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Production of Listeriolysin by Beta-Hemolytic Strains of *Listeria monocytogenes*

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Listeriolysin was isolated from target rabbit erythrocyte membranes after lysis of the cells with partially purified toxin derived from a culture supernatant of *Listeria ivanovii*. The membrane form of the toxin exhibited properties similar to those previously found for streptolysin O. Detergent-solubilized, delipidated listeriolysin was found to comprise a heterogeneous population of partially and fully circularized, amphiphilic oligomers whose embedment within the lipid bilayer generated large transmembrane pores. The molecular weight of the toxin monomer was estimated to be 55,000 to 60,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunological cross-reactions between the toxin and streptolysin O were demonstrable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. An immunoblot assay for detecting listeriolysin in agar-incorporated, lysed erythrocyte membranes was developed, and 28 defined, clinical isolates of *Listeria monocytogenes* were examined for toxin production. These isolates caused β-hemolysis on the agar plates and had previously been regarded as listeriolysin producers. However, we found that only two isolates produced genuine listeriolysin, since the sensitive immunoblot assay entirely failed to detect the toxin in all other cases. We excluded that this finding derived from proteolytic degradation of membrane-bound toxin. Thus, the great majority of human pathogenic *Listeria* strains appear to produce one or several hemolysins that are immunologically and, by inference, molecularly distinct from the streptolysin O-related listeriolysin. We propose that the streptolysin O-related toxin be designated α-listeriolysin and that the other hemolysin(s) be termed β-listeriolysin.

Virtually all pathogenic strains of *Listeria* produce zones of β-hemolysis on blood agar (10, 17–20, 22). The current belief among toxicologists is that these zones derive in the main from the production and release of one major, molecularly defined hemolysin, termed listeriolysin, from the cells (1, 21). Work aimed at defining this protein at a molecular level commenced over 20 years ago. Listeriolysin was successfully isolated from culture supernatants of certain strains of *Listeria monocytogenes* and shown to be a sulfhydryl-activated toxin sharing properties that are common to other proteins of this group (9, 11, 17). A molecular weight estimate for the toxin of 170,000 was made (12). An antiserum to the toxin cross-neutralized streptolysin O (SLO) (11), and the toxin appeared to be reversibly inactivated by atmospheric oxygen (11, 17). Since the toxin damaged phagocytic cells and lysosomes (14, 15, 24) and also exerted cardiotoxic and lethal effects in mice (16), there has been a tendency to assume that listeriolysin could represent a relevant pathogenic factor of these bacteria. Indeed, nonhemolytic listerial strains are generally nonpathogenic.

The assumption that β-hemolysis on blood agar invariably derives from the production of genuine listeriolysin is, however, somewhat debatable since no rigorous criteria have been applied to the actual identification of the toxin produced by the individual strains. In fact, human pathogenic *Listeria* strains all appear to react positively in the CAMP test (10, 20), and there is no known causal relationship between a positive CAMP test and production of sulfhydryl-activated toxins. In this connection, it is noteworthy that phospholipase production has also been reported in *L. monocytogenes* strains (12, 13).

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We now report the isolation of listeriolysin in its membrane-derived form and confirm its close similarity to SLO. A sensitive immunoblot assay is then described that permits direct detection of the toxin bound to agar-incorporated, target erythrocyte membranes. Using this method, we have found that β-hemolysis induced by 26 of 28 tested human pathogenic strains of *L. monocytogenes* somewhat surprisingly was not due to production of the genuine toxin, which we designate α-listeriolysin, but appeared to be caused by one (or several) other, as yet undefined toxin, termed β-listeriolysin. The latter, rather than the classical hemolysin, may play a pathogenic role in human listerial infections.

