gel filtration chromatography. The elution curve of the 40,000-×-*g* supernatant from an ACA/44 column is shown in Fig. 6. The relative amount of antigen in each fraction was determined by rocket immunoelectrophoresis with the monospecific anti-antigen 2 serum. Fractions 30 to 52, which contained antigen 2, corresponded to a molecu-

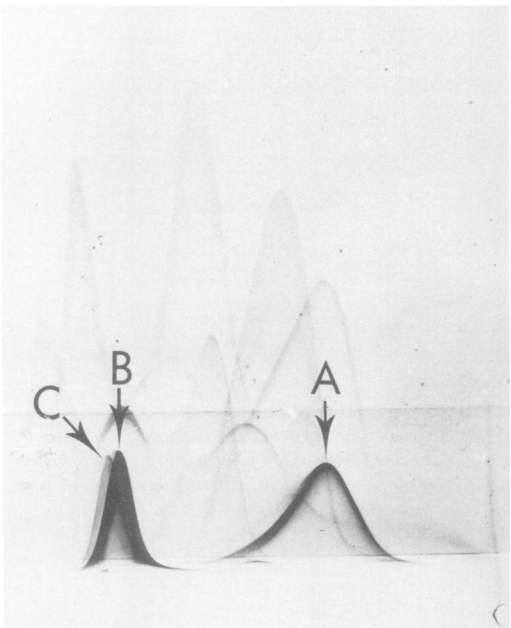

FIG. 3. *Crossed immunoelectrophoresis with intermediate gel. Antigen, 10 μl of a 20-mg/ml solution of L. monocytogenes 4b CSE; reference antiserum, 9 μl of anti-CE 4 serum per cm²; intermediate gel, 17 μl of anti-whole bacteria serum per cm². Compare and note the identity of antigens A and B (Fig. 1) with antigens 1 and 2 (Fig. 2); the intermediate gel reveals the antigens A and B, since the antigens 1 and 2 disappear in the reference gel.*

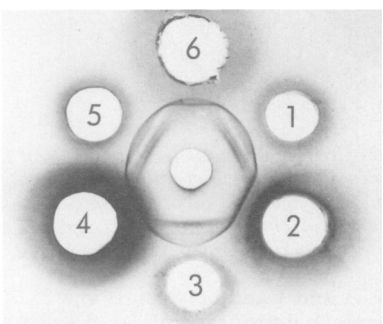

FIG. 4. *Agar gel double-diffusion test. Center well, L. monocytogenes serovar. 4b CSE. Wells 1, 3, and 5, anti-CE 4 serum; wells 2, 4, and 6, five-times-concentrated positive human sera.*

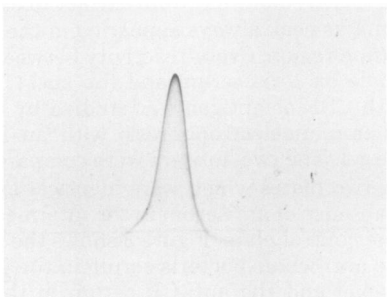

FIG. 5. *Crossed immunoelectrophoresis of L. monocytogenes 4b CSE against rabbit anti-antigen 2 serum, showing the specificity of this antiserum. Antigen, 10 μl of a 20-mg/ml solution; antiserum, 6 μl/cm².*

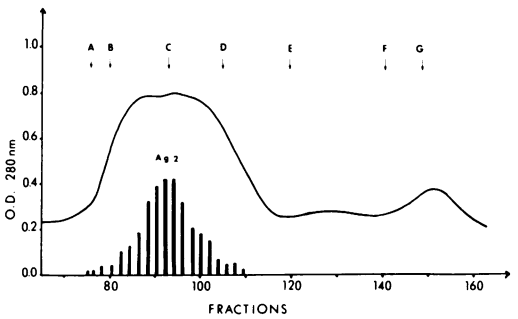

FIG. 6. *Chromatographic elution pattern on ACA/44 of 40,000-×-g CSE supernatant in tris(hydroxymethyl)aminomethane-NaCl buffer, pH 8.2. The flow rate was 8 ml/h; the glass column was 1 meter by 15 cm. Each fraction contained 2 ml. The height of each black bar corresponds to the rocket peaks obtained by testing the eluted fractions against anti-antigen 2 monospecific serum. The arrows indicate the elution points of the reference standards determined previously in the same column: A, exclusion; B, catalase (molecular weight, 240,000); C, aldolase (158,000); D, bovine albumin (67,000); E, ovalbumin (45,000); F, chymotrypsinogen (25,000); G, cytochrome c (12,500). O.D., Optical density.*

