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Chemical and Immunological Composition of Surface Structures of *Listeria monocytogenes*

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ABSTRACT

OSEBOLD, JOHN W. (University of California, Davis), OLE AALUND, AND CLARENCE E. CHRISP. Chemical and immunological composition of surface structures of *Listeria monocytogenes*. J. Bacteriol. **89**:84–88. 1965.—A proteinlike surface substance was demonstrated on *Listeria monocytogenes* when an explanation was sought for the inagglutinability of some somatic antigens. The serological behavior of live bacteria and organisms subjected to heat, formalin, and trypsin was compared. The agglutination-inhibiting phenomenon was most pronounced with heat-killed (100 C) antigens. Trypsinization eliminated inagglutinability and increased sensitivity. Substances released by the enzyme had an ultraviolet-absorption peak at 260 mμ and showed a spot on paper chromatograms compatible with polypeptide. Inagglutinable cells combined with antibody because they could readily absorb antibodies from serum. After reaction with anti-*Listeria* serum, inagglutinable cells could be agglutinated by the addition of antiglobulin serum. It was hypothesized that heat inactivation of cells denatured the proteinaceous surface layer which interferred with the formation of a visible agglutination product but did not eliminate antigen-antibody reaction.

The nature of surface structures on *Listeria monocytogenes* needs clarification in view of unresolved aspects of the immunological behavior of this bacterium. It is suspected that heterogenetic antigens are present, which might explain the high percentage of apparently normal vertebrates that have serum antibodies reactive with *L. monocytogenes* (Seeliger, 1961). Strains of *Staphylococcus aureus*, *Streptococcus faecalis*, and *Corynebacterium pyogenes* have been reported to be serologically cross-reactive with *L. monocytogenes* (Seeliger and Sulzbacher, 1956; Welshimer, 1960; Seeliger, 1955, 1961; Potel, 1956). *L. monocytogenes* has also been described as possessing the nonspecies-specific bacterial substance reported by Rantz, Randall, and Zuckerman (1956; Neter, Anzai, and Gorzynsky, 1960). A factor affecting serological behavior is a variable tendency toward inagglutinability of some somatic antigens, alluded to by Robbins and Griffin (1945).

This study was instigated by the observation that some somatic antigens would not react more strongly than 1+ or 2+ at any serum dilution. The possibility that the bacterial surface contained some substance which interfered with the formation of a visible agglutination product was considered.

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MATERIALS AND METHODS

Cultures. The strains of *L. monocytogenes* were originally isolated in this laboratory from natural infections in ruminant animals, and were designated 3-54, 4-59, and 4-61. All strains belonged to serotype 4b. Cultures were subjected to frequent mouse passage, which kept their virulence high and maintained them in the smooth colonial phase.

Antisera. Antisera to the live, virulent organism were obtained from sheep and cattle that had received a minimum of three inoculations of *L. monocytogenes*.

Antigen preparation. Tryptose Broth cultures incubated at 37 C for 24 hr were used to seed Roux bottles containing Tryptose Agar. The bottles were incubated for 48 hr at 37 C. Cells were harvested from the agar surface with a minimal volume of saline (0.145 M NaCl solution) and heated at 100 C for 1 or 2 hr. The suspensions were then washed three times in saline and preserved by the addition of 0.25% formalin. This concentrated stock antigen was diluted for testing to 50 to 53% optical transmission on a spectrophotometer at a wavelength of 450 mμ.

Trypsinization procedure. Cells to be trypsinized were grown and heat-killed as described for antigen preparation, and were then washed twice in phosphate buffer of pH 7.3 (Wheeler, Luhby, and

Scholl, 1950). A concentrated suspension of cells in buffer was prepared so that it read at 50 to 53% optical transmission (450 mμ) if diluted 1:20. The concentrated cell suspension was treated with trypsin by either of two procedures. (i) Treatment with 0.1% crude trypsin was carried out by adding one part of 1.0% trypsin solution (Difco, 1-250) to nine parts of cell suspension. The reaction proceeded for 15 min at 37 C, after which time the cells were washed twice in saline and preserved with 0.25% formalin. (ii) Purified trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio; 2X crystalline, 50% $MgSO_4$) was used in all analytical studies. To nine parts of concentrated cell suspension, one part of trypsin solution containing 0.5 mg/ml was added. The reaction time was 30 min at 37 C, after which the cells were washed and preserved with formalin. The treated antigen suspensions were diluted to 50 to 53% optical transmission (450 mμ) for testing.

