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Neutralizing Monoclonal Antibodies against Listeriolysin: Mapping of Epitopes Involved in Pore Formation

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Six different mouse monoclonal antibodies (MAbs) and a specific rabbit polyclonal antibody were raised against listeriolysin. Four of the MAbs also recognized seeligeriolysin, and five cross-reacted with ivanolysin. The hemolytic activity could be neutralized by the polyclonal antibody as well as by five of the MAbs. None of the neutralizing antibodies interfered with the binding of listeriolysin to the cellular membrane. The epitopes recognized by the MAbs were localized by using overlapping synthetic peptides between positions 59 and 279, a region hitherto not implicated in mediating hemolytic activity.

Listeriolysin, the cytolysin of the pathogenic bacterium *Listeria monocytogenes*, belongs to a family of toxins which are produced within the genus *Listeria* (*L. monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*) as well as by other gram-positive bacteria (19). These cytolysins are secreted into an aqueous environment but induce pore formation within lipid membrane bilayers. The mechanisms that evoke the transition of the toxin from a water-soluble to a hydrophobic lipid environment are not known. Oligomerization of the toxin into higher-order structures on the membrane seems to be a prerequisite in the cytolytic mechanism of these toxins. This is preceded by binding to a cellular receptor (3).

To understand more about this process, rabbits were immunized with purified listeriolysin (4) to produce polyclonal antibodies. In parallel, several monoclonal antibodies (MAbs) were generated against formaldehyde-treated, concentrated culture supernatant derived from *L. monocytogenes* EGD. Six listeriolysin-specific hybridomas were established and further studied. All reacted with a 40-kDa variant of listeriolysin with a truncated C terminus (5) and with fusion proteins between fragments of listeriolysin and the maltose-binding protein of *Escherichia coli* (data not shown), indicating binding to the N-terminal region of the molecule.

The MAbs were further characterized by determining their binding ability to overlapping synthetic peptides that covered the entire amino acid sequence of the listeriolysin molecule. A total of 166 peptides, each 12 amino acids in length, were synthesized as spots on specially derivatized cellulose paper the size of a microtiter plate as described by Frank (6). The first peptide corresponded to the amino acid positions 25 to 36 of mature listeriolysin. The other peptides had an offset of three amino acids and overlapped the following peptide by nine amino acids. Purified MAbs were incubated with the peptides immobilized on the cellulose sheet. Binding was detected with a peroxidase-conjugated secondary antibody and developed by chemoluminescence (ECL; Amersham). The sheet was reused several times after eluting the antibodies and stringent washings (6). A defined epitope could be assigned to each of the antibodies. The stretches recognized could be limited to six or eight amino acids (see Table 1) by their reactivity towards overlapping peptides, which was confirmed by blocking the reaction with a soluble form of the particular peptide.

The sequences of the hemolysins of the three *Listeria* species are closely related (10). Five of the MAbs reacted with ivanolysin of *L. ivanovii* (ATCC 19119), and four of them reacted with the toxin of *L. seeligeri* (SLCC 4113) (Table 1). Most of the epitopes in ivanolysin and seeligerolysin recognized by the cross-reactive MAbs contained very conservative amino acid replacements (e.g., L→V, I→V, and E→D, etc.), thus provid-

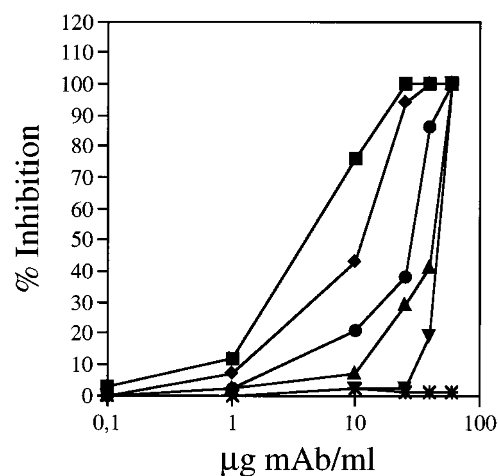

FIG. 1. Inhibition of hemolytic activity by anti-listeriolysin MAbs. Serial dilutions of different MAbs (M344 [■], M275 [◆], N20 [●], S23 [▲], S153 [▼], and S179 [✻]) were incubated with 1 μg of listeriolysin at room temperature for 1 h. Sheep erythrocytes were added to each well, and plates were incubated for an additional 30 min at 37°C. After centrifugation, 50 μl of supernatant was gently collected, and the amount of hemoglobin released was measured. Neutralization of listeriolysin hemolytic activity is expressed in percent inhibition compared with the lysis caused by distilled water (100%).

