INFECTION AND IMMUNITY, Dec. 1986, p. 641–645
0019-9567/86/120641-05$02.00/0

Vol. 54, No. 3

Use of a Monoclonal Antibody to Determine the Mode of Transmembrane Pore Formation by Streptolysin O

FERDINAND HUGO, JOHANNES REICHWEIN, MARDJAN ARVAND, SYLVIA KRÄMER, AND SUCHARIT BHAKDI*

Institute of Medical Microbiology, University of Giessen, D-6300 Giessen, Federal Republic of Germany

Received 26 June 1986/Accepted 21 August 1986

Murine monoclonal antibodies were generated against streptolysin O. One out of 10 tested immunoglobulin clones exhibited strong neutralizing activity; in solution, the presence of approximately two to four antibody molecules per toxin monomer effected 50% neutralization of hemolytic toxin activity. An enzyme-linked immunosorbent assay performed with target cell membranes that were treated with streptolysin O in the presence and absence of neutralizing antibodies showed that the antibodies did not block primary binding of the toxin to the cells. When membranes were solubilized in deoxycholate detergent and centrifuged in sucrose density gradients, those lysed with streptolysin O contained detergent-resistant, high-molecular-weight oligomers identical to the pore lesions, whereas those given toxin and neutralizing antibody contained the toxin exclusively in low-molecular-weight, nonoligomerized form. The process of pore formation by streptolysin O must thus involve two distinct steps, i.e., the primary binding of toxin molecules to the membrane followed by oligomerization of bound toxin monomers by lateral aggregation in the lipid bilayer to form the transmembrane pores.

Streptolysin O (SLO) is the prototype of a large group of bacterial cytolysins that have been denoted sulfhydryl-activated toxins (1, 3, 28). These proteins bind to cholesterol-containing target membranes and irreversibly damage bilayers of erythrocytes and nucleated mammalian cells. Several of these toxins have been shown to generate circular and semicircular "lesions" that can be visualized by electron microscopy (4, 13, 14, 16, 25, 28, 29).

In the course of our studies on the mode of membrane damage by SLO, we have recently demonstrated that the ultrastructural lesions represent noncovalently bonded toxin oligomers that can be isolated in delipidated form in deoxycholate detergent solution and reincorporated into artificial bilayers of phosphatidylcholine (11). Negative staining and freeze-etch electron microscopy were used to show that these toxin oligomers most probably are embedded in the target bilayer and generate true transmembrane pores, which we believe represent the primary toxin lesion in erythrocyte membranes. This concept stands in agreement with functional data on the toxin lesion (12, 15). Cholesterol molecules, although required for initial binding of the toxin to the cells, do not appear to significantly contribute to the formation of the pore structures (11). It seemed probable that other, if not all, sulfhydryl-activated toxins damage target membranes via a similar mode of action. In another study, morphologically identical lesions composed of alpha-listeriolysin oligomers were isolated and shown to be immunologically related to SLO (26).

The mode of membrane damage by sulfhydryl-activated toxins fits into the general concept of transmembrane pore formation through hydrophilic-amphiphilic transitions of protein molecules accompanying their oligomerisation on and in a membrane (9). Other proteins that damage bilayers via this process include staphylococcal alpha-toxin (6, 18), the terminal C5b-9 complement complex (7, 9), and cytolytic proteins of T lymphocytes (20). In view of the widespread occurrence of pore-forming proteins, it is of general interest to analyze the process of transmembrane pore formation in the available models.

Basically, there are two ways in which pore-forming oligomers might be generated. First, initial binding of one protein molecule to the membrane could lead to a conformational change that exposes a binding site for a second protomer, etc. In this case, binding of the first molecule would trigger the binding of the next protomers within a given pore. There is good evidence that such a mechanism is operative in the formation of the complement pore (8–10, 27). In the case of oligomeric bacterial cytolysins, studies conducted with both staphylococcal alpha-toxin and SLO indicate that a second mode of pore formation may dominate, i.e., the initial binding of monomers to the membrane, followed by lateral aggregation and, thereafter, oligomerization in the bilayer. Thus, some evidence exists that high levels of Ca^{2+} can inhibit pore formation by alpha-toxin (2, 19, 24), and it has been suggested that this is due to inhibition of the hexamerization process in the membrane (19) (however, this contention is not supported by data obtained with black lipid membranes [24]). In the case of SLO, it has been reported that nonlytic binding of toxin occurs at 0°C, and that erythrocytes treated with toxin at 0°C will subsequently lyse if warmed to 37°C (summarized in reference 1). These experiments are suggestive, but they were conducted at a time when methods for quantifying and characterizing cell-bound toxin were not available. As will be reported in this paper, it is also not possible to avoid hemolysis at 0°C at higher toxin doses.