MATERIALS AND METHODS

Partial purification of α-listeriolysin from bacterial culture supernatants. Partial toxin purification followed the method previously detailed for isolation of SLO (3). *Listeria ivanovii* (synonymous with *L. monocytogenes* serovar 5; see reference 17a for details) from the special listeria culture collection of the Institute of Medical Microbiology, University of Würzburg, was cultured for 18 to 24 h at 37°C in 2,000 ml of brain heart infusion broth supplemented with 0.5% glucose. Cells were then removed by centrifugation, the supernatant was concentrated at 4°C to approximately 100 ml, and the toxin was precipitated by the addition of solid ammonium sulfate (53 g/100 ml) and stirring for 30 min at 4°C. The ammonium sulfate precipitate was collected by centrifugation, dissolved in approximately 10 ml of distilled water, and dialyzed overnight against Veronal-buffered saline, pH 7.0 (4°C). Thereafter, the toxin was precipitated with 20% polyethylene glycol (3), the precipitate was dissolved in the Veronal-buffered saline, and the material was applied to a DEAE-Sephacel column in the same buffer. The protein

passing the column was pooled and directly utilized as the native toxin source. Hemolytic titers (assayed as described previously [3]) of the partially purified toxin preparations were in the range of 2,000 to 4,000 U/ml.

Isolation of the membrane form of α-listeriolysin. The procedures for isolation of the membrane form of α-listeriolysin followed exactly those previously described for the isolation of SLO from membranes. A 10-ml sample of a rabbit erythrocyte suspension (10^9 cells per ml in saline plus 5 mM dithiothreitol) received 25 ml of partially purified toxin. Lysis ensued instantaneously at 37°C. The membranes were pelleted, washed three times in 5 mM phosphate buffer (pH 8), and solubilized through the addition of solid deoxycholate to a final concentration of 250 mM detergent. A 1-ml sample of solubilized membrane material was then applied to a linear, 10 to 50% (wt/vol) sucrose density gradient containing 6.25 mM deoxycholate, and centrifugation was performed at 150,000 × *g* for 16 h at 4°C (Beckman ultracentrifuge, rotor type SW41 Ti). Ten equal fractions were subsequently collected from the bottom of the tubes and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane-derived toxin sedimentated to fractions 1 through 5 in an identical fashion as SLO (5) (see Results). The toxin-containing fractions were pooled and used as the antigen for immunization of rabbits and for electron microsopic analyses. The production of rabbit antibodies and SDS-PAGE immunoblotting were by published procedures (2, 23). SLO was isolated as described previously (3).

SDS-PAGE immunoblot assay for the detection of α-listeriolysin in agar cultures. A suspension of packed, washed rabbit erythrocyte ghosts (approximately 10^{10} ghosts per ml) obtained by hypotonic lysis of cells with 5 mM phosphate (pH 8) was added to DST agar (Diagnostic Sensitivity Agar; Oxoid, Wesel, Federal Republic of Germany) so that final concentrations of approximately 12% (vol/vol) of the membranes in the agar were obtained. Washed, intact rabbit erythrocytes were additionally included at a final concentration of approximately 1% (vol/vol). Bacteria were cultured on these agar plates in vacuum jars for 30 to 40 h at 37°C. Cultures were also performed in a normal oxygen atmosphere with the same results, although hemolysis zones were narrower under the latter conditions. Thereafter, the bacteria colonies were carefully removed from the agar surface with cotton swabs. Samples of the underlying agar containing lysed erythrocytes and membranes were then sectioned and removed from the plates. They were given 4% SDS and boiled briefly (15 s), and 100-μl samples of the still molten agar were then applied directly to SDS gels. Noninoculated agar was applied as a control in every experiment. Immunoblotting of the SDS gels was performed as described above by using the anti-listeriolysin antisera and peroxidase-labeled second antibodies. The peroxidase reaction, developed with aminocarbazole, was terminated by immersing the blots in 10 mM Tris (pH 8) containing 50 mM NaN_3.

