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Inhibition of In Vitro Human Lymphocyte Response by the Pneumococcal Toxin Pneumolysin

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The effects of pneumolysin, a sulfhydryl-activated cytolytic toxin produced by *Streptococcus pneumoniae*, on the in vitro human lymphocyte response was examined. The toxin, at concentrations of one to five hemolytic units per ml, caused marked inhibition of the response of lymphocytes to concanavalin A, phytohemagglutinin, pokeweed mitogen, and protein A. The response was assessed by measuring both [^{3}H]thymidine incorporation and the ability of lymphocytes to produce immunoglobulins and lymphokine activity. The effects of pneumolysin were irreversible, could be prevented by pretreatment of the toxin with cholesterol, and were not related to a direct cytotoxic effect on the lymphocytes. Pneumolysin appeared to act at the initiation phase of the immune response and had no effect on lymphocytes committed to DNA synthesis or to the synthesis and secretion of immunoglobulins. Furthermore, pneumolysin-mediated inhibition of the lymphocyte response was not due to the inhibition of binding of mitogens to leukocytes and is likely to be related to effects on membrane-mediated signals essential for lymphocyte triggering. This may be one means by which pneumolysin plays a role in the pathogenesis of pneumococcal infections.

Pneumolysin is a sulfhydryl-activated cytolytic toxin which is produced by *Streptococcus pneumoniae* and is known to bind cholesterol in host cell membranes (9). It has recently been demonstrated that pneumolysin plays a role in the pathogenicity of this organism in a mouse model (13) and has been suggested that pneumolysin reduces host resistance by inhibiting the antimicrobial properties of neutrophils and the opsonic activity of serum (12, 13).

An important event in the development of resistance to *S. pneumoniae* is the acquisition of type-specific antibodies to capsular polysaccharide (1, 10). The possible interaction of pneumolysin with cholesterol in the plasma membrane could conceivably interfere with events leading to lymphocyte stimulation and hence production of antibodies. Accordingly, the present study was initiated to examine the effects of pneumolysin on the in vitro immune response of human lymphocytes.

MATERIALS AND METHODS

Purification of pneumolysin. The toxin was purified from a type 1 *S. pneumoniae* strain as previously described (13). The final preparation migrated as a single major protein species (which accounted for >97% of the total protein) when analyzed by polyacrylamide gel electrophoresis in either the presence or the absence of sodium dodecyl sulfate. The purified material had a specific activity of at least 5×10^5 hemolytic units (HU) per mg of protein (13) (1 HU per ml will lyse 50% of a 1% suspension of human erythrocytes in 30 min at 37°C).

Preparation of MNLs. Mononuclear leukocytes (MNLs) were prepared from the blood of healthy volunteers by low-speed centrifugation in Hypaque-Ficoll medium (density, 1.114 g/ml) as previously described (5, 6). MNLs were suspended in RPMI 1640 medium (RPMI) (Flow Laboratories, Inc., McLean, Va.) with or without 10% heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Inc.).

Lymphocyte transformation. Lymphocyte transformation studies were conducted by a microtechnique in RPMI as previously described (15). Briefly, 2×10^5 lymphocytes were cultured in wells of microtiter plates in the presence or absence of mitogens and with or without pneumolysin. The total volume per well was 0.2 ml, and the cultures contained 10% FCS. The cells were incubated at 37°C for 72 h in an atmosphere of 5% CO_2–95% air at a high humidity and were pulse-labeled with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) 6 h before being harvested. The cells were harvested by aspiration onto glass fiber filters with a multiple-sample cell harvester, and the radioactivity was measured by liquid scintillation spectrometry. The mitogens phytohemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogen (PWM), and protein A were used at concentrations that resulted in the optimal stimulation of [^{3}H]TdR incorporation. These were 0.5 μg/ml, 12.5 μg/ml, a 1/200 dilution, and 12.5 μg/ml, respectively. PHA, ConA, PWM, and protein A were obtained from Wellcome Pharmaceuticals and Reagents Pty., Ltd. Sydney, Australia, Calbiochem-Behring, Sydney, Australia, GIBCO Laboratories, Grand Island, N. Y., and Sigma Chemical Co., St. Louis, Mo., respectively. Fluorescein isothiocyanate-labeled ConA was obtained from Sigma Chemical Co.

