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Role of Outer Envelope Contamination in Protection Elicited by Ribosomal Preparations Against *Neisseria gonorrhoeae* Infection

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A recent report (Cooper et al., Infect. Immun. **28**:92-100, 1980) demonstrated that immunization of guinea pigs with ribosomal preparations was protective (~90%) against chamber infections with *Neisseria gonorrhoeae*. Similar protection has been demonstrated with other cellular immunogens such as outer membranes (OM) (92%) or purified lipopolysaccharide (LPS) (83%). Protection by LPS (5 to 100 μg) was dose dependent (83% with 100 μg). Treatment of LPS with pronase reduced the protection by 50%. Ribosomal preparations contained LPS contamination (3.9%) based on dry weight determinations by 2-keto-3-deoxyoctonate analysis. Analysis of ribosomal preparations isolated from cells after lactoperoxidase-mediated ^{125}I labeling indicated a major OM contamination (Protein I). The ribosomal preparation also contained low levels of succinic and lactic dehydrogenase. Passive hemagglutination tests revealed that sera from guinea pigs immunized with ribosomal preparations also demonstrated antibody to OM proteins and LPS. LPS was able to absorb one line of precipitation seen in immunodiffusion reactions as well as the bactericidal activity of such sera. OM preparations were unable to absorb the remaining precipitin line or remove the bactericidal activity. It appears that LPS is the major antigen responsible for the bactericidal activity seen in ribosome-immune sera.

Previous work in our laboratory demonstrated that immunization of guinea pigs with gonococcal ribosomal preparations is protective against chamber infections with a homologous strain of *Neisseria gonorrhoeae* (2). This work established that the crude ribosomal preparation was a heterogeneous complex containing a proteinaceous moiety as a major protective immunogen as well as endotoxin and ostensibly other outer membrane (OM) components.

Isolated gonococcal outer envelope components have been used by other investigators as protective immunogens in experimental animals. Buchanan and Arko (1) reported the immunogenic role of purified OMs in protecting guinea pig subcutaneous chambers against homologous infection by *N. gonorrhoeae*. Diena et al. (3) passively immunized chicken embryos against both homologous and heterologous challenge with *N. gonorrhoeae* using anti-lipopolysaccharide (anti-LPS) antibodies.

Evidence that ribosomal preparations from other gram-negative bacteria are contaminated with outer envelope components has been reported from several laboratories (5, 6, 8, 16). Lieberman (12) reported that LPS-containing fractions from *Pseudomonas* ribosomal preparations were more immunogenic (protective) than fractions which were devoid of LPS. Thus, envelope contaminants may be important in the immune response generated by these preparations.

The present investigation was undertaken to determine the presence of outer envelope contaminants in the gonococcal ribosomal preparations and their role in protection.

MATERIALS AND METHODS

Organisms. Colony type 1 (T_1) of *N. gonorrhoeae* 120-94 was used throughout the investigation. The cultures were maintained on GC agar base (Difco Laboratories, Detroit, Mich.) supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.).

N. gonorrhoeae T_1 cells for ribosomal preparation and challenge of animals were grown in a liquid medium as previously described (2).

These organisms (T_1) grew as opaque colonies according to the classification scheme described by Swanson (19).

Animals. Female Hartley guinea pigs weighing approximately 600 g were obtained from Elm Hill Co., Chelmsford, Mass. Each guinea pig was implanted

with two dorsal subcutaneous chambers of modified polyallomer tubes (2).

Preparation of ribosomes. The ribosomal preparations were prepared by a modification of the method of Youmans and Youmans (26) as previously described (2).

Isolation of the OM components. Gonococcal OMs were isolated by the method of Wolf-Watz et al. (24). Membranes were also prepared by the method of Johnston et al. (10). In brief, washed cells were spheroplasted using 10 mg of lysozyme in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride containing 25% (wt/wt) sucrose per 5 g (wet weight) of gonococci. The spheroplasts were disrupted by sonication (80 W) for two 5-min intervals followed by centrifugation at 1,500 × *g* for 20 min. The supernatant fluid was centrifuged at 40,000 × *g* for 60 min, and the pellet was suspended in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) containing 1% (wt/vol) sodium lauryl-sarcosinate (Sarkosyl, Pfaltz and Bauer, Stamford, Conn.). The mixture was incubated at room temperature for 30 min, followed by centrifugation at 40,000 × *g* for 60 min. The Sarkosyl solubilization was repeated twice more, and the resulting pellet was suspended in 10 mM Tris-hydrochloride (pH 7.5) containing 0.02% (wt/vol) NaN_3 and 0.001% (wt/vol) phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) and exhaustively dialyzed against the same buffer. The dialyzed sample was sedimented at 40,000 × *g* for 60 min and suspended in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5).