Analysis of mixed bacterial cultures. Bacteria were first cultured in brain heart infusion broth to the log phase. When the absorbance of the culture at 620 nm approached 0.2, 1 volume of an α-listeriolysin-producing strain was mixed with 9 or 60 volumes of a nonproducer. The mixed culture was then plated out on membrane-containing agar, and immunoblot analyses were performed as detailed above. These experiments were conducted to determine whether the absence of detectable α-listeriolysin production in the second strain derived from cosecretion of a protease that degraded the membrane-bound toxin.

SDS-PAGE immunoblot assays of SDS bacterial extracts and culture supernatants. Bacteria were cultured in brain heart infusion broth and collected by centrifugation either in the late log phase or after overnight culture. The supernatants were tested for hemolytic activity as described above. The washed cell pellets were suspended in 1/20 of their original volume and given 20 μg of lysozyme per ml and 20 μg of lysostaphin per ml for 20 min at 37°C. They were then boiled in 4% SDS for 1 min and centrifuged (Eppendorf table centrifuge). The SDS extracts were analyzed by SDS-PAGE immunoblotting. These experiments were conducted to determine whether nonproducers might harbor membrane-bound, nonsecreted α-listeriolysin.

A final set of experiments was conducted wherein an overnight culture supernatant of *L. monocytogenes* serovar 3a/b, strain 157 (a producer of α-listeriolysin, see Results) was incubated for 1 h at 37°C with an equal volume of an overnight culture supernatant of three nonproducers and then examined by SDS-PAGE immunoblotting. These experiments were designed to test whether nonproducers might secrete proteases that could rapidly degrade genuine α-listeriolysin.

Electron microscopy of dialyzed preparations obtained after sucrose density gradient centrifugation was performed as described previously (5).

Clinical isolates. Twenty-eight defined clinical isolates from the special listeria culture collection of the Institute of Medical Microbiology, University of Würzburg, were analyzed in this study.

RESULTS

Isolation of α-listeriolysin from target membranes. α-Listeriolysin was produced in large quantities by *L. ivanovii*, and overnight culture supernatants of this bacterial strain were used as the starting material for toxin isolation. Rabbit erythrocytes were lysed with partially purified α-listeriolysin, and the detergent-solubilized membranes were centrifuged through linear, detergent-containing sucrose density gradients. Ten equal fractions were collected, and Fig. 1 shows the SDS-PAGE patterns obtained through the analy-

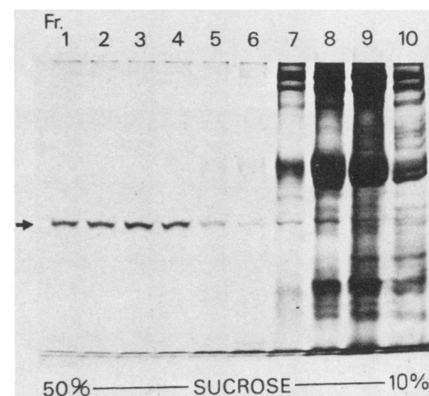

FIG. 1. Isolation of α-listeriolysin oligomers from rabbit target erythrocyte membranes. Toxin-treated membranes were solubilized in 250 mM deoxycholate and centrifuged through linear, deoxycholate-containing sucrose density gradients (direction of sedimentation, right to left). Ten equal fractions were collected, and samples were analyzed by SDS-PAGE. The α-listeriolysin bands are observed (arrow) in the high-molecular-weight regions (predominantly fractions 1 through 5), separated from the bulk of the contaminating erythrocyte membrane proteins.

sis of these fractions. A single protein band of M_r 55,000 to 60,000 is seen in fractions 1 through 5, corresponding to $s_{20,w}$ of 20 to 40 S, separated from the residual membrane proteins in fractions 7 through 10. The pattern was virtually identical to that previously observed with SLO (5). From the results obtained with the latter toxin (5), it was apparent that the fast-sedimenting protein most probably represented α-listeriolysin that had oligomerized in the membrane to form noncovalently bonded, supramolecular aggregates. The dissociation of such aggregates in SDS has been repeatedly shown (4, 5). Electron microscopy confirmed the expected presence of arc and ring structures in the given sucrose density gradient fractions (Fig. 2). These structures were micromorphologically indistinguishable from the SLO lesions. These results conformed to the general consensus that α-listeriolysin damages target membranes in the same manner as SLO, i.e., by oligomerization in the membrane to form large transmembrane pores.

