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Production and Characterization of Neutralizing and Nonneutralizing Monoclonal Antibodies against Listeriolysin O

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Listeriolysin O (LLO) is a thiol-activated toxin secreted by the facultative intracellular pathogen *Listeria monocytogenes*. LLO is essential for the survival of the bacterium in the infected cell because it promotes lysis of the phagosome membrane and escape of the bacterium into the cytosol. LLO was used as an antigen for the production of nine monoclonal antibodies (MAbs) in mice. Three of these could inhibit the hemolytic activity of LLO. One of them inhibited binding of LLO to erythrocyte membranes. The two other antibodies blocked the activity of LLO at a step subsequent to membrane binding. Only two of the nine MAbs recognized three other purified SH-activated toxins, streptolysin O, alveolysin, and pneumolysin. Western blot (immunoblot) analysis of culture supernatants of *Listeria ivanovii* and *Listeria seeligeri*, two hemolytic species of the genus *Listeria*, revealed that two MAbs recognized ivanolysin and seeligerolysin. The latter was also recognized by two other MAbs, including one of the neutralizing antibodies. MAbs raised against a peptide, ECTG LAWEWWR, present in all thiol-activated toxins sequenced to date, recognized all toxins and were not neutralizing. Taken together, these results demonstrate the existence of regions important for hemolytic activity that are unique to hemolysins of the genus *Listeria* and show that regions outside the conserved peptide are important for activity of LLO.

Thiol-activated toxins are a family of biologically and antigenically related membrane-damaging toxins. This group includes at least 18 proteins produced by gram-positive bacteria belonging to the genera *Bacillus*, *Clostridium*, *Listeria*, and *Streptococcus* (1, 2, 30). These toxins have no known enzymatic activity and exert their cytolytic activity by a mechanism involving either pore formation (4) or lipid-phase transition in the membrane due to cholesterol sequestration (30). Cholesterol is assumed to be the toxin binding site on the surface of a eucaryotic cell, since membranes lacking this compound are insensitive to the action of toxins. As the name given to these toxins indicates, thiol groups were assumed to play a crucial role in activity. Indeed, thiol alkylation and oxidation were shown to inhibit hemolytic action of these proteins, while maximal activity was obtained in the presence of reducing agents.

Genes for five of these toxins, streptolysin O (SLO) (17), pneumolysin (PLY) (35), listeriolysin O (LLO) (21), perfringolysin (33), and alveolysin (ALV) (14) have been sequenced. Comparison of the deduced amino acid sequences revealed that all five toxins share extensive primary sequence homologies and contain a single cysteine residue in the longest conserved region of identity, the undecapeptide ECTGLAWEWWR. This finding suggested that the unique cysteine and residues of the conserved peptide might be functionally important. To test this hypothesis, mutants affecting the cysteine or tryptophan residues of SLO (25), PLY (29), and LLO (23) were obtained and analyzed. The striking result of these studies is that, in all three cases, the cysteine residue was not absolutely required for hemolysis. However, changing the tryptophan residues of LLO in the conserved undecapeptide strongly affected the hemolytic activity (23), providing evidence that the conserved peptide was important for activity.

The role that these toxins play in vivo is currently under investigation. In the case of the intracellular bacterium *Listeria monocytogenes*, genetic studies (6, 7, 10, 26) have shown that LLO, a secreted protein of 58 kDa, is a virulence factor essential for survival in the infected host. It has been proposed that after phagocytosis of the bacterium by the mammalian cell, LLO damages the phagolysosomal membrane, allowing release of the bacterium into the cytosol of the host cell (9). The recent discovery that *L. monocytogenes* is able to spread directly from cell to cell (24, 31) has recently questioned the role of LLO in this step of the infectious process. However, demonstration of this function cannot be approached by simple genetic means such as use of nonhemolytic mutants, since these do not escape from the phagosome. Microinjection of neutralizing antibodies or immunocytochemical techniques could be strategies for dissecting the problem. With that aim, as well as to understand the basic properties and the mode of action of LLO, we prepared monoclonal antibodies (MAbs) against LLO and the conserved undecapeptide and tested their neutralizing activity as well as their specificity for other thiol-activated toxins, especially toxins produced by two other species of the genus *Listeria*, *Listeria ivanovii* and *Listeria seeligeri* (13, 20).