LPS was extracted from whole cells (50 g [wet weight]) by the hot phenol method of Westphal and Jann (22).

Gonococcal LPS was digested with pronase (50 μg/ml, from *Streptomyces griseus* [Sigma Chemical Co.]) at 37°C for 6 h. The LPS was treated at 100°C for 5 min to terminate the reaction. The sample was centrifuged at 100,000 × *g* for 30 min, washed twice in 10 mM phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$, and resuspended in the same buffer.

Enzyme assays. Succinate and D-lactate dehydrogenase were measured by coupling the enzymes via phenazine methosulfate to the reduction of thiazolyl blue as described by Miller and Morse (15).

Chemical analysis. Protein was measured by the method of Lowry et al. (13) using crystalline bovine serum albumin as a standard. Ribonucleic acid and deoxyribonucleic acid were measured by the orcinol (18) and diphenylamine (4) reactions, respectively. Carbohydrates were measured by the phenol-sulfuric acid reaction (23). The assay for 2-keto-3-deoxyoctonate was performed as described by Karkhanis et al. (11).

^{125}I surface labeling. Cells harvested from broth culture were washed in phosphate-buffered saline (pH 7.2) containing 10 mM $MgCl_2$ and suspended to a concentration of approximately 10^{10} cells per ml. The suspension was cooled to 4°C, and 1 mCi of carrier-free sodium ^{125}I (New England Nuclear Corp., Boston, Mass., specific activity 17 Ci/mg) was added along with lactoperoxidase (18 mU/ml, Sigma Chemical Co.) and glucose oxidase (18 mU/ml, Sigma Chemical Co., *Aspergillus niger*). The reaction was initiated by the addition of glucose (5 μmol/ml). The reaction mixture was removed from the ice bath and immediately stirred for 30 min at room temperature. The reaction was terminated by the addition of L-cysteine (10 mM final concentration). The cells were washed once in phosphate-buffered saline (pH 7.2) containing 10 mM $MgCl_2$ and 30 mM KI and processed for either OMs or ribosomal preparations as described above.

SDS-PAGE. Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by solubilization in 10 mM phosphate buffer containing 1% (wt/vol) SDS and 1% (wt/vol) β-mercaptoethanol and by incubation at 100°C for 5 min. Electrophoresis in 10% gels was performed essentially as described by Weber and Osborn (21). Gels were stained for 2 h using 0.25% Coomassie brilliant blue R-250 in methanol-glacial acetic acid (5:1). Gels were destained using 7.5% acetic acid and 5% methanol at 50°C. Molecular weights were estimated using Dalton-Mark VI standards (Sigma Chemical Co.).

Radioactivity measurement. SDS-PAGE gels of ^{125}I-labeled OMs and ribosomal preparations were cut into 2-mm slices, and the ^{125}I counts per minute were determined directly for each slice in a Beckman model 310 gamma counter (Beckman Instrument Co., Palo Alto, Calif.).

Immunization of guinea pigs. Guinea pigs were immunized subcutaneously with ribosomal preparations as previously described using Freund incomplete adjuvant (2). OM preparations (200 μg of protein) were used without Freund incomplete adjuvant to immunize the animals. LPS was suspended in physiological saline and administered in graded doses. With the exception of the ribosomal preparations, all doses were given subcutaneously in a total volume of 1 ml. The evaluation of the immune response was determined as previously described (2).

Tests for antiribosomal antibody. The passive hemagglutination and immunodiffusion tests were performed as previously described (2).