Lymphocyte culturing for immunoglobulin production. MNLs (10^6/ml) were suspended in RPMI with FCS and cultured in LUX tissue culture tubes (16 by 125 mm; Miles Laboratories Inc., Naperville, Ill.) with or without the T-cell dependent B-cell mitogen PWM at the optimal mitogen concentration (1/200 dilution). The cultures were incubated at 37°C in an atmosphere of 5% CO_2–95% air at a high humidity. The supernatants were harvested after 7 days of incubation and examined for quantities of immunoglobulin M (IgM), IgG, and IgA by an enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay for quantitation of immunoglobulins. Antibodies to human IgM, IgG, and IgA (heavy-chain specific) were bound to a solid phase as follows. A 200-μl volume of μ-chain-, γ-chain-, or α-chain-specific antisera (diluted 1/200, 1/200, and 1/150, respective-

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ly, in 0.1 M carbonate buffer, pH 9.6) was added to wells of microtiter plates. Sheep antisera to human μ-chain and human γ-chain, were obtained from Silenus Laboratory, Victoria, Australia. Rabbit antiserum to human α-chain was obtained from Calbiochem-Behring. After incubation for 18 h at 4°C, the plates were washed five times with phosphate-buffered saline supplemented with 0.05% Tween 20 (pH 7.5). A 100-μl volume of 5% sheep serum in phosphate-buffered saline, 0.05%–Tween 20 was then added, and the plates were incubated at 37°C for 1 h. After a further washing step, 50 μl of various dilutions of immunoglobulin standards or culture supernatant were added to the wells. Standard human IgG was obtained from Cappel Laboratories, Cochranville, Pa., and standard human IgM and IgA were obtained from Calbiochem-Behring. The plates were incubated at 37°C for 1 h and washed again, and then 50 μl of horseradish peroxidase-linked sheep antihuman immunoglobulin (1/2,500 dilution in phosphate-buffered saline–0.05% Tween 20) (Amersham, Australia, Pty., Ltd.) was added. After incubation at 4°C for 18 h, the wells were washed, 100 μl of a 1 mM solution of the substrate 2, 2′-azino-di(3-ethylbenzthiazoline sulfate) (Amersham) was added, and the plates were incubated at 37°C for 1 h. The absorbance at 414 nm was then measured, and the immunoglobulin concentration in each test sample was determined by comparison with a standard curve.

Conditions for the stimulation of N-MIF. The stimulation of neutrophil migration inhibitory factor (N-MIF) activity was brought about as previously described (3). MNLs at a concentration of 2×10^6 cells per ml were incubated in RPMI with FCS in either the presence or the absence of 0.5 μg of PHA per ml. The leukocytes were incubated at 37°C for 24 h in an atmosphere of 5% CO_2–95% air at a high humidity. The MNLs were washed, resuspended in RPMI with FCS, and incubated for 48 h. After incubation, the supernatants were prepared by centrifugation of cells and filtration of the supernatant through a 0.22-μm-pore-diameter membrane (Millipore Corp., Bedford, Mass.).

Measurement of N-MIF. N-MIF activity was measured by examining the ability of MNL culture supernants to inhibit the random migration of neutrophils under agarose. The migration technique used was essentially that described by Nelson et al. (11), with some modifications (2). Briefly, 2.5-mm-diameter wells were cut in 1% agarose (made up in RPMI with FCS). Neutrophils (4×10^7/ml) were incubated at 37°C for 1 h with an equal volume of test supernatant, and then 5-μl volumes were added to each well. The plates were incubated at 37°C in an atmosphere of 5% CO_2–95% air at a high humidity. After 2.5 h, the distance of neutrophil migration was measured with an inverted microscope under phase contrast.

Cell viability. Cell viability was assessed by the trypan blue dye exclusion method under previously described conditions (4).

Statistics. Data were analyzed by Student's paired *t* test (two-tailed).

