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Immunogenic Activity of Gonococcal Protein I in Mice with Three Different Lipoidal Adjuvants Delivered in Liposomes and in Complexes

WIM JISKOOT,[1] TOM TEERLINK,[2] MONIQUE M. M. VAN HOOF,[1] KEES BARTELS,[1] VISHNA KANHAI,[2] DAAN J. A. CROMMELIN,[1] AND E. COEN BEUVERY[2]*

Department of Pharmaceutics, State University of Utrecht, 3511 GH Utrecht,[1] and Departments of Bacterial Vaccines and Inactivated Viral Vaccines, National Institute of Public Health and Environmental Hygiene, 3720 BA Bilthoven,[2] The Netherlands

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For several reasons the major outer membrane protein from *Neisseria gonorrhoeae* (gonococcal protein [PI]) is an attractive component for a gonococcal vaccine. This paper describes the influence of two different physical forms of PI on its immunogenic activity. To this end PI was delivered in liposomes and in protein-detergent complexes. In both forms PI was present in a multimeric form. The liposomes were composed of phosphatidylcholine and cholesterol. The effect of dicetylphosphate as a negatively charged amphiphile and three lipoidal adjuvants was investigated. Two lipoidal adjuvants (Avridine and dimethyldioctadecylammoniumbromide) were positively charged amphiphiles, whereas the third one (tridecyl *N*-acetylmuramyl-L-alanyl-D-isoglutaminate) was neutral. The protein-detergent complexes were also tested in the presence of the lipoidal adjuvants and in an $AlPO_4$-adsorbed form. The liposome preparations were characterized for their size, charge, and residual amount of detergent. The immunogenic activity of PI in all forms was tested in mice. The results of the antibody assays showed that PI in the liposomes was more immunogenic than PI in the complexes. A second dose with liposomes induced only a small booster effect, whereas such a dose with the complexes produced pronounced booster effects. The incorporation of the positively charged lipoidal adjuvants in the liposomes resulted in enhanced booster effects. The highest immunogenic activity of PI after two injections, however, was observed in the complexed form adsorbed to $AlPO_4$.

Gonorrhea is the most commonly reported infectious disease in the United States and in a number of other countries. The high incidence and the increasing antibiotic resistance of *Neisseria gonorrhoeae* is a source of growing concern. Therefore, efforts have been made to develop an effective vaccine (9). During the last decade a great deal of our knowledge of the surface structures of the gonococcus was collected (15, 16). One of these structures is the major outer membrane protein (PI). PI is a porin protein and is responsible for the sieving properties of the outer membrane. Several things make PI an attractive component of a multicomponent gonococcal vaccine (4, 15, 16). PI is surface exposed, does not show isogenic variation, may play a role in the endocytosis of the gonococcus by epithelial cells, and antibodies against it may protect humans against salpingitis. Protein I can be subdivided into two classes according to peptide mapping and susceptibility to proteolysis (6, 32). PIA molecules have one of their termini exposed to the surface, whereas PIB molecules have both termini buried in the membrane, leaving a central portion of the molecule exposed to the surface (1). This division was found to correlate with serogroup patterns (29, 30) and with pathogenesis. Gonococci expressing PIA are associated with systemic infections, whereas gonococci expressing PIB are associated with localized infections (8, 18). Recently, Blake and Gotschlich described an isolation procedure for gonococcal PII (5). We have adapted the method to obtain purified PI (34). This purified PI may be appropriate as a vaccine component for human use.

* Corresponding author.

Morein and co-workers (22–24) indicated that the physical form of viral membrane proteins has a considerable influence on their immunogenic activity. They found that the membrane proteins in a multimeric form, as for example in liposomes, iscoms, and protein micelles, have higher activity than the membrane protein in a monomeric form. The protein micelles were composed only of protein (devoid of lipids and detergent), whereas the monomers were found when both protein and detergent were present in a mixed micellar structure. Like viral membrane proteins, PI can be incorporated in liposomes (9, 36). The application of the Blake and Gotschlich isolation procedure results in a PI preparation that may resemble the monomeric form described by Morein et al. (22). Protein-detergent complexes are formed by diluting PI in a mixed micellar condition below the critical micellar concentration of the detergent (34). We assume that PI is present in such a complex in a multimeric form.