Test procedure. Sera were diluted by doubling serially in saline followed by addition of an equal volume of antigen. The standardized incubation was 50 C for 2 hr, followed by a 48-hr holding period at 4 C. Tests were read by holding the tubes near the hood of a fluorescent lamp. Degrees of opacity in the test fluid could be observed easily when the transmitted illumination of the tube was viewed against a darkened background. Tubes were then tilted to observe the amount and character of aggregated cells.

Ultraviolet-absorption spectra and paper chromatography. Supernatant fluid from trypsinized *Listeria* cells was concentrated threefold by lyophilization. Ultraviolet-absorption spectra were determined with a Beckman DB recording spectrophotometer.

For paper chromatography, spots of the above-described test samples and their appropriate controls were placed on Whatman no. 1 filter paper. A solvent of *n*-butanol-acetic acid-water (4:4:1) was used with the ascending chromatographic technique. Spots were located by spraying with 0.2% ninhydrin in 60% ethanol.

Antibody absorption. Two-step absorptions were made by diluting the serum 1:6.25 in saline and mixing with an equal volume of concentrated absorbing antigen (heated or heated-and-trypl sinized *Listeria* cells).

Anti-γ-globulin. Antisheep γ_2-globulin serum was prepared by immunizing rabbits with protein from the first peak obtained by anion-exchange column chromatography on diethylaminoethyl (DEAE) Sephadex (Fahey, McCoy, and Goulian, 1958).

RESULTS

Effects of heat and formalin. Partial inagglutinability of antigen was seen when the bacterial cells were heated for 1 hr at 100 C, suggesting that the bacterial surface contained some interfering substance. This was a consistent result with antigens made from all three strains of *L. monocytogenes*. A marked increase in agglutinability was usually achieved when such antigens were autoclaved for 15 min at 120 C (Table 1). It was of interest that the high temperature did not destroy the capacity of the bacteria to combine with antibody. However, auto-aggregation of the cells frequently occurred with autoclaved antigens.

TABLE 1. *Agglutinability comparison of a somatic Listeria antigen before and after autoclaving**

Antigen treatment	Serum dilution				
	1:25	1:50	1:100	1:200	1:400
100 C	2†	2	2	1	0
100 C plus autoclaving	4	4	4	2	0

* Prepared from *L. monocytogenes* strain 4-59.

† Scored by visual estimation: 4 = 100% cells agglutinated; 2 = 50%; 1 = 25%; and 0 = no agglutination.

The effects of formalin inactivation of bacterial cells were compared with changes obtained by heat treatment. Tests with five batches of cells inactivated with 0.5% formalin indicated that they did not have the tendency toward inagglutinability displayed by heated cells.

Effects of trypsin. When 20 different heat-killed cell preparations were trypsinized prior to testing, a striking enhancement of agglutinability was observed. Complete agglutination occurred with sparkling clear supernatant fluids and closely packed aggregates of clumped cells in the bottom of the tubes. These more sensitive antigens usually produced an increased serum titer of one or two doubling dilutions. In an experiment on kinetics, the reaction with purified trypsin was stopped at the appropriate time by the addition of soybean trypsin inhibitor. The study indicated that the degree of agglutinating enhancement was directly proportional to increased reaction time and enzyme concentration. The strongest trypsin treatment used was 25 μg of trypsin per ml of cell suspension for 2 hr. This, or milder enzyme exposures, did not appear to affect the cells adversely. However, physical changes had occurred, since the cell pellet was easier to break up and resuspend after centrifugation than that of untreated cells. Once washed and resuspended, the trypsinized organisms regularly made smooth-cell suspensions which were free from the tendency to auto-aggregate. The ultraviolet absorption spectrum of the supernatant fluids obtained from trypsinized *Listeria* cells showed an absorption maximum at approximately 260 mμ, whereas the trypsin control had

a small absorption peak at 275 mμ, and the cell control (second washing from untreated cells) showed only minimal ultraviolet absorbancy (Fig. 1).