MATERIALS AND METHODS

Antigens. (i) LLO. LLO was purified from culture supernatants of *L. monocytogenes* LO28 (21). Bacteria were grown without shaking at 37°C in brain heart infusion medium (Difco) supplemented with 1% glucose to an optical density at 600 nm (OD_{600}) of 0.9 (5 to 6 h). The supernatant was supplemented with glycerol (5% final concentration), acetic acid (50 mM final concentration), and β-mercaptoethanol (5 mM final concentration), and the pH was adjusted to

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4.95 with NaOH (buffer E). Buffered supernatant was chromatographed on 4 S/P Zetaprep 60 cartridges (CUNO, Meriden, Conn.) mounted in series and activated according to the manufacturer's instructions and equilibrated with buffer E at a flow rate of 3 to 4 ml/min. Cartridges were washed with buffer E, and LLO was eluted with 500 ml of 1 M NaCl–buffer E. Fractions (4 ml) were collected at a flow rate of 3 ml/min. Peak fractions were concentrated to approximately 60 ml on a YM 30 membrane (Amicon) and desalted on a G-50 Sephadex column (5 by 20 cm; Pharmacia) preequilibrated in 50 mM morpholineethanesulfonic acid (MES)–5 mM β-mercaptoethanol–5% glycerol, pH 6.5. The final step of the purification was chromatography on a fast-protein liquid chromatography MonoS HR 5/5 column (Pharmacia). LLO adsorbed to the column was eluted with a linear gradient of 0 to 1,000 mM NaCl in 50 mM MES–5% glycerol–1 mM β-mercaptoethanol, pH 6.5, with an increase of 25 mM NaCl per min. LLO eluted at 300 mM NaCl. Purity was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Peak fractions were pooled, concentrated, aliquoted, and frozen. LLO was stable for 2 weeks at 4°C and retained full activity for at least 6 months upon storage at −80°C. Final yield was approximately 0.5 to 1 mg of purified protein per liter of original culture supernatant.

(ii) Synthetic peptide coupled to BSA. An 11-amino-acid peptide, ECTGLAWEWWR, was purchased from Neosystem (Strasbourg, France) and coupled to glutaraldehyde-activated bovine serum albumin (BSA) according to the method of Briand et al. (5). The ratio of peptide to protein, determined by amino acid analysis, was estimated to be 8 mol of peptide per mol of BSA.

(iii) Other thiol-activated toxins. SLO was purchased from Sigma. PLY was kindly supplied by M. K. Johnson (Tulane Medical School, New Orleans, La.), and ALV was prepared as described previously (11).

(iv) *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii* culture supernatants. *L. monocytogenes* (strain LO28) (18), *L. seeligeri* (type strain CIP 100/100), and *L. ivanovii* (strain CIP 7842, from the collection of the Institut Pasteur) were grown in brain heart infusion (Difco) for 8 to 24 h at 37°C under agitation. Bacteria were centrifuged. Supernatant was recovered and filtered on 0.22-μm Millex filters. In the case of *L. seeligeri*, supernatant was concentrated on a PM10 membrane (Amicon) in a stirred cell concentrator.

MAb production. Four C57BL/6 mice and four BALB/c mice were injected subcutaneously with 20 μg of LLO and conjugated peptide, respectively, in Freund's complete adjuvant. Mice were boosted with 10 μg of antigen in Freund's incomplete adjuvant every 10 days. After eight injections, mice were bled and the polyclonal response was tested by enzyme-linked immunosorbent assay (ELISA). The mouse which gave the best response against LLO or the coupled peptide was sacrificed 3 days after a last intravenous booster injection for splenic B-cell fusion according to the method of Köhler and Milstein (18). Supernatants were screened for antibody production by ELISA (see below). Ascites production was performed by injecting 5×10^6 cells intraperitoneally into histocompatible mice previously primed with 0.5 ml of Pristane (2,6,10,14-tetramethylpentadecane; Sigma). Ascitic fluid was precipitated with 40% ammonium sulfate. Total protein concentration was evaluated by measuring the OD_{280}. All MAbs containing solution were adjusted to 1 mg/ml. Monoclonality was verified by isoelectrofocalization. Antibody preparations were more than 98% pure, and all characterizations were performed with these ammonium sulfate-purified preparations.