For these reasons, we sought another approach that would permit clear dissection of the pore-forming process by SLO. With the use of a monoclonal antibody, we demonstrate that this toxin first binds in monomer form to erythrocyte targets. Oligomerization follows in a second step, which can be selectively inhibited by the monoclonal antibody. The results establish that pore formation by SLO, although phenomenologically similar to that elicited by complement, follows a distinct and different pattern of events.

* Corresponding author.

MATERIALS AND METHODS

SLO was generally used in partially purified form, obtained by polyethylene glycol precipitation of concentrated culture supernatants of *Streptococcus* sp. strain Richards and one passage through a DEAE-column at pH 7 (5). The toxin preparations were concentrated to yield solutions with hemolytic activities of 25,000 to 100,000 hemolytic units (HU) per ml, corresponding to toxin protein concentrations of 30 to 120 μg/ml. Titrations for hemolytic activities were performed as described previously (5) at 0 or 37°C.

The membrane form of SLO was isolated from target erythrocytes as described previously (11). Toxin solutions recovered from sucrose density gradients were chromatographed over Sephadex G-25 (commercially available PD 10 columns; Pharmacia Fine Chemicals, Uppsala, Sweden) to remove detergent and sucrose and concentrated to approximately 0.2 mg/ml. The toxin thus obtained was used to immunize mice for the production of monoclonal antibodies.

Production of monoclonal antibodies. BALB/c, C57BL/6Han, and the F_1 hybrids of these mouse strains were obtained from the Zentralinstitut für Versuchstiere, Hannover, Federal Republic of Germany. The immunization procedure followed a published schedule (30). For primary immunization, antigen was admixed with Freund complete adjuvant and injected into both hind footpads and subcutaneously at different sites of the ventral surface. They were boostered 3 to 5 weeks later with subcutaneous injections of the antigen in incomplete Freund adjuvant. Final immunizations were performed approximately 4 weeks later with the antigen applied without adjuvant intraperitoneally and intravenously 4, 3, and 2 days before fusion of the cells.

The myeloma cell line X63-Ag 8.6.5.3. was chosen as the fusing partner for the spleen lymphoid cells. These myeloma cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (all from Biochrom, Berlin, Federal Republic of Germany). The myeloma cells and the spleen cells from immunized mice were fused in the conventional manner (21, 22) with a 50% PEG 4000 solution (E. Merck AG, Darmstadt, Federal Republic of Germany) as the fusing agent. Fused cells were diluted in culture medium containing 1 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine and distributed into 4 by 96-well Nunc trays (Nunc, Wiesbaden, Federal Republic of Germany). Hybrid cultures producing specific antibodies were cloned thrice by limiting dilution (23). Large-scale production of monoclonal antibodies was carried out in culture plates of 14-cm diameter. Samples of approximately 1 liter of culture supernatants were concentrated 10-fold (Amicon concentration chamber), and the immunoglobulins were precipitated by the addition of sodium sulfate (18% final concentration). Final purification of the antibodies was achieved with the use of protein A affinity chromatography (17). The purity of the immunoglobulin preparations was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the antibodies were concentrated to final preparations containing 0.5 to 2.0 mg of protein per ml.

Neutralization tests. Initial experiments showed that unfractionated cell culture supernatants containing fetal calf serum exhibited spontaneous toxin-neutralizing properties due to the presence of serum proteins, probably lipoproteins. Therefore, it was essential to use purified immunoglobulin G (IgG) preparations in all neutralization tests. Samples (50 μl) of antibody solutions containing 0.5 to 1 mg of protein per ml were doubly diluted in microtiter plates or Eppendorf tubes and incubated with 50 μl of SLO (20 ng of toxin) for 30 min at 22°C. SLO preparations were passed over a PD 10 column (Pharmacia) to remove dithiothreitol. To each tube was added 50 μl of 2.5% rabbit erythrocytes, and hemolysis was read after incubation for 60 min at 37°C.