lar weight of approximatively 160,000, as indicated by the Combitek kit. Fractions were pooled and were five times concentrated by filtration. When this procedure was used, 200 mg of CSE yielded approximately 30 mg (dry weight) of material. A relative substantial enrichment of antigen 2 was noticed by comparing crossed immunoelectrophoresis patterns obtained with material before and after gel filtration with anti-whole bacteria and anti-CE sera. Nevertheless, the antigen 2 preparation was still contaminated with other antigens.

(ii) Anti-antigen 2 immunosorbent. An anti-antigen 2 immunosorbent was prepared with the monospecific anti-antigen 2 serum and allowed to react with the concentrated material separated by chromatography. The eluate of the anti-antigen 2 immunosorbent was tested by crossed immunoelectrophoresis, using anti-CSE serum in the indicating gel and anti-antigen 2 serum in the intermediate gel (Fig. 7). The pattern obtained showed that antigen 2 was obtained in a form apparently cleared of all antigenic contaminants except for a small nonmigrating peak which may represent low-solubility antigen. The approximate yield of antigen 2 was 0.4 mg from 4 mg of the material separated by chromatography.

Location of antigen 2 in the bacterial cell. Since whole bacteria induced anti-antigen 2 antibodies, it could be expected that this antigen might be a surface component. To test this hypothesis, anti-antigen 2 serum was reacted against fixed *L. monocytogenes* serovar. 4b and stained with a fluorescent anti-rabbit serum. Controls were carried out as indicated above. Figure 8 shows a marked fluorescence located on the bacterial surface. Control tests were negative (data not shown).

Specificity of antigen 2. As pointed out above, the anti-antigen 2 serum did not react with CSEs from *S. aureus*, *S. epidermidis*, *S. faecalis*, *B. subtilis*, and *C. pseudodiphtheriticum*, and conversely, purified antigen 2 was not precipitated by the following rabbit antisera: anti-*S. aureus*, anti-*S. epidermidis*, anti-*S. fae-*

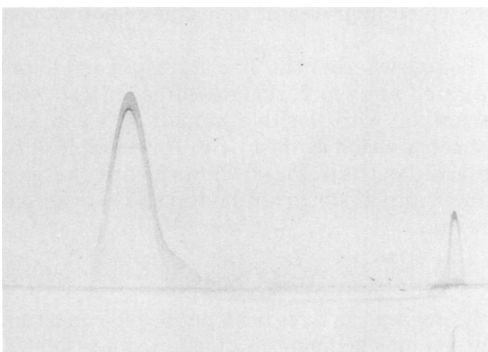

FIG. 7. *Crossed immunoelectrophoresis. Antigen, 40 μl of a 1-mg/ml antigen 2 immunosorbent eluate; intermediate gel, anti-antigen 2 serum (6 μl/cm²); reference gel, anti-crude extract serum (9 μl/cm²). Note that the peaks obtained with the two sera indicate immunological purity since the second peak shows identity with the first.*

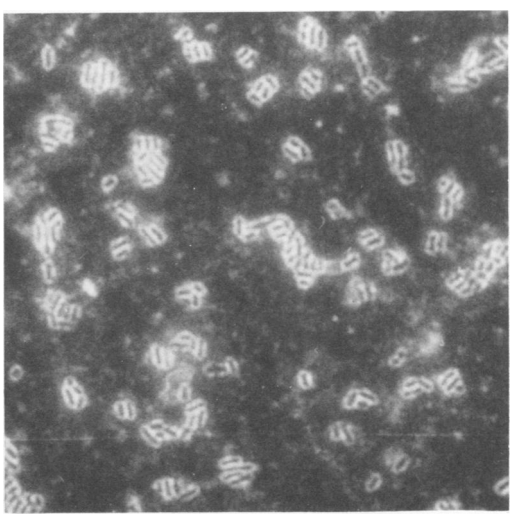

FIG. 8. *Indirect immunofluorescence. Antigen, whole L. monocytogenes serovar 4b; reference serum, anti-antigen 2 serum (1/200); conjugate, sheep anti-rabbit fluorescent immunoglobulin (1/5). ×1,500.*

calis, anti-*B. subtilis*, and anti-*C. pseudodiphthericum*. CSEs of the *Listeria* strains listed above were tested against the anti-antigen 2 serum by crossed immunoelectrophoresis. Precipitation patterns showed that antigen 2 was present in all serovariants of *L. monocytogenes* and in *L. grayi* (data not shown). The results strongly suggest that antigen 2 is genus specific for *Listeria*.