Immunological cross-reactions between SLO and α-listeriolysin. Sulfhydryl-activated cytolysins have been reported to be antigenically related to one another (1, 21). SDS-PAGE immunoblot analyses indeed confirmed the immunological cross-reactions between α-listeriolysin and SLO. Figure 3 depicts the results obtained upon analysis of an unfractionated culture supernatant of *L. ivanovii* (lane a); the purified, membrane form of α-listeriolysin (pooled fractions 1 through 4 of Fig. 1 [lane b]); and purified SLO (SLO_b is the hemolytically active, degraded form of SLO; M_r, 57,000 [lane c]). The immunoblot in Fig. 3 was developed with anti-SLO antiserum; a similar blot but with markedly weaker staining of the SLO band was obtained with anti-listeriolysin antiserum (data not shown). One major band stained in the unfractionated culture supernatant of *L. ivanovii* that exhibited exactly the same M_r as the purified α-listeriolysin (lane b). The lower-molecular-weight bands most likely represent degradation products of the protein (analogy to SLO [3]). The significance of the faint band of slightly higher M_r is presently unknown. The results indicate that native listeriolysin is produced as a single-chain polypeptide of M_r 55,000 to 60,000, that the toxin is not markedly degraded during the partial purification procedure, that, as found for SLO, membrane-binding is not accompanied by any detectable cleavage of the molecule, and that the toxin is antigenically related to SLO. At the same time, these data clearly identify the isolated protein as the classical, SLO-related listeriolysin.

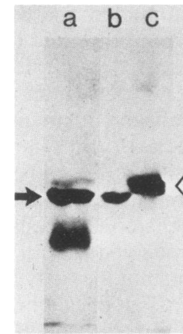

FIG. 3. Immunological cross-reactivity between α-listeriolysin and SLO. An unfractionated culture supernatant of *L. ivanovii* (lane a), purified α-listeriolysin (fraction 2 of Fig. 1, lane b), and purified SLO_b (M_r 57,000 [3], lane c) were subjected to SDS-PAGE immunoblotting with anti-SLO antibodies. The antibodies recognized the listeriolysin (solid arrow) in addition to SLO (open arrow). The faint band of slightly higher M_r in lane a may represent a precursor form or the true native form of the toxin; the lower-M_r band most likely represents a degradation product present in the supernatant of *L. ivanovii*.