Similar neutralization tests were performed with unfractionated culture supernatants of *Listeria ivanovii* and with supernatants of *Bacillus cereus* containing 4 to 8 HU of the respective toxins per ml.

ELISA for membrane-bound SLO. For the enzyme-linked immunosorbent assay (ELISA), 1 ml of a 10% suspension of rabbit erythrocytes in saline was incubated with 10 μl of SLO (32,000 to 64,000 HU/ml) in Eppendorf tubes for 30 min at 37°C, centrifuged, and washed thrice in 5 mM phosphate (pH 8), and the membrane pellets were suspended in 1 ml of saline containing 2% bovine serum albumin. Purified monoclonal antibodies were then added to a final concentration of 20 μg/ml. After 60 min at 37°C the membranes were washed thrice and suspended in saline-bovine serum albumin, and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts immunoglobulins, Copenhagen, Denmark) were added at final dilution of 1:1,000. After 60 min at 37°C the membranes were washed thrice and suspended in 1 ml of substrate solution (1 mg of *p*-nitrophenylphosphate dissolved in 1 ml of diethanolamine, pH 9.8). The reaction was stopped with 3 N NaOH after 20 min, and the absorbance was read at 405 nm.

Neutralization experiments were performed by preincubating the toxin with the monoclonal antibody for 10 min at 37°C, after which the erthrocytes were added. No hemolysis ensued under these conditions. The erthrocytes were subsequently washed twice, lysed with 5 mM phosphate (pH 8), and washed thrice in this buffer. The ELISA for membrane-bound SLO was then performed as described above.

Characterization of membrane-bound SLO. Erythrocyte membranes that had been treated with SLO in the presence or absence of neutralizing antibody were washed thrice in saline and thrice in 5 mM phosphate (pH 8). They were solubilized with 10% deoxycholate and centrifuged through sucrose density gradients containing 6.25 mM detergent as described previously (11). Ten equal fractions were collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Western blotting. The immunoblots were developed with polyclonal rabbit anti-SLO antibodies as described previously (11).

RESULTS

Lysis of rabbit erythrocytes by SLO at 0 and 37°C. When a toxin solution was assessed for hemolytic activity at 0 and 37°C, titers at 0°C were approximately 2% of those obtained at 37°C. When erythrocytes were incubated with subcytolytic toxin doses at 0°C (e.g., with 32 HU/ml) and the supernatants were retested for hemolytic activity at 37°C, no toxin activity could be found. This indirectly indicated that toxin had bound to but not lysed the cells. When erythrocytes receiving 8 to 32 HU of toxin per ml at 0°C were washed at 4°C and suspended in saline at 37°C, these cells lysed. These results confirmed earlier reports that SLO can bind to target erythrocytes at low termperature without inducing hemolysis, and that functional toxin lesions become apparent when such cells are warmed to 37°C (1). However, hemolytic activity is not totally abolished at 0°C. The present results also do not relate the observed functionality to a defined molecular form of membrane-bound toxin molecules. Even at 0°C, erythrocytes tolerated only rather low

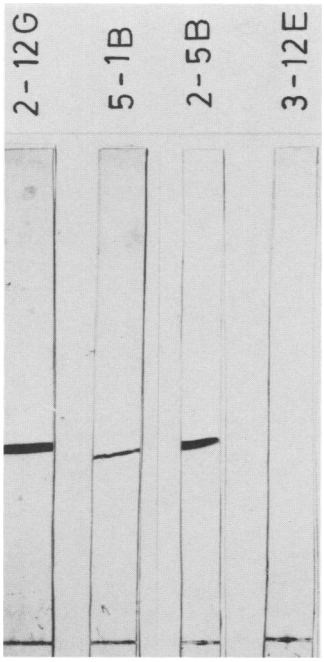

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Western blots of SLO probed with four monoclonal-antibodies (clone designations given at top of respective lanes). Three of the four antibody clones stained the sodium dodecyl sulfate-denatured protein.

toxin levels. The amount of nonlytic, membrane-bound toxin was too low to permit subsequent detection with the presently available methods.

Monoclonal antibodies to SLO. Ten monoclonal antibodies were generated against the nontoxic, membrane-bound form of SLO. Only four clones stained the SDS-denatured protein in Western blots (Fig. 1). Only one antibody clone was found to exhibit neutralizing properties, and the neutralizing capacity of these antibodies was approximated by titration (Fig. 2). We found that approximately 2.5 μg of purified IgG effected 50% neutralization of 0.4 μg of SLO. From the known molecular weight of SLO (69,000 [5]), the antibody toxin ratio required for 50% neutralization under the given experimental conditions was thus in the order of 2:1 to 4:1.