ELISA. Mouse sera or culture supernatants of hybridomas were screened for the presence of anti-LLO antibodies by ELISA according to the method of Voller et al. (34). Reactivity of other toxins or peptides with MAbs was also tested by ELISA. Either antigens (100 ng per well of LLO or other purified toxins) or BSA-coupled peptide (500 ng) in phosphate-buffered saline (PBS) was used to coat wells of a microtiter plate (Nunc). Culture supernatants, dilutions of antisera, or MAb preparations were incubated for 2 h at 37°C. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Igs) (Diagnostics Pasteur, Marnes-la-coquette, France) were then added and revealed with freshly prepared 0.2% orthophenylenediamine containing 0.02% hydrogen peroxide. ELISA allowed definition of the relative titers of the MAbs, expressed as the amount of antibody preparation which gives an OD of 0.5.

Subtyping of MAbs was also performed by ELISA. Peroxidase-conjugated rabbit antibodies specific for different isotypes of mouse Igs (Miles Laboratories) were used according to the manufacturer's instructions.

Antibody binding sites determined by antibody competition assay. An antibody competition assay was used to measure the ability of the different MAbs to compete for the same binding site on the antigen. Conjugation of MAbs to peroxidase was performed with glutaraldehyde according to the method of Avrameas (3). LLO (100 ng per well) was coated onto 96-well microtiter plates (Nunc). The plates were then saturated with 0.5% gelatin. Peroxidase-conjugated antibody at a subsaturating concentration (50 μl) and various concentrations of nonconjugated MAbs (50 μl) were simultaneously added. After incubation for 1 h at 37°C and four washes, color was developed as described above. Optimization of this assay requires subsaturating levels of labeled antibody, which were determined in preliminary tests.

Determination of dissociation constants. Binding affinities of MAbs were determined according to the method of Friguet et al. (8). Briefly, various concentrations of LLO were incubated in solution overnight at 4°C with a defined quantity of MAb until equilibrium was reached. To be in excess of antigen, the antibody concentration used had been deduced from preliminary ELISA calibrations. Each mixture (100 μl) was transferred to a well of a microtiter plate previously coated with various concentrations of antigen and was incubated for 15 min at 4°C. After washing with PBST, bound antibodies were detected by the addition of β-galactosidase-conjugated goat anti-mouse Igs. The enzyme activity was revealed by 0.5 μM 4-methylumbelliferyl-β-D-galactopyranoside (Sigma) in 0.1 M phosphate buffer, pH 7.0, containing 0.1 M β-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid magnesium dipotassium salt, 1 mM $MgSO_4$, and 0.2 mM $MnSO_4$ and read with a Fluoroskan (Dynatech) microtiter plate reader. The K_d was estimated by slope measurement of the regression line obtained by plotting the reciprocal of the fraction of bound antibody versus the reciprocal of the molar concentration of antigen.

SDS-PAGE and Western blot (immunoblot) analysis. Electrophoresis in 11.25% polyacrylamide gels was performed according to the method of Laemmli (19). Samples (200 ng of LLO or 0.5 ml of trichloroacetic acid-precipitated culture supernatant) were denatured in 1× sample buffer for 5 min at 100°C. Proteins were transferred to nitrocellulose according to the method of Towbin et al. (32). After saturation, the membrane was incubated with MAbs. After four to five washes with PBST, the membrane was incubated for 2 h