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TABLE 1. Characterization of anti-listeriolysin specific MAbs

MAb	Subclass	Epitope	Amino acid positions in hemolysin	Neutralization of hemolytic activity[a,b]	Reaction with ivanolysin[b,c]	Reaction with seeligerolysin[b,c]
S23	IgG1	DEIDKYIQ	59–66	+	−− (not conserved)	−− (not conserved)
M275	IgG1	LTLSIDLP	152–159	++	++ (VTLSIDLP)	+ (LTLSVDLP)
M344	IgG2b	LTLSIDLP	152–159	+++	++ (VTLSIDLP)	+ (LTLSVDLP)
N20	IgG1	TLVERW	184–189	+	++ (TLVDRW)	++ (TLVERW)
S179	IgG1	WNEKYAQ	189–195	−−	+ (WNNKYSE)	Not detectable (WNDKYSK)
S153	IgG1	GKAVTKEQL	271–279	+ (at high concn)	+ (GKSVTKENL)	Weak (GGSVTKEQL)

[a] See Fig. 1.
[b] Symbols: +++, very strong; ++, strong; +, intermediate; −−, not detectable.
[c] The homologous amino acid sequence of the cross-reacting toxin is shown in parentheses.

ing a reasonable explanation for the cross-reactive binding activity.

The ability of the MAbs to neutralize the hemolytic activity of listeriolysin was further investigated. As demonstrated in Fig. 1, five of the six MAbs neutralized the hemolytic activity of listeriolysin. Between 2- and 15-fold molar excess of the MAbs was necessary to obtain 50% neutralization, possibly reflecting differences in the affinities of the MAbs. One MAb (S179) failed to neutralize the hemolytic activity of listeriolysin at any concentration tested, suggesting that its epitope is not involved in the pore-forming process.

To distinguish whether the neutralizing antibodies interfere either with the binding step or with the subsequent steps leading to pore formation, we designed a sensitive binding assay. Various concentrations of listeriolysin were incubated with the mouse fibroblast cell line CA 36.2.1 (17) on ice for 30 min. Binding was detected by incubation with 2 μg of polyclonal anti-listeriolysin antibodies and a fluorescent secondary antibody. Analysis was performed by flow cytometry. Binding of listeriolysin to CA 36.2.1 cells could be readily measured at concentrations of 0.1 μg/ml (Fig. 2), thus providing a simple, sensitive binding assay.

To test whether the MAbs inhibited the binding of the toxin to the cells, listeriolysin was incubated with MAbs at neutralizing concentrations. Half of this mixture was tested for neutralization of hemolysis. The other half was incubated with CA 36.2.1 cells, and binding of listeriolysin was revealed by staining for the mouse MAb prebound to listeriolysin. As can be seen in Fig. 3, all MAbs bound to the cells via listeriolysin despite the fact that the hemolytic activity was inhibited in the parallel control assays by the five neutralizing antibodies. This demonstrated that neutralization did not interfere with the binding of listeriolysin to the cellular membrane.

Similarly, at concentrations where neutralization of the hemolytic activity was achieved by the polyclonal antibodies, listeriolysin still bound significantly to the target cells (Fig. 4).

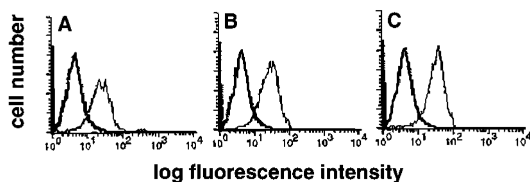

FIG. 2. Binding of listeriolysin to eucaryotic cellular membrane analyzed by flow cytometry. Different concentrations of listeriolysin (2.5 [A], 1 [B], and 0.1 [C] μg/ml) were incubated with the mouse fibroblast cell line CA 36.2.1. Specific polyclonal rabbit anti-listeriolysin antibodies and fluorescein isothiocyanate-labelled secondary antibodies were used to detect the bound listeriolysin. Thick lines represent the background fluorescence in the absence of listeriolysin. Thin lines represent the binding in the presence of listeriolysin.

However, increasing the antibody concentration completely abolished the binding of listeriolysin to the fibroblasts (Fig. 4). This indicated that antibodies directed towards the site involved in binding to the membrane were present, although in lower concentrations than the antibodies inhibiting pore formation.