Paper chromatography indicated a trace amount of ninhydrin-reacting substance in the untreated cell control, largely confined to the spot of application. The trypsin control also gave a reaction near the point of application but was more intense. The test sample had the same appearance, but, in addition, another spot was seen which had a discrete advancing border and a trailing portion which was confluent with the spot of application. The limited migration of the material in the test sample suggested a sizable molecule, probably a polypeptide.

It was considered probable that the stable somatic antigens remaining after heat and enzyme treatments would be polysaccharide. Some experiments were performed with periodic acid oxidation of cells that had been heated at 100 C. Whether trypsinized or not, these cells were strongly gram-positive and retained all their normal morphological characteristics. When subjected to the periodic acid-Schiff (PAS) reaction, the cells were shown to be strongly PAS-positive (Pearse, 1960). The cells were initially gram-positive, but exposure to periodic acid rendered them completely gram-negative and perhaps somewhat smaller in outline.

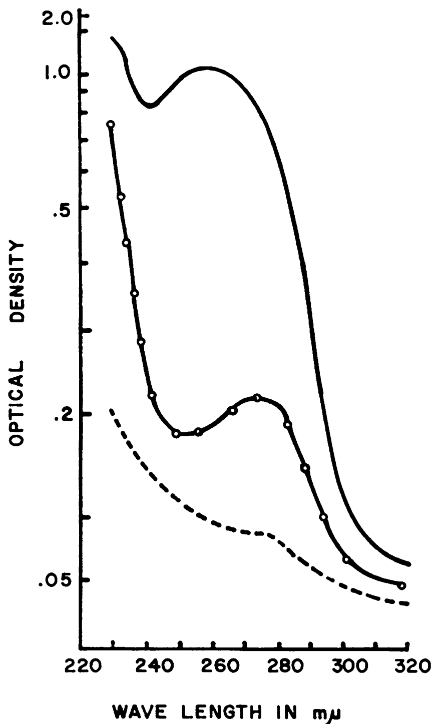

FIG. 1. *Ultraviolet-absorption spectrum of supernatant fluid from trypsinized Listeria monocytogenes. Solid line = trypsinized Listeria cell supernatant fluid; ○ = trypsin control; broken line = untreated Listeria cell control.*

Antibody absorption experiments yielded the surprising result that heated and partially inagglutinable cells were as efficient in absorbing antibodies from serum as were trypsinized *Listeria* cells. Two-step absorptions with either trypsinized or nontrypsinized antigen nearly exhausted antibody activity, and antibody titers after the first step of absorption were essentially equal. The results indicated that *Listeria* cells in a test showing inagglutinability had, nevertheless, combined with antibodies. This was demonstrated with an antibody reagent to sheep γ_2-globulin. The initial step was to perform the usual test procedure with heat-killed *Listeria* cells (100 C) and anti-*Listeria* serum. The portion of cells that did not agglutinate was decanted and saved. This was only done in serum dilutions known to contain sufficient antibody to aggregate all cells when trypsinized antigen was used. The inagglutinable cells were washed three times in saline to remove serum components, and were then allowed to react with rabbit antisheep γ_2-globulin. Complete agglutination occurred with the cells in tight, granular masses characteristic of somatic agglutination. The antiglobulin serum did not agglutinate cells unless they had first reacted with anti-*Listeria* serum.

The question of reduced serological specificity of the *Listeria* cells after trypsinization was examined. Antisera to the following microorganisms were tested against trypsinized and nontrypsinized *Listeria* cells: *Salmonella typhimurium, Staphylococcus aureus, Pasteurella haemolytica, Brucella abortus*, and *Leptospira icterohaemorrhagiae*. Antibodies to these pathogenic organisms did not appear to be reactive with the *Listeria* cells.