TABLE 1. Properties of anti-LLO murine MAbs

MAb	Relative titer (μg/ml)[a]	Isotype	K_d	Epitope grouping[b]	Neutralizing activity	Recognition of purified hemolysins			Recognition of hemolysin from culture supernatant	
						SLO	ALV	PLY	*L. seeligeri*	*L. ivanovii*
H14-3	0.39	IgG1	4.0×10^{-8} M	A	+	−	−	−	−	−
B8B20-3-2	0.30	IgG1	1.1×10^{-8} M	A	+	−	−	−	+	−
A4-8	6.00	IgG1	3.0×10^{-9} M	B	+	−	−	−	−	−
B3-19	0.21	IgG2b	6.2×10^{-8} M	C	−	+	+	+	+	+
E4-3	0.14	IgG1	1.2×10^{-7} M	C	−	+	+	+	+	+
B8B20-1-5	14.0	IgG1	ND[c]	D	−	−	−	−	−	−
A3-1	4.00	IgG2b	5.0×10^{-7} M	E	−	−	−	−	−	−
B18-14	1.60	IgG2b	ND	F	−	−	−	−	−	−
D21-1-4	0.02	IgG2b	ND	G	−	−	−	−	+	−

[a] The relative titer is expressed as the concentration of total protein which gave an OD of 0.5 in ELISA. This technique measures directly the interaction of the antibody with the immobilized antigen and does not permit the measurement of the true equilibrium dissociation constant (8).
[b] Letters correspond to groups of specificity, as determined by competition assay (see Materials and Methods).
[c] ND, not determined.

with ^{125}I Bolton Hunter (2,000 Ci/mmol; Amersham)-labeled goat anti-mouse IgG (Biosys) and washed with PBST, dried, and autoradiographed.

Test for inhibition of hemolytic activity by MAbs. MAbs were screened for inhibition of hemolytic activity by a modification of our standard microtiter plate hemolysis assay (23). Briefly, a V-shaped, 96-well microtiter plate was first saturated with a solution of 2% BSA in PBS. One-hundred-microliter serial dilutions of MAb (6 μg/ml to 6 ng/ml) and 10 ng of LLO in 50 μl of PBS, pH 6.7, were added to each well. After incubation for 1 h at room temperature, 50 μl of washed diluted sheep erythrocytes (SRBC) (fresh SRBC diluted such that the OD_{541} of hypotonically lysed cells = 1.85) was then added to each well. The plate was incubated at 37°C for 30 min and centrifuged at 1,500 rpm for 5 min. The amount of LLO used in the assay was sufficient to lyse all the added erythrocytes, and neutralizing MAbs were scored by the appearance of a red pellet of unlysed erythrocytes at the bottom of the well after centrifugation.

Membrane binding. The ability of the neutralizing MAbs to inhibit binding of LLO to SRBC was tested by incubating 50 ng of LLO at 0°C with the minimum quantity of antibody required for inhibition (60 ng for H14-3 or B8B20-3-2 and 250 ng for A4-8). Washed SRBC preincubated at 0°C were then added to this mixture. Binding of LLO to the cells was allowed to proceed for 20 min on ice. SRBC were then washed twice in cold PBS and hypotonically lysed. The SRBC membranes were then washed twice in PBS, and the presence of membrane-bound LLO was determined by Western analysis with rabbit IgG anti-LLO antibodies and ^{125}I-labeled anti-rabbit IgG.

RESULTS

Production and characterization of nine anti-LLO MAbs. Nine anti-LLO MAbs were obtained by the procedures described in Materials and Methods. Their properties are summarized in Table 1. Five MAbs (A4-8, H14-3, E4-3, B8B20-3-2, and B8B20-1-5) were of the IgG1 isotype, while the remaining four (B3-19, A3-1, B18-14, and D21-1-4) were of the IgG2b isotype. They were of different specificities and affinities (Table 1). In the Western blot analysis, all MAbs reacted strongly with purified LLO and gave a single band with *L. monocytogenes* culture supernatants (data not shown).