RESULTS

Effect of pneumolysin on the mitogen-induced lymphocyte response. In these experiments, pneumolysin was added simultaneously with the lymphocyte mitogens at concentrations ranging from 0.01 to 10 HU/ml. The results showed that pneumolysin inhibited the ability of lymphocytes to proliferate in the presence of any of the four mitogens (ConA, PHA, PWM, and protein A) (Table 1). The response to PWM and protein A was, however, more sensitive to pneumolysin than that to ConA and PHA. The minimal concentrations of pneumolysin at which significant depression of the lymphoproliferative response occurred were 0.1 HU/ml for protein A, 1.0 HU/ml for PWM, and 5.0 HU/ml for ConA and PHA. At concentrations of up to 10 HU/ml, pneumolysin had no effect on the viability of MNLs (as assessed by the trypan blue dye exclusion method), after either 1 h or 3 days of incubation.

DNA synthesis was also observed in the absence of the above mitogens. This is possibly related to the nonspecific stimulation of human lymphocytes by factors in FCS. Pneumolysin also inhibited DNA synthesis in these cultures.

Reversibility of the effects of pneumolysin. The binding of pneumolysin to cholesterol in the plasma membranes of MNLs may cause irreversible changes in the membranes. To examine this possibility, we cultured MNLs for 1 h at 37°C with 5 HU of pneumolysin per ml, washed the cultures three times with medium, and then tested the cultures for their ability to respond to mitogens, as described above. The washing procedure failed to alleviate the inhibitory effects of pneumolysin on lymphoproliferation in response to any of the four mitogens (data not shown).

Effect of delayed addition of pneumolysin to cultures. The inhibitory effects of pneumolysin could be related to an action on either the initiation or the proliferative phase of the immune response. To examine this possibility we studied the effects of adding pneumolysin to the cultures at various times. After the initiation of cultures, 5 HU of the toxin per ml was added to the cultures at 0, 4, 24, or 48 h. The results showed that pneumolysin inhibited the lymphocyte response

TABLE 1. Effect of pneumolysin treatment on [^{3}H]TdR incorporation by lymphocytes in response to mitogens

Pneumolysin concn (HU/ml)	% of [^{3}H]TdR incorporated by lymphocytes in response to[a]:				
	Medium	ConA	PHA	PWM	Protein A
0.01	95.6 ± 6.9	101.8 ± 5.0	108.0 ± 7.8	96.0 ± 6.1	95.0 ± 14.4
0.1	89.7 ± 8.9	104.1 ± 7.8	111.9 ± 17.0	95.3 ± 10.6	82.0 ± 14.9[b]
0.5	85.0 ± 14.6	102.9 ± 13.9	98.1 ± 13.8	94.3 ± 14.0	59.5 ± 18.6[c]
1.0	75.1 ± 21.8	94.4 ± 7.4	95.2 ± 15.5	76.0 ± 16.0[d]	44.0 ± 21.7
5.0	25.2 ± 7.2[e]	17.5 ± 3.0[b]	12.7 ± 9.0[b]	4.8 ± 2.3[b]	5.8 ± 3.1[b]
10.0	18.0 ± 5.4[b]	6.1 ± 4.5[b]	12.7 ± 15.2[b]	2.2 ± 1.5[b]	4.3 ± 1.3[b]

[a] The data represent the amount of [^{3}H]TdR incorporated in the presence of various concentrations of pneumolysin expressed as a percentage of that incorporated by diluent controls (means ± standard deviations of six experiments). The mean [^{3}H]TdR incorporation in these controls was 2,768 dpm in the absence of a mitogen, 177,953 dpm with ConA, 196,747 dpm with PHA, 116,037 dpm with PWM, and 31,603 dpm with protein A. The *P* values (see footnotes *b–e*) are for results that were significantly different from the results for the respective diluent controls.

[b] $P < 0.001$.

[c] $P < 0.01$.

[d] $P < 0.02$.

[e] $P < 0.05$.

TABLE 2. Effect of delayed addition of pneumolysin on the lymphocyte response

Addition time (h)	% of [^{3}H]TdR incorporated by lymphocytes in response to[a]:				
	Medium	ConA	PHA	PWM	Protein A
0	57.2 ± 8.4[b]	21.9 ± 8.6[b]	71.9 ± 12.4[b]	21.1 ± 12.8[b]	6.1 ± 2.5[b]
4	93.2 ± 11.2	96.1 ± 3.6	96.9 ± 1.7	90.4 ± 8.2	101.3 ± 9.4
24	95.8 ± 9.7	97.1 ± 3.0	97.5 ± 2.4	96.1 ± 3.2	97.9 ± 3.6
48	99.7 ± 8.3	99.9 ± 2.2	96.3 ± 3.0	96.6 ± 2.6	101.5 ± 5.4

[a] See Table 1, footnote *a*. The pneumolysin concentration was 5 HU/ml.
[b] $P < 0.001$.