Agglutination of live Listeria cells. Agglutinability of live *Listeria* cells was determined by testing at room temperature; visible agglutination was barely perceptible after 4 hr. Stronger, but incomplete, agglutination was seen at 24 hr, and complete reactions were obtained at 48 hr. Titers were approximately one doubling dilution lower than those obtained from trypsinized cells tested under the same conditions. When live cells were trypsinized, they had essentially the same degree of reactivity seen in untreated live cells. The surfaces of live cells may have been unaffected by exposure to enzyme, since they could be resuspended after centrifugation in a

manner typical of untreated cells, rather than resuspending easily as did heat-killed trypsinized cells.

DISCUSSION

This investigation indicated that the release of a substance containing amino acids from the cell surface of *L. monocytogenes* by interaction with trypsin increased the amount of visible product after reaction with antibodies.

It may be that more than one substance was released after the trypsin attack, since analysis of supernatant fluid from treated cells revealed an ultraviolet-absorption spectrum ranging from 240 to 320 mμ with a peak at 260 mμ. The position of the peak suggested that nucleic acids could have been present to form a composite peak with peptide. The latter was additionally demonstrated by paper chromatography.

Examination of the fine structure of *L. monocytogenes* by Edwards and Stevens (1963) showed an irregular surface on the cell wall which could have been adsorbed material of foreign origin, but they also considered it reasonable that this was material excreted by the bacterium. It is likely that the substances thus observed were the same as those dealt with in this report. The importance of polysaccharides as somatic antigens in *L. monocytogenes* was indicated by their thermostability, resistance to proteolysis by trypsin, and the positive reaction of trypsinized *Listeria* cells with the Schiff reagent.

One could hypothesize the following explanation for the phenomena observed in these studies. The surface of *L. monocytogenes* contains a variable amount of protein or polypeptide, but usually this does not interfere with antigenic determinants of the cell soma, since the live organisms will agglutinate with antibody. When somatic antigens are prepared by heating at 100 C, the surface proteins are denatured which may cause them to swell, thus thickening the surface layer. The cells continue to combine with antibody, but the thickness of the expanded surface layer may sometimes make it impossible for antibody molecules to combine with two cells. The addition of anti-γ_2-globulin molecules allows the completion of a lattice, and agglutination results (Fig. 2).

It is not unique to find a protein at or near the cell surface of gram-positive bacteria. This has been demonstrated with *S. pyogenes*, *C. diphtheriae*, and *S. aureus* (Barkulis and Jones, 1957; Cummins, 1954; Oeding, 1960). In those instances, however, there seemed to be no difficulty in demonstrating the antigenicity of the proteinaceous material. Analogous antigens have not

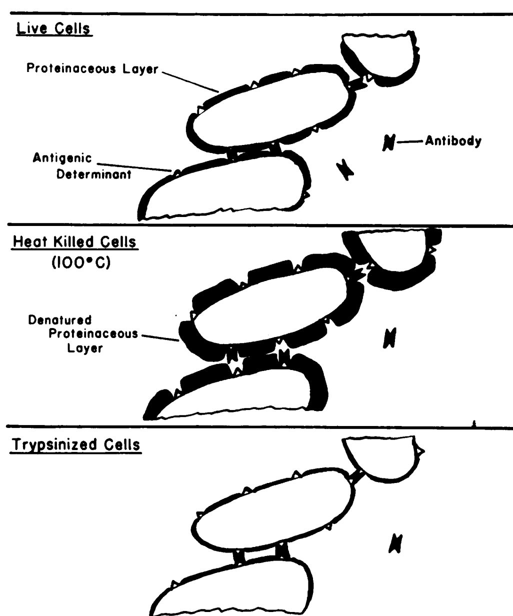

FIG. 2. *Hypothesis to rationalize observed phenomena related to the surface of Listeria monocytogenes.*

been described for *L. monocytogenes*, but the results of this study are indicative of a proteinaceous surface that is altered by heat. Trypsinization will be of advantage to serologists when seeking the greatest reactivity with stable somatic factors of *L. monocytogenes*.

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