Relationship between human antibodies and anti-antigen 2 serum. It was observed that positive human sera tested against CSE from *L. monocytogenes* serovar 4b by immunoelectrophoresis produced a distinct precipitation line which migrated in the same region as peak 2.

To test whether this line corresponded to precipitated antigen 2, double-diffusion tests were performed with positive human sera and anti-antigen 2 serum against CSE. An example of the observed pattern (Fig. 9) shows identity between human anti-*Listeria* antibodies and anti-antigen 2 serum.

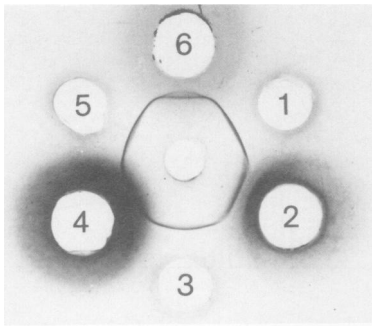

FIG. 9. *Agar gel double-diffusion test. Center well, L. monocytogenes serovar 4b CSE; wells 1, 3, and 5, monospecific anti-antigen 2 serum; wells 2, 4, and 6, five-times-concentrated positive human sera.*

DISCUSSION

Many reports have been published concerning the serological diagnosis of listeric infections (8). Serological findings are, however, still difficult to interpret (8, 20), as *L. monocytogenes* may cross-react with a number of commonly occurring bacteria (8, 17, 20). Since serological methods currently employed use preparations composed of many antigenic determinants, it appears obvious, therefore, that one way to avoid cross-reactions would be to use a purified antigen preparation.

In the present paper, the identification and purification from *L. monocytogenes* serovar 4b of a soluble antigen, referred to as antigen 2, are described. To our knowledge, this report presents the first successful attempt to obtain a serologically homogeneous, surface-specific antigen preparation from *L. monocytogenes*. An immunoprecipitation system which has been shown to give a distinct reaction with minimal cross-reaction with other bacteria (13, 20) was chosen to study the antigen composition of a soluble extract of *L. monocytogenes* 4b. Enhanced resolution of formed immunoprecipitate complexes was obtained by using the crossed immunoelectrophoresis system originally developed by Laurell (11). The antigenic patterns of CSE obtained with anti-whole bacteria serum and anti-CE serum were different, with the number of immunoprecipitates formed being 9 and 17, respectively. The results indicated that the antigenic composition of *L. monocytogenes* 4b is more complex than that previously described by Armstrong and Sword (1). Three major antigens were detected, one of which, antigen 2, appeared the most antigenic in humans and rabbits.

By using a monospecific anti-antigen 2 serum, enrichment and immunological purification of the antigen were completed by gel filtration and immunoadsorption. Extracts of all serovariants of *L. monocytogenes* and of *L. grayi* contained the antigen in the same proportion. *L. murrayi* and *L. denitrificans* were not tested, but it is likely that the first species, which appears strongly related if not identical to *L. grayi* (21), contains antigen 2. The antigen is probably genus specific since no cross-reactions were observed with hyperimmune rabbit sera prepared against bacterial species which share common antigens with *Listeria* and since monospecific anti-antigen 2 serum did not react with extracts from these bacterial species.

Since whole bacteria induce anti-antigen 2 antibody, the antigen might be a major surface component. As expected, immunofluorescent tests performed with the monospecific anti-antigen 2 serum suggest its surface location. However, it could not be determined whether the antigen is associated with the cell wall or the capsule. It is also not known whether antigen 2 corresponds to the surface antigen described by Jaeger and Myers (20).

Experiments to detect anti-antigen 2 human antibodies by the passive agglutination reaction and by the enzyme-linked immunosorbent assay are under way. The first results suggest a good relationship between bacteriological and serological data.

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