Absorption of immune sera. Antisera from guinea pigs immunized with ribosomes were absorbed with OMs prepared by the method of Wolf-Watz et al. (24), Sarkosyl-extracted proteins, or LPS. Antisera (0.5 ml) were absorbed with 250 μg of LPS or membrane proteins at 37°C for 1 h. This mixture was allowed to further incubate at 4°C overnight, and the complex precipitated was removed by sedimentation at 48,246 × *g* for 60 min. Any unprecipitated LPS was removed by centrifugation at 100,000 × *g* for 60 min, and the supernatant was concentrated to the original volume using a YM 10 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). The resulting absorbed sera were then used in either immunodiffusion tests or in the bactericidal assays.

Serum bactericidal assay. The bactericidal assay employed in these studies was a modification of the method of Wong et al. (25): microtiter plate wells were blanked with 25 μl of diluent (0.006% $MgCl_2 \cdot 6H_2O$, 0.004% $CaCl_2 \cdot 2H_2O$, and 1% bovine serum albumin [pH 7.2]). Heat-inactivated (56°C, 30 min) test serum (25 μl) was added to the first well and serially diluted. Immediately after dilution, 25 μl of a 1/10 dilution of guinea pig sera previously tested as being negative for antigonococcal activity was added to each well as a

complement source. The wells were inoculated with 25 μl of a 10^7-colony-forming units per ml inoculum of gonococci and incubated at ambient temperature for 90 min. After the incubation, 10 μl of the reaction mixture was plated on GC agar plates (Difco) containing VCN inhibitor (BBL Microbiology Systems), allowed to air dry for 20 min with the cover on, and incubated overnight at 37°C in a 5% CO_2 atmosphere. Killing was ascribed to those dilutions of antisera which had at least a 75% reduction in growth when compared to the number of organisms present in the wells with normal or heat-inactivated normal guinea pig sera.

RESULTS

Immunogenicity of ribosomal preparations and outer envelope components. A comparison of the efficacy of ribosomal preparations and that of isolated outer envelope preparations was made to determine the relative immunogenicity of the isolated components. Immunization with 1,000 μg of ribosomal preparation induced 92% protection ($P \leq 0.01$) (Table 1). This protection was comparable to immunization obtained using an OM complex (200 μg) prepared by the method of Wolf-Watz et al. (24) and was better than, although not statistically different from, protection provided by an equal dose of Sarkosyl-extracted proteins. A series of graded doses of LPS (5, 20, 50, and 100 μg) demonstrated a dose-dependent response resulting in up to 83% protection ($P \leq 0.01$). LPS treated with pronase (50 μg/ml) demonstrated a diminished ability to induce protection.

TABLE 1. *Comparative protection of guinea pigs by gonococcal ribosomal preparations and envelope components when challenged with 10^7 CFU[a] of N. gonorrhoeae*

Immunogen	Dose[b] (μg)	Protection at 10 days postchallenge		
		Ratio[c]	%	P[d]
Ribosomal prepn	1,000	12/13	92	≤0.01
OM complex (Wolf-Watz [24])	200	11/12	92	≤0.01
Sarkosyl extract	200	8/10	80	≤0.01
LPS	100	10/12	83	≤0.01
	50	9/14	64	≤0.01
	20	8/15	53	≤0.01
	5	5/13	38	≤0.01
LPS + protease[e]	100	5/12	42	≤0.01
	20	4/14	28	≤0.01
Controls (Freund incomplete adjuvant + saline)	0	0/22	0	

[a] CFU, Colony-forming units.

[b] Ribosomal preparations, OM complex, and Sarkosyl extracts are expressed in micrograms of protein; LPS is expressed in micrograms of dried material.

[c] Number of noninfected chambers/total number of chambers.

[d] Level of significance when compared with the controls (saline + Freund incomplete adjuvant) by one-way analysis of variance.

[e] LPS was digested with pronase (50 μg/ml) for 6 h, followed by heating at 100°C for 5 min and sedimentation at 100,000 × g for 30 min.

Detection of envelope component antibodies. Sera from guinea pigs immunized with 1,000 μg of ribosomal preparations were tested by passive hemagglutination for the presence of antibodies to contaminating envelope components (Table 2). When sheep erythrocytes were coated with OM complex or LPS and tested against sera from ribosome-immunized animals, titers to the envelope components were observed. However, these titers were lower than those obtained when ribosomes were used as the coating antigen. This indicated a contamination of the ribosomal preparation by enough outer envelope components to stimulate antibody production.