Epitope specificity was determined by competition assays. This assay measures the competitive binding of two MAbs for the antigen. All combinations of the nine MAbs were tested. H14-3 and B8B20-3-2, two neutralizing antibodies, competed (80%) with each other, suggesting that they recognize the same epitope or overlapping epitopes. Similarly, B3-19 and E4-3 competed for the same epitope (90% competition). For MAbs A4-8, D21-1-4, B18-14, and A3-1, significant competition was observed only with the homologous, unconjugated antibody. These results suggest that the nine anti-LLO MAbs can be divided into seven groups, A to G, each group reacting with a different antigenic determinant on LLO (Table 1).

We then tested, by ELISA, the recognition of the BSA-coupled peptide ECTGLAWEWWR by these MAbs. Even with repeated attempts, none of them recognized the peptide.

Reactivity of anti-LLO antibodies with other thiol-activated toxins. The specificity of the nine anti-LLO MAbs for three other members of the family of thiol-activated hemolysins was evaluated by ELISA with purified SLO, PLY, and ALV. Only two MAbs, B3-19 and E4-3, demonstrated cross-reactivity with these three toxins (Table 1). Reactivity of the nine MAbs with ivanolysin and seeligerolysin present in culture supernatant of *L. seeligeri* and *L. ivanovii* was tested by ELISA and Western blot. Two antibodies, B3-19 and E4-3, reacted with *L. ivanovii* supernatant. They also reacted with culture supernatants of *L. seeligeri*, which were also recognized by two other antibodies (B8B20-3-2 and D21-1-4).

Identification of three neutralizing antibodies. Three of the nine MAbs, H14-3, B8B20-3-2, and A4-8, were able to inhibit the LLO-mediated lysis of SRBC (Fig. 1 and Table 1). Ten to 20 ng of H14-3 or of B8B20-3-2 was sufficient for neutralizing 10 ng of LLO, while 60 ng of A4-8 was necessary for neutralizing the same amount of the hemolysin (Table 1). B8B20-3-2, which recognizes seeligerolysin in culture supernatants, also inhibited its hemolytic activity.

Further characterization of the neutralizing antibodies was performed by testing for the ability to inhibit membrane binding by LLO (Fig. 2). LLO was incubated on ice with one of the three neutralizing antibodies or with one of the other antibodies or buffer alone as controls. SRBC were added, and the mixture was divided into two parts: one part was incubated at 37°C to test for hemolysis; the other was subjected to hypotonic lysis. In the latter case, membranes were further recovered by centrifugation and tested for the

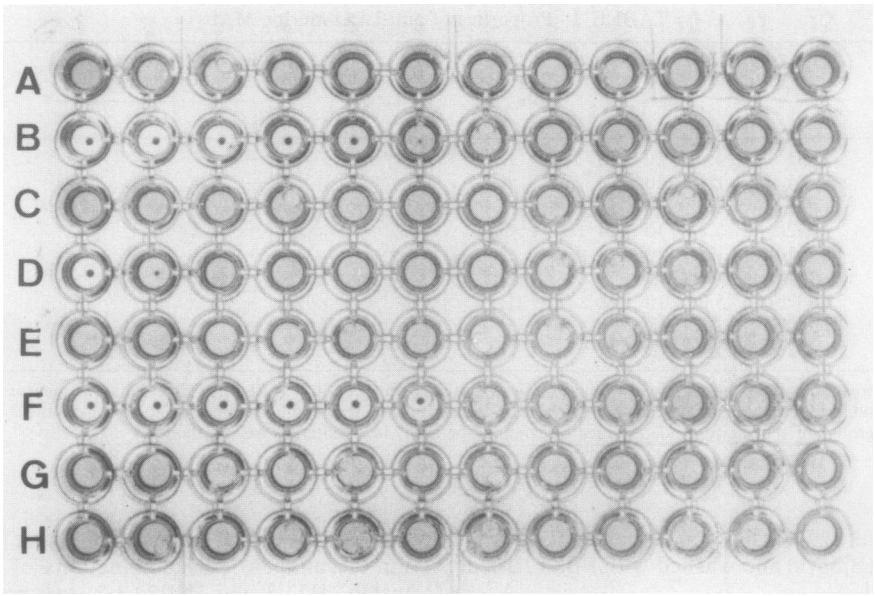

FIG. 1. Inhibition of hemolytic activity by anti-LLO MAbs. Serial dilutions of different MAbs (600 ng in the first column) were incubated with 10 ng of LLO at 37°C for 1 h. SRBC were added to each well, and the plate was further incubated for 30 min at 37°C and centrifuged. Rows are as follows: A, anti-tetanus toxoid antibody; B, H14-3; C, B3-19; D, A4-8; E, B8B20-1-5; F, B8B20-3-2; G, E4-3; H, D21-1-4.