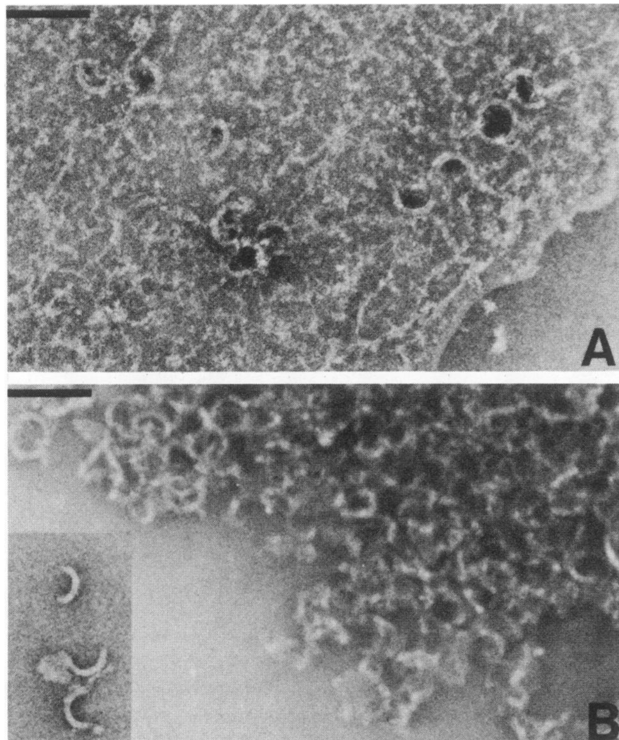

FIG. 2. (A) Fragment of rabbit erythrocyte membrane with large circular and semicircular lesions induced by treatment with listeriolysin. (B) Isolated oligomers of α-listeriolysin recovered after dialysis of the purified protein (fraction 2 of Fig. 1). The inset at lower left shows three copies of unaggregated toxin oligomers. Sodium silicotungstate negative staining. Bars, 100 nm.

Sensitivity of the SDS-PAGE immunoblot assay. A solution of α-listeriolysin recovered from a sucrose density gradient (fraction 2, Fig. 1) and containing 50 μg of protein per ml was doubly diluted and applied to SDS-PAGE immunoblotting to test the sensitivity of the detection system. This approach has recently been described for *Staphylococcus aureus* α-toxin (2). We found that the detection limit for the toxin given with the presently available reagents was on the order of 30 ng of protein in a sample.

Absence of detectable α-listeriolysin in culture supernatants of β-hemolytic clinical isolates of *Listeria*. A total of 28 defined, clinical isolates of β-hemolytic *Listeria* were tested for production of α-listeriolysin. *L. ivanovii* and *Listeria inocua* (an apathogenic, nonhemolytic strain) served as positive and negative controls, respectively. Brain heart infusion cultures of the bacteria were prepared, and overnight or late-log-phase supernatants were tested for the presence of α-listeriolysin by hemolytic functional assays and by SDS-PAGE immunoblotting. We found that the culture supernatants derived from only two strains (*L. monocytogenes* serovar 3a/b, strain 157, and *L. monocytogenes* serovar 1/2a, strain 2371) displayed hemolytic activity, and only here were we able to detect α-listeriolysin by SDS-PAGE immunoblotting. All of the other strains displayed no hemolytic activity, nor could α-listeriolysin or any

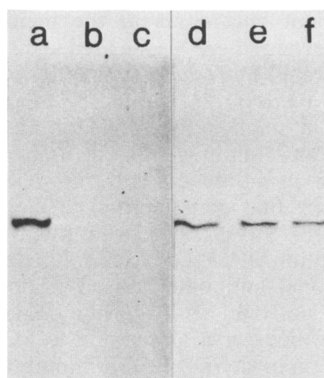

FIG. 4. Unfractionated culture supernatants (100 μl) of *L. monocytogenes* serovar 3a, strain 157 (lane a), *L. monocytogenes* serovar 3a, strain 1191 (lane b), and serovar 4b, strain 4527 (lane c), were analyzed from the presence of α-listeriolysin by SDS-PAGE immunoblotting with anti-α-listeriolysin antibodies to develop the blots. Toxin was only detected in the supernatant of strain 157. Supernatant a was mixed with brain heart infusion broth (control, lane d) or with supernatant b (lane e) or supernatant c (lane f) for 60 min at 37°C. Note the persistence of the α-listeriolysin band indicating the absence of toxin-degrading proteases in the culture supernatants of strains 1191 and 4527.

cleavage product thereof be detected (Fig. 4). Moreover, incubation of negative culture supernatants with the positive supernatant of strain 157 did not lead to rapid degradation of the toxin through the action of fluid-phase proteases (Fig. 4).