None of the tested monoclonal antibodies cross-reacted with alpha-listeriolysin or cereolysin in Western blotting or neutralized these toxins in the functional assay.

Neutralizing antibody does not inhibit SLO binding to erythrocytes. Neutralization by the monoclonal antibody could be due either to inhibition of toxin binding to the cells or to inhibition of pore formation by bound toxin molecules. An ELISA system for quantitation of membrane-bound toxin was utilized to differentiate between these possibilities.

In the positive control experiment, cells were first lysed with toxin, washed, and incubated with monoclonal antibodies, and the amount of bound monoclonal antibody was estimated by incubation of the membranes with phosphatase-conjugated second antibodies and measurement of substrate formation. In parallel, cells were treated with SLO in the presence of the same monoclonal antibody and processed similarly. Similar absorbance values were registered irrespective of the monoclonal antibody used (Fig. 3). Thus, the monoclonal antibody 2-5B, although it entirely inhibited hemolysis in the depicted experiment, did not prevent binding of the toxin to the erythrocytes. The results also indicated that the binding sites on SLO for the neutralizing monoclonal antibody were not concealed on lytic toxin oligomers.

Neutralizing monoclonal antibody inhibits formation of SLO oligomers on the membrane. Cell membranes receiving lytic doses of SLO in the presence and absence of the protective antibody were washed, solubilized in detergent, and centrifuged through sucrose density gradients. Western blotting of the harvested fractions revealed the presence of high-molecular-weight toxin oligomers in the positive controls (Fig. 4A) (11). As anticipated from the results of Fig. 3, toxin was also readily detected in solubilized membranes that had been protected by the monoclonal antibody. However, the toxin molecules recovered in this experiment sedimented exclusively to low-molecular-weight regions corresponding to approximately 8S, presumably as dimeric antigen-antibody complexes (Fig. 4B). Hence, the observed neutralization of bound toxin was directly correlated to the absence of oligomer formation in the membrane. When membranes were lysed in the presence of nonneutralizing antibody 5-1B, oligomers were again observed (data not shown).

DISCUSSION

Several basic conclusions may be drawn from these studies. First, the membrane-damaging function of SLO (1, 3, 5, 28) has clearly been shown to be related to the process of oligomer formation in the membrane. This conclusion is based on the observation that monoclonal antibody clone 2-5B totally inhibits hemolysis without detectably affecting

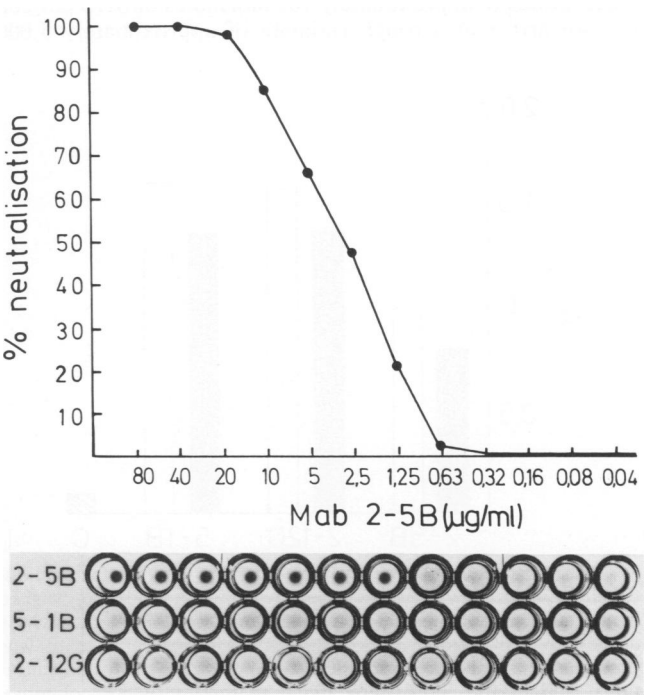

FIG. 2. Neutralization of SLO-hemolytic activity by monoclonal antibody clone 2-5B. Samples (50 μl) of a purified IgG preparation were doubly diluted, and then 20 ng of SLO (0.4 μg/ml) was added to each probe. After preincubation with the antibodies for 30 min, erythrocytes were added, and the ensuing lysis was recorded. The reciprocal values of percent hemolysis were taken as percent neutralization. The lower panel shows a microtiter neutralization assay performed in parallel with two other, nonneutralizing monoclonal antibodies.