In previous studies on other members of the thiol-activatable toxins tested, binding was localized to the C-terminal halves of the molecules (13, 22). Furthermore, mutations within the undecapeptide motif located close to the C terminus (7, 9, 11, 21, 23) which severely reduce or abrogate hemolysis did not abolish binding to cholesterol and toxin oligomerization (15, 16). The neutralizing MAbs recognized sequences within the N-terminal half of the toxin between positions 59 and 66, 152 and 159, 184 and 189, and 271 and 279. This is consistent with the finding that binding of listeriolysin to the membranes was still observed in the presence of the neutralizing antibody. It is in agreement with previous findings by Nato et al. (12), who also observed that neutralization of listeriolysin by MAbs does not necessarily inhibit its binding to erythrocyte membranes. Thus, the MAbs could recognize epitopes on the protein surface involved in pore formation. On the other hand, these MAbs could be inhibiting a conformational change that facilitates insertion of monomeric toxin into the membrane, or alternatively, the MAbs may be

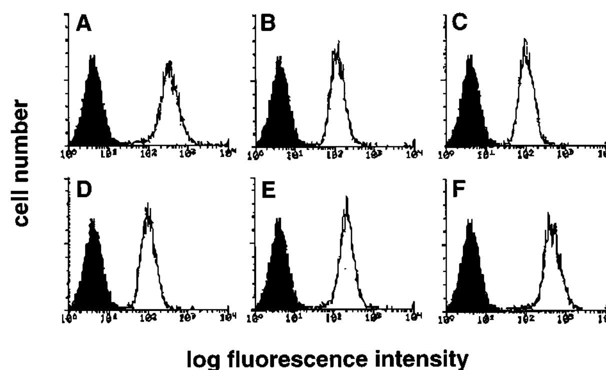

FIG. 3. Binding of listeriolysin to eucaryotic cellular membrane in the presence of MAbs. Different MAbs (33 μg/ml; M344 [A], M275 [B], N20 [C], S23 [D], S153 [E], and S179 [F]) were incubated with 1 μg of listeriolysin at room temperature for 30 min. The mixture was then added to mouse fibroblast cell line CA 36.2.1. Listeriolysin bound to the fibroblasts was detected by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin. Solid profiles represent the background fluorescence in the absence of listeriolysin. Open profiles represent the binding in the presence of listeriolysin and MAbs. It is important to note that despite that five of the MAbs inhibited hemolysis in the parallel assay (Fig. 1), binding of listeriolysin to the cells as a complex of MAb-listeriolysin was not affected.

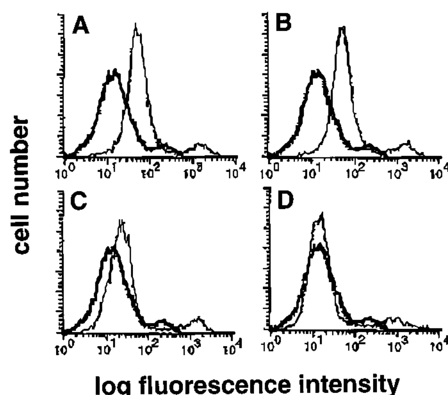

FIG. 4. Binding of listeriolysin to eucaryotic cellular membrane in the presence of specific polyclonal rabbit anti-listeriolysin antibodies. Different concentrations of specific polyclonal rabbit anti-listeriolysin antibodies (2.5 [B], 5 [C], and 50 [D] μg/ml) were incubated with 1 μg of listeriolysin at room temperature for 30 min. The mixture was then added to the mouse fibroblast cell line CA 36.2.1. Bound listeriolysin was detected by fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin. Thick lines represent the background fluorescence in the absence of listeriolysin. Thin lines represent the binding in the presence of listeriolysin. Panel A represents the control binding of untreated listeriolysin to the fibroblasts detected with a primary rabbit anti-listeriolysin and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin.

inhibiting the formation of oligomeric structures leading to pore formation. Their relative neutralization capacities may reflect the various steps at which they operate to inhibit hemolysis.

It is conceivable that conformational changes after binding could expose hydrophobic surfaces that facilitate insertion. The regions defined by the MAbs can now be selectively mutagenized to examine these steps in greater detail.

Besides exhibiting pore formation, thiol-activatable toxins often display diverse additional functions even at sublytic concentrations (1, 2, 8, 14, 18, 20). Hence, listeriolysin may interact with other membrane components contributing to signal transduction apart from its primary receptor.

The MAbs produced and characterized in this study can now be tested for their influence on these additional activities of listeriolysin. Furthermore, the novel binding assay described here can be used to isolate other MAbs specifically blocking these processes. By designing appropriate screening assays for additional MAbs, it should be possible to further characterize the regions involved in the various functions of listeriolysin. This should help to increase our understanding of its interaction and elicitation of signals in the eucaryotic cell membrane.

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