Absence of detectable α-listeriolysin in agar-incorporated target membranes. A method was next devised to test for the presence of α-listeriolysin in the target erythrocyte membranes themselves. Because hemolytic activity could not be recovered in the bacterial culture supernatants, we incorporated erythrocyte membranes into DST agar to trap the secreted hemolysin. One percent intact erythrocytes were added as indicator cells. We thereby assumed that toxin binding to the membranes as well as to intact cells would occur, since this has been found for all channel formers in the past (4, 6–8). After bacterial culture, bacteria were removed from the agar surface, and 100-μl samples of the underlying agar containing the lysed cells were boiled in SDS and directly applied to SDS gels. This method guaranteed that toxin-lysed erythrocyte membranes were truly being examined in every case.

TABLE 1. *L. monocytogenes* clinical isolates

Serovar	Strain no.	β-Hemolysis on agar[a]	Listeriolysin immunoblot test	Lane no. in Fig. 5
3a	157	++++	+	1
3a//	696	+	0	2
1/2a	2371	++++	+	3
1/2a	3110	(+)	0	
1/2a	4956	+	0	4
1/2a	5132	+	0	5
1/2a	5156	(+)	0	
1/2b	4525	+	0	6
1/2b	4526	(+)	0	
1/2b	4528	(+)	0	
1/2b	5957	+	0	7
1/2b	6124	++	0	8
1/2c	5078	+	0	9
1/2c	5956	+	0	10
3a	1191	++	0	11
3a	3106	+	0	12
3b	5885	+	0	13
3	1205	+	0	14
4b	3551	+	0	15
4b	4013	+	0	16
4b	4324	+	0	17
4b	4524	+	0	18
4b	4527	++	0	19
4b	4621	+	0	20
4b	4631	+	0	
4b	5381	+	0	
4b	5489	+	0	
4d	3207	+	0	

[a] ++++, Strong; ++, moderate; +, weak; (+), very weak hemolysis.

Figure 5 depicts the results of an experiment. Lane I was loaded with agar on which *L. ivanovii* had been cultured; note the appearance of a heavily stained immunoblot band corresponding to α-listeriolysin. A faint, lower-M_r band representing a proteolytic cleavage product of the toxin was also observed. The higher-molecular-weight bands represented nonspecific staining of the major erythrocyte membrane proteins (the "spectrins" and band 3 protein) and were present on control gel blots (lane Ø). Lanes 1 through 20 were loaded with agar cultured with 20 β-hemolytic clinical isolates (Table 1). Only in the case of strains 3a/157 and 1/2 a/2371 was α-listeriolysin detected. Thus, although the agar-incorporated erythrocytes were clearly lysed in the

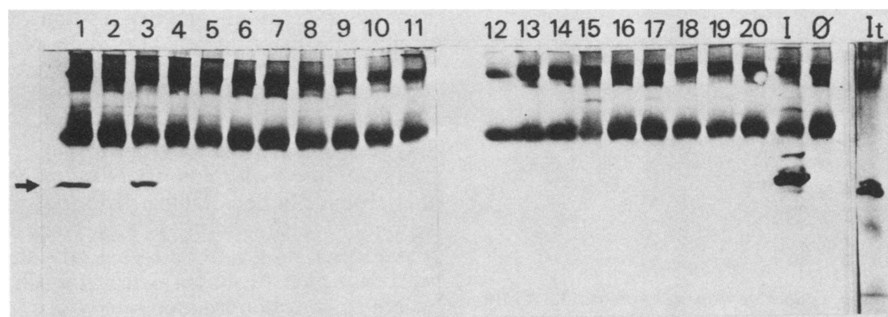

FIG. 5. SDS-PAGE immunoblotting of 100-μl samples of DST agar containing approximately 1.5×10^8 lysed erythrocyte membranes after bacterial culture. *L. monocytogenes* strains numbered 1 through 20 (Table 1) were analyzed; only *L. monocytogenes* serovar 3a, strain 157, and serovar 1/2a, strain 2371, were found to produce α-listeriolysin with this method. Lane I is a blot obtained from an agar culture of *L. ivanovii*. For lane It, membranes lysed with listeriolysin from *L. ivanovii* were trypsinized for 60 min at 37°C; note the only slight degradation of the toxin, but almost complete removal of the unspecifically staining erythrocyte membrane protein bands. Lane Ø is a control blot of noninoculated agar.

residual 18 cases, the membranes carried no detectable α-listeriolysin.