Absorption of immune sera and bactericidal activity. The unabsorbed antiribosomal sera, when tested by immunodiffusion against homologous ribosomal preparations, formed at least two lines of precipitation. Adsorption of this serum with either OM prepared by the method of Wolf-Watz et al. (24), Sarkosyl OM preparations, or LPS removed the antibody forming the precipitin line closest to the antiserum wells. Bactericidal activity of the absorbed sera is shown in Table 3. Sera absorbed with OM protein complexes had reductions in their bactericidal activity whereas sera absorbed with LPS exhibited a marked reduction in activity. This strongly suggests that antibody to LPS is the predominant component of the bactericidal activity demonstrated by the antiribosomal sera.

Chemical and enzymatic analysis. Contamination of the ribosome preparations with membrane components was also examined by chemical and enzymatic analyses (Table 4). On

TABLE 2. *Passive hemagglutination titers of ribosome-immune sera when ribosomal preparations or outer envelope components were used as sensitizing antigens*

Immunogen	Animal no.	PHA titer using sheep erythrocytes sensitized with:[a]		
		Ribosomal prepn	OM complex	LPS
Ribosomal prepn (1,000 μg)	1	2560	640	40
	2	5120	80	10
	3	10240	160	320
	4	10240	80	320
	5	1280	320	10
	6	5120	40	20

[a] The numbers indicated are the reciprocals of the serum dilutions tested.

TABLE 3. *Bactericidal activity of ribosome-immune sera absorbed with OM components*

Animal	Bactericidal titer[a]			
	Unabsorbed sera	Sera absorbed with:[b]		
		Wolf-Watz proteins	Sarkosyl proteins	LPS
1	128	64	128	16
2	128	32	16	≤2
3	256	64	128	≤2

[a] Titer is defined as a 75% reduction in colony-forming units when compared to the organisms present in wells containing normal or heat-inactivated normal sera. The numbers expressed are the reciprocals of the serum dilutions tested.

[b] Antisera (0.5 ml) were absorbed with 250 μg of LPS or membrane proteins at 37°C for 1 h and sedimented after overnight incubation at 4°C.

TABLE 4. *Chemical and enzymatic analysis of outer envelope and ribosome components of N. gonorrhoeae 120-94 T_1*

Analysis	OM complex		Ribosomal prepn
	Wolf-Watz	Sarkosyl	
Protein[a]	54.5	75.0	24.0
RNA[a]	ND[b]	ND	68.0
LPS[a]	19.2	13.5	3.9
Total carbohydrate[a]	4.8	6.7	7.1
Enzyme activities[c]			
Succinate dehydrogenase	2.3 (3.3)[d]	4.1 (5.9)	6.9 (9.8)
Lactate dehydrogenase	2.6 (1.5)	3.5 (2.0)	54.0 (30.9)

[a] Expressed as percent of dry weight.

[b] ND, Not determined. (Preparations were pretreated with nucleases during the extraction procedure.)

[c] Specific activity expressed as nanomoles of the electron acceptor reduced (thiazolyl blue) per minute per milligram of protein.

[d] Expressed as percent of the activity in the cytoplasmic fraction.

a dry weight basis, the ribosomes contained 24% protein, 68% ribonucleic acid, and 7.1% carbohydrate, and the composition was similar to that reported for other gram-negative bacteria (14). These components accounted for 99% of the dry weight of the ribosomes. The ribosomal preparations also contained 3.9% LPS as well as significant levels of the specific cytoplasmic membrane enzymes, lactate and succinate dehydrogenase (30.9 and 9.8% of cytoplasmic membrane levels, respectively). As anticipated, both the Wolf-Watz and Sarkosyl OM preparations contained large amounts of protein and LPS (Table 4), although the Sarkosyl preparation appeared to be composed of a significantly greater amount of protein based on percent of dry weight. Both preparations also contained small but detectable levels of cytoplasmic membrane enzyme activity.