(as measured by [^{3}H]TdR incorporation) only when added at time 0 (Table 2). It had no effect when added 4 h or more after the initiation of cultures.

Effect of pneumolysin on lymphoblasts. The time-addition studies presented above suggested that pneumolysin had no effect on lymphoblasts. To examine this possibility directly, we stimulated lymphocytes with mitogens, washed them, and then incubated them in either the presence or the absence of pneumolysin. The results showed that pneumolysin at 5 HU/ml failed to prevent the division of lymphoblasts (Table 3). Viability studies showed that noncultured lymphocytes and lymphoblasts were equally sensitive to the cytotoxic effects of pneumolysin (in 1-h assays). Cytotoxic concentrations were found to be >100 HU/ml.

Effect of pneumolysin on mitogen binding. One possible reason for the effects of pneumolysin on the lymphocyte response could be the prevention of binding of mitogens to MNLs. To test this possibility, we incubated MNLs treated with or without 10 HU of the toxin per ml at 37°C for 60 min with fluorescein isothiocyanate-labeled ConA (Sigma Chemical Co.) or ^{125}I-labeled protein A (Amersham). After incubation, the cells were washed, and bound label was assessed by fluorescence microscopy or gamma spectrometry, respectively. The results (data not shown) showed that MNLs bound similar amounts of ConA and protein A in either the presence or the absence of pneumolysin.

Effect of cholesterol on the inhibitory properties of pneumolysin. To examine whether the inhibition of lymphoproliferation by pneumolysin was due to its hemolytic activity, we preincubated the toxin for 10 min at 37°C with 2 μg of cholesterol per 1,000 HU, causing >98% inhibition of hemolytic activity. The addition of this cholesterol-inactivated pneumolysin to lymphocyte cultures did not inhibit the lymphocyte response to any of the four mitogens, as measured by [^{3}H]TdR incorporation (data not shown).

Effect of pneumolysin on N-MIF production. Pneumolysin at 5 HU/ml markedly inhibited the production of N-MIF by MNL cultures (Table 4).

Effect of pneumolysin on immunoglobulin synthesis. The ability of pneumolysin to inhibit the synthesis of IgM, IgG, and IgA by lymphocytes stimulated with PWM was examined. Significant inhibition ($P < 0.01$) of the synthesis of all immunoglobulin classes by stimulated cells was observed at toxin doses as low as 1 HU/ml (Fig. 1). At 5 HU/ml, the synthesis of IgM, IgA, and IgG was inhibited by 99.3, 97.0, and 96.0%, respectively.

Effect of pneumolysin on immunoglobulin secretion by immunoglobulin-producing cells. To test whether the effect of the toxin on immunoglobulin production was due to a direct effect on the plasma membranes of the lymphocytes, thereby preventing secretion, we stimulated MNLs with PWM and incubated them for 7 days. The cells were then washed and reincubated for 24 h in either the presence or the absence of 5 HU of pneumolysin per ml. This concentration of pneumolysin, which caused marked inhibition of immunoglobulin production when added at the initiation of cultures, failed to depress the secretion of IgA, IgG, or IgM when added to cells committed to synthesizing immunoglobulins (data not shown).

DISCUSSION

The data presented showed that a highly purified preparation of pneumolysin caused marked inhibition of the response of human peripheral blood lymphocytes to the mitogens ConA, PHA, PWM, and protein A (as measured by [^{3}H]TdR incorporation). The effects of pneumolysin were irreversible, as prior short-term exposure of lymphocytes to the toxin followed by washing of the cells did not alleviate the inhibitory effects. The inactivation of pneumolysin by cholesterol removed the inhibitory properties of the toxin for the lymphocyte response. It is most unlikely that the de-

TABLE 3. Effect of pneumolysin on lymphoblasts

Lymphocyte stimulator	% of [^{3}H]TdR incorporated by lymphoblasts in response to[a]:
ConA	101.7 ± 5.3
PHA	105.6 ± 5.4
PWM	98.2 ± 6.3
Protein A	102.5 ± 7.3