Analysis of mixed bacterial cultures. The results continued to support the notion that β-hemolysis derived from all but two of the tested clinical isolates of *L. monocytogenes* was caused by a hemolysin distinct from α-listeriolysin. The possibility was next considered that failure to detect the toxin might be due to proteolytic degradation of the protein after its insertion into membranes, through secretion of proteases by the given strains. We therefore prepared mixtures of α-listeriolysin producers (strain 3a/157) with a series of putative nonproducers and cocultured the bacteria on membrane-enriched agar. When SDS-PAGE immunoblotting of these agar samples was performed, we invariably detected the α-listeriolysin even when nonproducers outnumbered producers in a 60:1 ratio at the commencement of cultures (data not shown). Thus, secondary degradation of toxin was not an acceptable explanation for the absence of the toxin band in the immunoblot analyses of Fig. 5.

This contention was strengthened by additional experiments wherein erythrocyte membranes lysed with α-listeriolysin were trypsinized in suspension with 20 μg of trypsin per ml (final concentration) for 60 min at 37°C. SDS-PAGE immunoblotting of such membrane samples continued to show the presence of a toxin band of only slightly lower molecular weight (Fig. 5, lane It). Thus, α-listeriolysin shares with other channel formers the property of trypsin resistance once it becomes incorporated into the target bilayer. Native toxin, in contrast, was found to be highly sensitive to tryptic degradation (data not shown).

Analysis of SDS extracts of whole bacteria. A final possibility was considered, i.e., that α-listeriolysin might be tightly bound to the cell membrane and not be released in detectable amounts. Therefore, whole bacteria were treated with lysostaphin plus lysozyme and then boiled in SDS. The detergent extracts were analyzed by SDS-PAGE immunoblotting. α-Listeriolysin was again detectable in detergent extracts of *L. ivanovii* and *L. monocytogenes* serovar 3a, strain 157, but entirely absent in detergent extracts derived from all the α-listeriolysin-negative strains (Fig. 6).

DISCUSSION

In the first part of this study, we isolated a protein from target membranes that, on the basis of several independent criteria, could not but represent the membrane form of classical listeriolysin, here designated α-listeriolysin. The protein was secreted by *L. ivanovii* and by two strains of *L. monocytogenes* (serovar 3a, strain 157, and serovar 1/2 a, strain 2371) as a single polypeptide of M_r approximately 60,000. Membrane binding was accompanied by oligomerization of toxin monomers to form supramolecular arc and ring structures that were micromorphologically identical to those formed by SLO. These structures could be isolated in detergent solution after membrane solubilization in deoxycholate. Polyclonal antibodies raised against the isolated protein reacted not only with the homologous antigen but also with SLO. Vice versa, antiserum to SLO also reacted with α-listeriolysin in an SDS-PAGE immunoblot assay. The collective results conform to the general consensus that SLO and α-listeriolysin are closely related (1, 11, 21).

With the availability of satisfactory immunological methods for identification of α-listeriolysin, we then sought to reexamine whether the appearance of zones of β-hemolysis on blood agar induced by human pathogenic strains of *L. monocytogenes* was invariably related to production of α-listeriolysin. These investigations were prompted by the initial observation that no hemolytic activity could be detected in the culture supernatants of most of these strains. Findings similar to these were indeed reported in early studies (17, 19). In contrast, hemolytic activity was discernable in culture supernatants of genuine α-listeriolysin producers such as *L. ivanovii*. SDS-PAGE immunoblotting of culture supernatants strengthened our suspicion that α-listeriolysin was indeed not being produced in the majority of cases; thus, no toxin or degradation products thereof could be detected by this sensitive method. Further, coincubation of an α-listeriolysin-positive culture supernatant with a negative culture supernatant did not lead to visible degradation of the toxin.