^{125}I surface labeling and SDS-PAGE analysis of OM components and ribosomal preparations. To confirm the presence of OM protein contamination in the ribosomal preparations, intact *N. gonorrhoeae* were surface labeled with ^{125}I, using a lactoperoxidase-catalyzed reaction, before the extraction of the OMs and ribosomal preparations. This enzyme does not penetrate lipid bilayers and is too large to penetrate known membrane pores (9). After SDS-PAGE of these preparations, gels were sliced and the radioactivity was measured. Comparison of the location of the radioactivity with the duplicate Coomassie blue-stained gels is shown in Fig. 1. The Sarkosyl gel contained only one peak of radioactivity which was associated with the 34,000 molecular weight major OM protein. This band had a relatively simple protein composition as seen in Coomassie blue stains, with a major band at 34,000 molecular weight and a few additional bands. The majority of the Wolf-Watz-extracted proteins contained no detectable radioactivity although there was a small amount of radioactivity associated with the 34,000 molecular weight protein and two minor bands of higher molecular weights. This membrane complex, when stained with Coomassie blue, contained numerous protein bands; because of the large numbers, direct comparison with corresponding bands in the ribosomal gels was not meaningful. Analysis of the ribosomal preparation showed one large peak of radioactivity which corresponded with the 34,000 molecular weight protein. Coomassie blue staining revealed major bands which corresponded to subunit molecular weights of 82,000, 73,000, 58,000, 34,000, 30,000, 24,000, 20,000, and 14,000, respectively. The major band (34,000 molecular weight) containing the majority of the radioactivity corresponds to the principal OM protein observed by others (1, 10). This corresponding protein is found in all the OM and ribosomal preparations. Thus, these data imply that there is a significant contamination of the ribosomal preparation by envelope components.

DISCUSSION

Previous results in our laboratories have shown that immunization of guinea pigs with ribosomal preparations conferred protection against chamber infection with *N. gonorrhoeae* (2). This work implied that surface contaminants were present in the ribosomal preparation and might contribute to the immunity induced by the ribosomes. The present study demonstrated that the gonococcal ribosomal preparations were

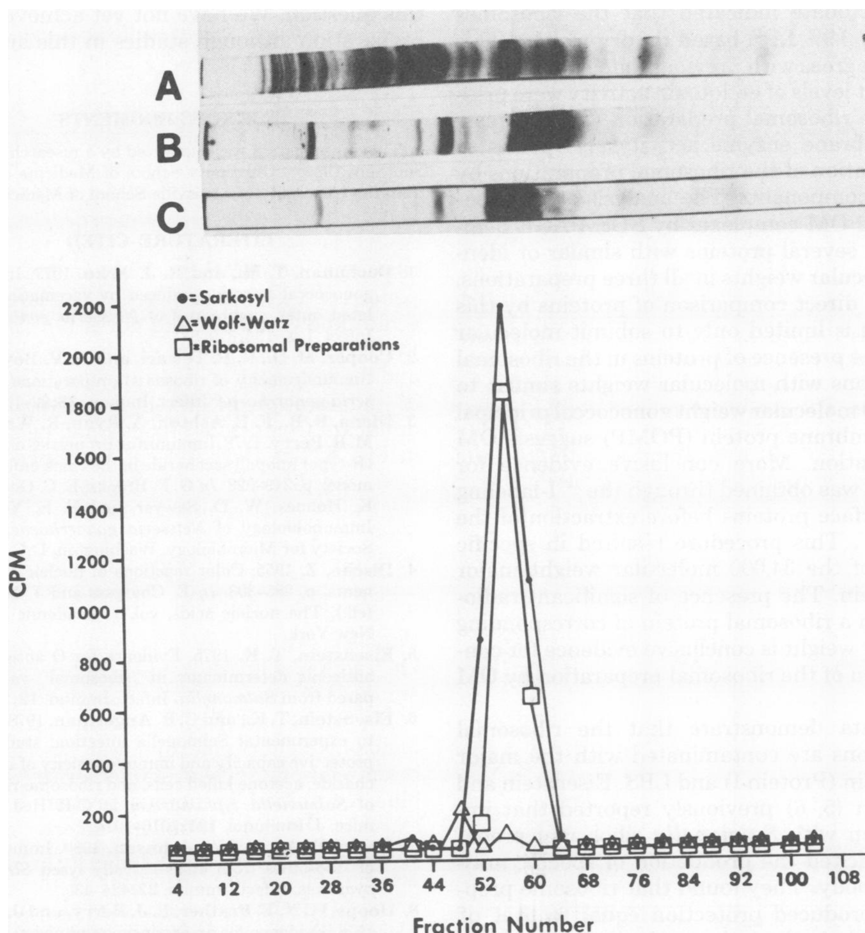

FIG. 1. *Radioactivity profile of SDS-PAGE gels of OM proteins and ribosomal preparations. (A) Wolf-Watz OM complex; (B) Sarkosyl-extracted proteins; (C) ribosomal preparation.*