[a] Lymphoblasts were produced by culturing MNLs for 24 h at 37°C with the various indicated mitogens. After this incubation, the leukocytes were washed, and [^{3}H]TdR incorporation was then measured in the presence of 5 HU of pneumolysin per ml (as described previously) and expressed as a percentage of the incorporation in diluent controls. The mean [^{3}H]TdR incorporation in these controls was 304,495, 230,819, 187,244, and 13,632 dpm for PHA-, ConA-, PWM-, and protein A-induced lymphoblasts, respectively. The results are expressed as the means ± standard deviations of five experiments.

TABLE 4. Effect of pneumolysin on N-MIF production by PHA-stimulated human lymphocytes

Pneumolysin concn (HU/ml)	% Inhibition of migration[a]
0	55.9 ± 1.2
1	57.0 ± 0.6
5	21.8 ± 2.1[b]
10	10.7 ± 8.6[b]

[a] Results are expressed as the means ± standard deviations of three experiments, and N-MIF activity was measured as the percentage of inhibition of random neutrophil migration (as compared with random migration in controls in which the neutrophils were incubated with supernatants from MNL cultures incubated with medium containing FCS or with the respective diluent concentration). No significant difference was observed between the supernatants from control cultures incubated in the presence or absence of the diluent.
[b] $P < 0.001$.

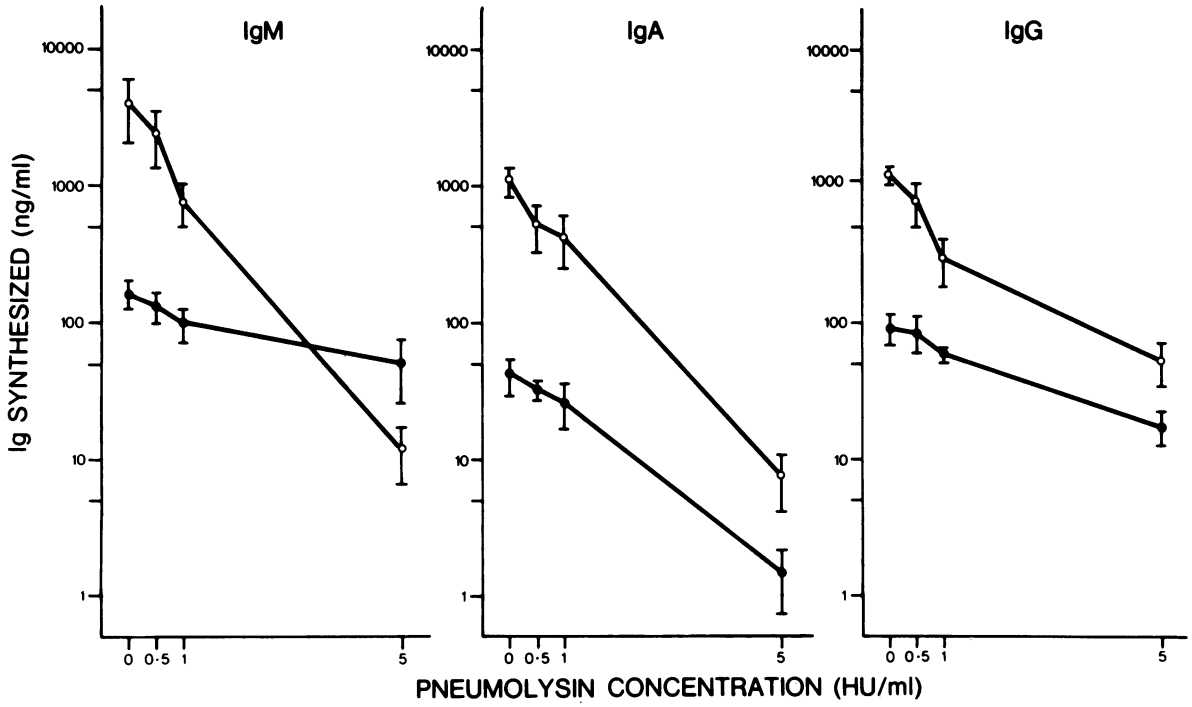

FIG. 1. Effect of pneumolysin on in vitro immunoglobulin (Ig) production by lymphocytes. Pneumolysin-treated lymphocytes were incubated in the presence (○) or absence (●) of PWM. The results show the IgM, IgG, and IgA concentrations in culture supernatants on day 7 of culturing. The results are expressed as the means ± standard deviations of three experiments.

creased [^{3}H]TdR incorporation was related to changes in the pool size of unlabeled intracellular thymidine or in the activities of thymidine kinase or the plasma membrane thymidine carrier. This view is supported by our findings that the production of lymphocyte products, namely, N-MIF and immunoglobulins, was similarly inhibited by the toxin.