presence of LLO. Clearly, LLO incubated with MAb A4-8, H14-3, or B8B20-3-2 failed to lyse SRBC, while LLO incubated with buffer or MAb B3-19 efficiently lysed the added cells (data not shown). Concerning interaction with membranes, the three MAbs behave differently: as shown in Fig. 2, MAb A4-8 prevented LLO membrane binding, while MAbs H14-3 and B8B20-3-2 did not.

Production and characterization of MAbs against the conserved peptide ECTGLAWEWWR. Seven MAbs against the undecapeptide, present in all thiol-activated toxins, were produced. These MAbs were of the IgG1 isotype and recognized both the peptide and LLO in ELISA. Western immunoblotting as well as ELISA revealed that all the MAbs tested reacted with LLO, PLY, SLO, ALV, seeligerolysin, and ivanolysin, but no neutralizing activity could be demonstrated for any of the seven antipeptide antibodies.

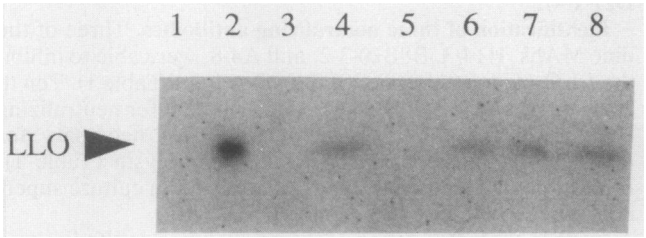

FIG. 2. Western blot analysis of LLO membrane binding. Fifty nanograms of LLO was incubated at 0°C with different MAbs. SRBC were then added to this mixture. After a further incubation for 20 min on ice, the SRBC were washed, hypotonically lysed, and subjected to SDS-PAGE-Western blot analysis with a rabbit polyclonal anti-LLO serum. Lanes: 1, no LLO; 2, LLO; 3, no LLO; 4, LLO incubated with buffer; 5, LLO incubated with A4-8 (250 ng); 6, LLO incubated with H14-3 (60 ng); 7, LLO incubated with B8B20-3-2 (60 ng); 8, LLO incubated with B3-19 (250 ng).

DISCUSSION

LLO is a key protein in the cellular infectious process of the intracellular pathogen *L. monocytogenes*. A series of genetic studies have converged to demonstrate that this protein mediates lysis of the phagolysosomal membrane, allowing escape of the bacterium in the cytoplasm (6, 7, 9, 10, 11, 26). This model was nicely correlated to the fact that LLO has an optimal activity at pH 5.5, a pH value thought to be that of the phagosome compartment (12). Recently, it was shown that *L. monocytogenes* is able to spread directly from cell to cell (24, 31). Bacteria protrude from the cell surface of the infected cell at the tip of a pseudopod which is then engulfed in the neighboring cell. Shortly after, bacteria are present in double-membrane vacuoles which have to be lysed to liberate the bacterium in the cytosol. The discovery of this phenomenon has led to questioning of the role of LLO in the lysis of the two-membrane vacuoles. Since nonhemolytic mutants do not escape from the phagosome, the genetic approach may not be appropriate for clarifying this question. In contrast, microinjection of anti-LLO neutralizing antibodies in the infected cell could be a powerful strategy. Moreover, careful examination of LLO production within the infected cell by immunocytochemistry might also allow evaluation of the various roles of LLO, if any. For these purposes, as well as to understand the basic properties of LLO, the only thiol-activated toxin (along with ivanolysin) to be produced by an intracellular bacterium, we raised MAbs against LLO and the conserved peptide ECTG LAWEWWR, which is present in all thiol-activated toxins sequenced to date (14, 17, 21, 33, 35).