primary binding of toxin molecules to the membrane. However, all bound toxin is present in nonoligomerized form. This finding provides direct support for the previously advanced concept that SLO-induced membrane damage is mediated through formation of toxin oligomers in the bilayer, presumably because these oligomers generate transmembrane pores. Whether the antibody directly interacts with oligomerizing surfaces, or whether it acts more indirectly by sterically hindering the apposition of toxin molecules, cannot be answered at present. However, the fact that the antibody binds to both oligomerized and nonoligomerized, membrane-bound toxin tends to speak for the latter case. It is of interest that the epitope reacting with this (and other tested) monoclonal antibody is not present on alpha-listeriolysin and cereolysin, despite the fact that alpha-listeriolysin pores are micromorphologically indistinguishable from SLO oligomers (26).

Another point of interest relates to the mode of oligomer formation by SLO and, by inference, by other bacterial cytolysins that act similarly. Since toxin binding precedes oligomerization, the latter process must be due to lateral aggregation of toxin molecules in the membrane and not to direct interaction of toxin monomers in the water phase with membrane-bound primers. Monoclonal antibody 2-5B obviously inhibits the process of toxin aggregation. In this respect, pore formation by SLO differs basically from that induced by C9 (8, 10, 27). We also confirmed that toxin binding can be dissociated from pore formation at low temperature if toxin doses are limited. Lysis does ensue when toxin levels exceed a critical threshhold corresponding to approximately 50 to 100 HU/ml. If we consider that 1 HU corresponds to approximately 100 molecules of SLO per cell (5), we arrive at a rough estimate of approximately 5,000

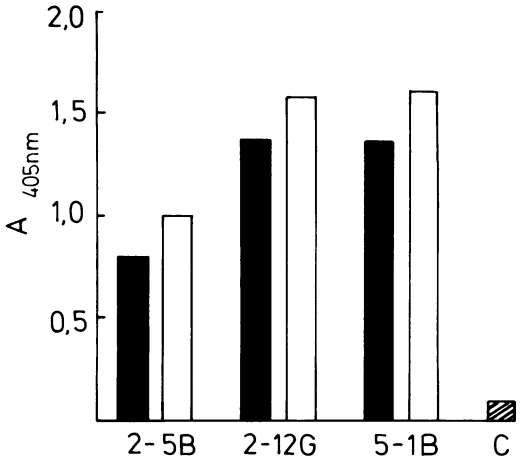

FIG. 3. ELISA for membrane-bound SLO with monoclonal antibodies. Erythrocytes were lysed with SLO, and the washed membranes were incubated with three monoclonal antibodies (clone designations in figure; black columns). Alternatively, toxin was preincubated with the antibodies at exactly the same concentrations, and erythrocytes were added thereafter (white columns). Preincubation of toxin with antibody clone 2-5B totally inhibited hemolysis under the given experimental conditions, whereas no neutralization occurred with clones 2-12G and 5-1B. When the ELISAs were developed with enzyme-labeled second antibodies, cell membranes protected by antibody clone 2-5B carried similar amounts of toxin compared to those first lysed with toxin. Nonneutralizing antibodies did not inhibit toxin binding to cells. C indicates the control, which was hypotonically lysed membranes probed with antibody clone 2-5B. A_{405nm}, Absorbancy at 405 nm.