Pneumolysin inhibited the ConA- and PHA-induced lymphocyte response to a much lesser degree than it did that to PWM and protein A. As these polyclonal ligands display a certain degree of specificity for either T- or B-lymphocytes, the results suggest that pneumolysin may have a preferential effect on B-lymphocytes; ConA and PHA stimulate T-cells, whereas PWM and protein A stimulate both T- and B-lymphocytes.

The inhibitory effects on the lymphocyte response appeared to be unrelated to a direct cytotoxic effect on MNLs; pneumolysin at 10 HU/ml failed to reduce leukocyte viability (as assessed by trypan blue dye exclusion). Indeed, a significant loss in viability was only observed at doses >100 HU/ml, with both noncultured lymphocytes and lymphoblasts being equally sensitive. The inhibitory effects of pneumolysin were related to the early events of the lymphocyte response. Concentrations of 5 HU/ml caused marked suppression of the lymphocyte response if added at the initiation of cultures, but no effect was observed if such amounts were added 4 h later. Further support for this conclusion was provided by the finding that DNA synthesis in both T- and B-lymphoblasts (produced by culturing with mitogens and washing the leukocytes after 24 h of incubation) was not inhibited by pneumolysin. This further supports our observation that the toxin is not cytotoxic for lymphocytes at the studied concentrations.

The mechanism by which pneumolysin influences the lymphocyte response is presumably related to its known affinity for cholesterol in the plasma membranes of host cells (8, 9). The inhibition of the lymphocyte response could be a consequence of the modification of mitogen receptors on the plasma membrane, thereby preventing mitogen binding or interfering with subsequent membrane-mediated signals essential for lymphocyte triggering or both. Our results show that the former is unlikely, as both fluorescein isothiocyanate-labeled ConA and ^{125}I-labeled protein A bound to both control and pneumolysin-treated MNLs to a similar degree. Previous studies have shown that for the triggering of lymphocytes to occur, plasma membrane lipids must be in a fluid state (7), and that cholesterol plays an important part in maintaining this fluidity (7). It is therefore conceivable that pneumolysin could interfere with lymphocyte triggering by altering plasma membrane fluidity through an interaction with cholesterol. Interestingly, such putative modifications to the plasma membranes of leukocytes did not appear to prevent the secretion of immunoglobulins by plasma cells.

It has previously been shown that the immunization of mice with a similar preparation of pneumolysin significantly increased survival time after challenge with virulent *S. pneumoniae* (13). The nature of this protection is not known, but one possibility that has been suggested is that immunization prevents pneumolysin from interfering with components of the immune system involved in the clearance and killing of pneumococci (12, 14). Antibody against the capsular polysaccharide antigens of *S. pneumoniae* provides the principal means of conferring protection against virulent pneumococci (1, 10). Pneumolysin may potentially prevent the establishment of an adequate antibody response against the surface antigens of the organism by primarily interfering with B-cell function. Although the amount of pneumolysin produced by *S. pneumoniae* in vivo is not known, the present finding that extremely low doses of the toxin (<1 HU [2 ng] per ml) caused the inhibition of the lymphocyte response suggests that these observations may have in vivo relevance. The

effects of pneumolysin on lymphocytes reported here complement previous findings that pneumolysin inhibits neutrophil chemotaxis and bactericidal function (8, 12) and depletes serum of opsonic activity by activating complement (14). These findings further support our credence that pneumolysin may contribute to the pathogenicity of *S. pneumoniae* by interfering with components of the immune system. It is therefore possible that incorporation of (inactivated) pneumolysin into the existing pneumococcal polysaccharide vaccine may improve its efficacy in preventing invasive pneumococcal disease.

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