In this study, we have produced nine MAbs against LLO and seven MAbs against the peptide ECTGLAWEWWR. Three of the nine anti-LLO MAbs were able to inhibit the hemolytic activity of LLO. This was not the case for the antipeptide antibodies.

It has long been known that thiol-activated toxins, in addition to sharing physical properties such as inhibition by cholesterol or thiol-alkylating agents (1, 2), share epitopes and that all thiol-activated toxins are recognized by a hyperimmune serum raised against SLO (1, 2). We were therefore expecting that not only the antipeptide antibodies but also some of the anti-LLO MAbs would recognize other toxins of the family, although the first polyclonal antiserum raised against LLO was able to recognize only ivanolysin and seeligerolysin, two toxins produced by two other *Listeria* species, *L. ivanovii* and *L. seeligeri* (12, 13). In fact, two anti-LLO MAbs recognized pure SLO, PLY, and ALV and the toxins present in the culture supernatants of *L. ivanovii* and *L. seeligeri*. Two additional anti-LLO antibodies reacted with *L. seeligeri* supernatant. The antipeptide antibodies recognized all toxins. Taken together, these results reinforce the hypothesis that thiol-activated toxins are members of a family but that the genes coding for these toxins have extensively diverged. In fact, at the DNA level, homologies with the gene coding for LLO have been detected only in the genus *Listeria*, i.e., in *L. ivanovii* and *L. seeligeri* (15, 20). Seven different epitopes were recognized by the nine MAbs, but none of the MAbs recognized the conserved peptide. This result suggests that this peptide is poorly immunogenic in the native form of LLO and could explain why the polyclonal anti-LLO serum recognized only proteins produced by *Listeria* species.

Three MAbs were shown to inhibit the hemolytic activity of LLO. In our competition assay, we had shown that of these three antibodies, H14-3 and B8B20-3-2 recognized identical or overlapping epitopes, while A4-8 recognized a distinct epitope. These data are in agreement with the further characterization of the MAbs. Indeed, only A4-8 was shown to inhibit binding of LLO to erythrocyte membranes, in contrast with the two others, which did not prevent binding. Since cholesterol present in the eucaryotic membrane is believed to be the receptor for thiol-activated toxins (30), it is possible that the epitope recognized by A4-8 is or lies in the region of the peptide interacting directly with cholesterol. The two other neutralizing MAbs, which allow binding of LLO but prevent lysis, must act at a later step in the hemolytic process. It has been suggested that after binding to the membrane, oligomerization of thiol-activated toxins is a prerequisite for their activity (4). It is possible that H14-3 and B8B20-3-2 prevent this oligomerization, but we cannot exclude the possibility that they could also act at an even further step since it has been shown that mutations in SLO or PLY which do not prevent binding and oligomerization can lead to inactive proteins (25, 29). This remains to be clarified. Other neutralizing MAbs (against perfringolysin O and SLO) which do not prevent binding of thiol-activated toxins but prevent lysis were isolated (16, 22, 27, 28). To our knowledge, no MAb preventing binding of a thiol-activated toxin on the membrane has been isolated to date, and A4-8 is the first example of this type of antibody.

The three neutralizing antibodies did not recognize the conserved peptide ECTGLAWEWWR, found in all the thiol-activated toxins, and the antipeptide MAbs did not inhibit the hemolytic activity. These results strongly suggest that the peptide is inaccessible to the antibody in solution when LLO is in its native structure but do not exclude the possibility that this peptide is involved in the activity of the proteins. Indeed, previous data obtained with mutations constructed in vitro (23, 25, 29) had shown that the thiol group of the unique cysteine of each toxin was not absolutely required for activity but that the undecapeptide region was important for activity. In conclusion, the results presented in this article indicate that epitopes lying outside the conserved peptide are also crucial for activity. This is under current investigation in our laboratory.

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