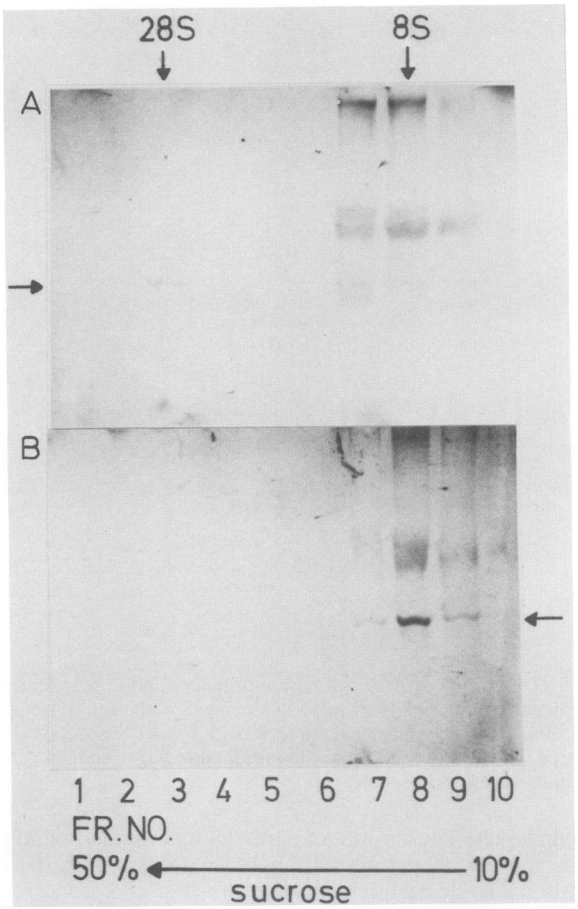

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Western blotting of sucrose density gradient fractions of deoxycholate-solubilized membrane samples. Membrane samples (0.3 ml) were applied to each 5-ml gradient. A, Positive control sample of membranes lysed with SLO in the absence of neutralizing antibody; B, SLO preincubated with antibody clone 2-5B to neutralize hemolytic activity and incubated with erythrocytes. Blots were probed with polyclonal rabbit antibodies to SLO. Arrows indicate SLO bands (5, 11). Higher-molecular-weight bands represent nonspecific background staining of erythrocyte emebrane proteins, primarily the spectrins and band 3 protein. Note the presence of SLO on membranes protected by the monoclonal antibody exclusively in the low-molecular-weight, ~8S form. In contrast, membranes that had been lysed with the toxin presented the high-molecular-weight oligomeric structures (11). The direction of sedimentation and approximate $s_{20,w}$ values are indicated.

toxin molecules that may be bound at 0°C without causing lysis. At high toxin levels, erythrocytes will also lyse at 0°C, and toxin oligomers become detectable (data not shown). Hence, collision, oligomerization, and membrane insertion of SLO can basically take place at low temperature.

In closing, it should be noted that since SLO is known to bind to cholesterol molecules (1, 3, 26a, 28), labeled complexes of the toxin with the neutralizing antibody could emerge as a very useful tool to probe the distribution, orientation, and mobility of cholesterol molecules in biological membranes.

ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft (Bh 2/2).