To corroborate these findings, we devised a method to directly analyze lysed erythrocyte membranes. Since we were unable to perform hemolysis experiments in solution, we incorporated washed membranes in addition to intact erythrocytes into the agar plates to trap the toxin when it was secreted by the cells. This approach appeared justified since numerous earlier investigations had shown that membrane-inserting channel formers generally bind equally well to lysed cell membranes. Samples of agar containing the lysed erythrocytes were then directly applied in SDS-PAGE immunoblotting analyses. The guarantee was thus given that membranes carrying the hemolytic factor were indeed under examination. These experiments confirmed that, of the 28 hemolytic *L. monocytogenes* strains examined, only two produced α-listeriolysin. The zones of β-hemolysis induced by the 26 residual strains appeared not to derive from α-listeriolysin but from an immunologically and, by inference, molecularly unrelated hemolytic factor or factors. Experiments in which α-listeriolysin producers were mixed and cocultured with nonproducers provided sound evidence that absence of detectable α-listeriolysin did not derive from secondary degradation of membrane-bound toxin, which was further shown to be trypsin resistant. Finally, we also were not able to detect α-listeriolysin in SDS extracts of whole cells of nonproducers.

The immunoblot assay permitted detection of approximately 30 ng of α-listeriolysin in a sample, corresponding to approximately 3×10^{11} molecules of the toxin. Each 100-μl agar sample contained approximately 1.5×10^8 cell membranes. A negative immunoblot analysis thus excluded the possibility that a mean of >2,000 molecules of α-listeriolysin

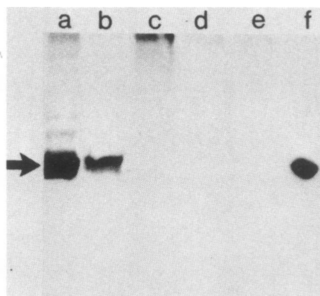

FIG. 6. *L. ivanovii* (lane a), *L. monocytogenes* serovar 3a, strain 157 (lane b), serovar 3a, strain 696 (lane c), serovar 1/2b, strain 6124 (lane d), and serovar 4b, strain 4527 (lane e) were treated with lysozyme and lysostaphin and boiled in SDS. The detergent extracts were analyzed for the presence of α-listeriolysin by immunoblotting. Whereas the toxin was detected in genuine producers, it was absent in the extracts of nonproducers. Lane f shows a control blot with purified α-listeriolysin.

was bound per cell. One should consider that the presence of approximately 100 molecules of SLO per cell is required to induce 60% hemolysis of erythrocytes in suspension; a considerably higher number is required to induce 100% hemolysis (3). Since membranes immediately adjacent to the bacteria in the agar will first absorb larger amounts of the toxin, relatively high concentrations would actually be required for the protein to successfully diffuse past the initial membrane barriers to the underlying cells to cause the observed zones of β-hemolysis. Therefore, we are confident that the negative immunoblot results truly indicate that α-listeriolysin is not the cause of the β-hemolysis zones. It follows that one or several molecularly distinct hemolysins must be produced by human pathogenic *L. monocytogenes* strains. Early workers in this field already observed heterogeneity in β-hemolysis patterns and indeed considered the possibility that some listerial strains might produce betalysins similar to those elaborated by staphylococci (17). Our data indicate that production of such β-listeriolysins, which are also probably responsible for the positive CAMP phenomenon (10, 20), actually predominates among human pathogenic strains. These, rather than the classical, sulfhydryl-activated α-listeriolysin, may eventually emerge as significant factors of listerial pathogenicity in humans.

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