contaminated with several envelope components which may contribute to the protective immunity elicited by the ribosomal preparations.

Immunization of guinea pigs with ribosomal preparations or outer envelope components resulted in comparable degrees of protection ($P \leq 0.01$). The LPS preparations were effective immunogens and gave a dose-dependent response. However, when the LPS was treated with protease, the protection was reduced by approximately 50%, suggesting that a protein-LPS complex may be a more effective immunogen. The envelope preparations gave protection comparable in degree to that of ribosomes even though smaller amounts of the immunogen were used without incorporation into adjuvant. We previously reported that ribosomal preparations, when not incorporated into adjuvant, give poor protective responses (2).

The passive hemagglutination data demonstrated that there was sufficient contamination of the ribosomal preparations with OM components to induce antibody production against them. Certain antibody populations could be absorbed from the antiribosomal sera with both OM protein complexes and purified LPS as evidenced by the immunodiffusion patterns. These antibody populations appeared to contain the bactericidal antibody directed against the gonococcus. These antibodies were primarily directed against LPS since most of the activity was removed by adsorption with LPS but not by the OM complex. This is in agreement with the reports of Tramont et al. (20) and Rice and Kasper (17), who showed that in human infections LPS appeared to be the predominant antigen against which bactericidal antibody was directed.

Direct chemical evidence for the contamination of ribosomal preparations with envelope components was demonstrated in several ways. The detection of the specific OM marker 2-keto-

3-deoxyoctonate indicated that the ribosomes contained 3.9% LPS based on dry weight. This evidence agrees with previous data showing that significant levels of endotoxin activity were present in the ribosomal preparation (2). Cytoplasmic membrane enzyme activity also points to contamination of the ribosomal preparations by multiple components. The analysis of the ribosomal and OM complexes by SDS-PAGE demonstrated several proteins with similar or identical molecular weights in all three preparations. Although direct comparison of proteins by this technique is limited only to subunit molecular weight, the presence of proteins in the ribosomal preparations with molecular weights similar to the 34,000 molecular weight gonococcal principal outer membrane protein (POMP) suggests OM contamination. More conclusive evidence for this point was obtained through the ^{125}I-labeling of the surface proteins before extraction of the ribosomes. This procedure resulted in specific labeling of the 34,000 molecular weight major OM protein. The presence of significant radioactivity in a ribosomal protein of corresponding molecular weight is conclusive evidence for contamination of the ribosomal preparation by OM protein.

Our data demonstrate that the ribosomal preparations are contaminated with the major OM protein (Protein I) and LPS. Eisenstein and Angerman (5, 6) previously reported that immunization with *Salmonella* ribosomal preparations elicited the production of specific anti-LPS antibody. They found that ribosome preparations produced protection equal to that of acetone-killed cells and superior to that of LPS; in addition, the ribosome preparations provided longer-lasting immunity. Lieberman (12) also showed that a protective fraction containing LPS has been isolated from *P. aeruginosa* ribosomal preparations. Further, *Streptococcus pyogenes* ribosomes have been shown to be contaminated with cell wall proteins (7).

Although major outer envelope contaminants such as POMP and LPS have been identified in the ribosomal preparation, it is not possible to identify a role in protective immunity for both of these components. It appears clear that LPS is a significant contributor to the bactericidal activity elicited by gonococcal ribosomal preparations. The role of POMP is less clear. It is possible that there are several antigens which are capable of producing immunity and which can act independently or in concert with one another to produce the immunity seen with ribosome preparations. The preparation of more highly purified ribosomes which are devoid of outer envelope contamination may help clarify this question. We have not yet achieved such a preparation although studies in this area are in progress.

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