LITERATURE CITED

1. **Alouf, J. E.** 1980. Streptococcal toxins (steptolysin-O, streptolysin-S, erythrogenic toxin). Pharmacol. Ther. **11**:661–717.
2. **Bashford, C. L., G. M. Alder, K. Patel, and C. A. Pasternak.** 1984. Common action of certain viruses, toxins, and activated complement: pore formation and its prevention by extracellular Ca^{2+}. Biosci. Rep. **4**:797–805.
3. **Bernheimer, A. W.** 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta **344**:27–50.
4. **Bernheimer, A. W., and B. Rudy.** 1986. Interactions between membranes and cytolytic peptides. Biochim. Biophys. Acta **864**:123–141.
5. **Bhakdi, S., M. Roth, A. Sziegoleit, and J. Tranum-Jensen.** 1984. Isolation and identification of two hemolytic forms of streptolysin O. Infect. Immun. **46**:394–400.
6. **Bhakdi, S., and J. Tranum-Jensen.** 1981. Staphylococcal α-toxin:oligomerization of hydrophilic monomers to form amphiphilic hexamers induced through contact with deoxycholate detergent micelles. Proc. Natl. Acad. Sci. USA **78**:5475–5479.
7. **Bhakdi, S., and J. Tranum-Jensen.** 1983.Membrane damage by complement. Biochim. Biophys. Acta **737**:343–372.
8. **Bhakdi, S., and J. Tranum-Jensen.** 1984. On the cause and nature of C9-related heterogeneity of C5b-9(m) complexes generated on erythrocyte targets through the action of whole human serum. J. Immunol. **133**:1453–1463.
9. **Bhakdi, S., and J. Tranum-Jensen.** 1984. Mechanism of complement cytolysis and the concept of channel-forming proteins. Philos. Trans. R. Soc. Lond. B Biol. Sci. **306**:311–324.
10. **Bhakdi, S., and J. Tranum-Jensen.** 1986. C5b-9 assembly: average binding of one C9 molecule to C5b-8 without poly-C9 formation generates a stable transmembrane pore. J. Immunol. **136**:2999–3005.
11. **Bhakdi, S., J. Tranum-Jensen, and A. Sziegoleit.** 1985. Mechanism of membrane damage by streptolysin O. Infect. Immun. **47**:52–60.
12. **Buckingham, L., and J. L. Duncan.** 1983. Approximate dimensions of membrane lesions produced by streptolysin-S and streptolysin-O. Biochim. Biophys. Acta **729**:115–122.
13. **Cowell, J. L., P. S. Grushoff-Kosyk, and A. W. Bernheimer.** 1978. Alteration by cereolysin of the structure of cholesterol-containing membranes. Biochim. Biophys. Acta **507**:230–241.
14. **Dourmashkin, R. R., and W. F. Rosse.** 1966. Morphologic changes in the membranes of red blood cells undergoing hemolysis. Am. J. Med. **41**:699–710.
15. **Duncan, J. L.** 1974. Characteristicsof streptolysin O hemolysis: kinetics of hemoglobin and ^{86}rubidium release. Infect. Immun. **9**:1022–1027.
16. **Duncan, J. L., and R. Schlegel.** 1975. Effect of streptolysin-O on erythrocyte membranes, liposomes and lipid dispersions. A protein-cholesterol interaction. J. Cell Biol.**67**:160–173.
17. **Ey, P. L., S. J. Prowse, and C. R. Jenkin.** 1978. Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry **15**:429–436.
18. **Füssle, R., S. Bhakdi, A. Sziegoleit, J. Tranum-Jensen, T. Kranz, and H.-J. Wellensiek.** 1981. On the mechanism of membrane damage by *S. aureus* α-toxin. J. Cell Biol. **91**:83–94.
19. **Harshman, S., and N. Sugg.** 1985. Effect of calcium ions on staphylococcal alpha-toxin induced hemolysis of rabbit erythrocytes. Infect. Immun. **47**:37–40.
20. **Henkart, P.** 1985. Mechanism of lymphocyte-mediated cytotoxicity. Annu. Rev. Immunol. **3**:31–58.
21. **Hugo, F., D. Jenne, and S. Bhakdi.** 1985. Monoclonal antibodies against neoantigens of the terminal C5b-9 complex of human complement. Biosci. Rep. **5**:649–658.
22. **Köhler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of pre-defined specificity. Nature (London) **256**:495–497.
23. **Lefkovits, I., and H. Waldmann.** 1979. Limiting dilution analysis of cells in the immune system. Cambridge University Press, Cambridge.
24. **Menestrina, G.** 1986. Ionic channels formedby staphylococcus aureus alpha-toxin: voltage-dependent inhibition by divalent and trivalent cations. J. Membr. Biol. **90**:177–190.
25. **Mitsui, K., T. Sekiya, S. Okamura, Y. Nozawa, and J. Hase.** 1979. Ring formation of perfringolysin O as revealed by negative staining electron microscopy. Biochim. Biophys. Acta **558**: 307–313.
26. **Parrisius, J., S. Bhakdi, M. Roth, J. Tranum-Jensen, W. Goebel, and H. R. P. Seeliger.** 1986. Production and non-production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes*. Infect. Immun. **51**:314–319.

26a. **Prigent, D., and J. E. Alouf.** 1976. Interaction of streptolysin-O with sterols. Biochim. Biophys. Acta **443**:288–300.

27. **Silversmith, R. E., and G. L. Nelsestuen.** 1986. Assembly of the membrane attack complex of complement on small unilamellar phospholipid vesicles. Biochemistry **25**:852–860.
28. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen labile) cytolysins, p. 129–183. *In* J. Jeljaszewicz and T. Wadström (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., New York.
29. **Smyth, C. J., J. H. Freer, and J. P. Arbuthnott.** 1975. Interaction of *Clostridium* perfringens theta-haemolysin, a contaminant of commercial phospholipase C with erythrocyte ghost membranes and lipid dispersions. Biochim. Biophys. Acta **382**:479–493.
30. **Stähli, C., T. Staehelin, V. Miggiono, J. Schmidt, and P. Häring.** 1980. High frequencies of antigen-specific hybridomas: dependence on immunization parameters and prediction by spleen cell analysis. J. Immunol. Methods **32**:297–304.