This report describes our studies on the incorporation of PIA into liposomes with dicetylphosphate and three lipoidal adjuvants. We were able to incorporate both PI and either dicetylphosphate or one of these adjuvants in the same liposome. The charge and size of the liposome preparations were determined. The immunogenic activity of PI present in liposomes with different compositions was compared with that of PI present in protein-detergent complexes. The results show that (i) after one dose, PI present in a liposomal form was more immunogenic than in a complexed form; (ii) after a second dose, the complexed form resulted in a clearly higher booster effect than the liposomal form, (iii) both positively charged lipoidal adjuvants (dimethyldioc-

tadecylammoniumbromide and Avridine) stimulated secondary response to PI, whereas the negative charge of the liposomes did not influence the activity of PI, and (iv) all adjuvants stimulated the secondary response to PI present in the complexes.

MATERIALS AND METHODS

Materials. Egg yolk L-alpha-phosphatidylcholine type V-E (PC), cholesterol, *n*-octyl-beta-D-glucopyranoside (octyl glucoside), and dicetylphosphate (DCP) were obtained from Sigma Chemical Company, St. Louis, Mo. Casamino Acids were from Difco Laboratories (Detroit, Mich.). *N*,*N*,dioctadecyl-N^1,N^1-(2-hydroxymethyl)-propanediamine (Avridine) was a gift of Keith Jensen from Pfizer Central Research (Groton, Conn.). Dimethyldioctadecylammoniumbromide (DDA) was obtained from Eastman Kodak, Rochester, N.Y. Tridecyl *N*-acetylmuramyl-L-alanyl-D-isoglutaminate (MDP-C13) was synthesized by Frits Tesser, Catholic University, Nijmegen, The Netherlands. *N*-Tetradecyl-*N*,*N*-dimethyl-3-ammonia-1-propanesulfonate (Z3-14) was supplied by Calbiochem, La Jolla, Calif.

Cultivation of bacteria. Gonococcal strain B2 (serotype 1) was cultivated at 35°C in Frantz medium (11) supplemented with 0.2% (vol/vol) yeast extract dialysate and 0.2% (wt/vol) Casamino Acids in a 40-liter fermentor with aeration. The pH was maintained at 7.0 and the pO_2 at 10%. The culture was inactivated by heating at 56°C for 30 min. After centrifugation the bacteria were pooled and lyophilized. The cell-free culture liquid was processed as described below.

Preparation of OMC. The cell-free culture liquid was concentrated 80-fold on an Amicon hollow-fiber cartridge (H10P10). Residual bacteria were removed by centrifugation (10,000 × *g*, 20 min). Outer membrane complexes (OMC) were pelleted (2 h at 100,000 × *g*), suspended in 300 mM NaCl–50 mM Tris, pH 7.2, pelleted again (2 h at 100,000 × *g*), and finally suspended in distilled water at a protein concentration of 2 mg/ml.

Purification of PI. The isolation procedure was based on the procedure used by Blake and Gotschlich to isolate PII (5). Lyophilized gonococci (2.5 g, dry weight) were extracted with 2.5 g of Z3-14 in0.5 M $CaCl_2$ at pH 4.0 in a total volume of 250 ml. After 1 h, intact cells and debris were removed by centrifugation (20 min, 10,000 × *g*). If necessary, the pH of the supernatant was readjusted to 4.0 with dilute HCl, and ethanol was added to a concentration of 20%. After 30 min, precipitated material was removed by centrifugation (20 min, 10,000 × *g*). The supernatant was concentrated to 150 ml by ultrafiltration (Amicon hollow-fiber H1Dx50); 150 ml of 50 mM Tris–10 mM EDTA–0.05% (wt/vol) Z3-14 (pH 8.0) was added, and the volume was reduced twofold. This procedure was repeated five times to ensure complete removal of $CaCl_2$ and ethanol. The protein solution was then applied to a DEAE-Sepharose column (50 by 1.8 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient from 0.0 to 0.6 M NaCl in the Tris buffer mentioned above (two times, 400 ml), at a flow rate of 50 ml/h. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and PI-containing fractions were pooled. Ethanol was added to a final concentration of 80% (vol/vol). After centrifugation (1 h, 3,000 × *g*), the protein pellet was dissolved in a small volume of 50 mM Tris–2.0% (wt/vol) Z3-14 (pH 9.0). Partially purified PI was applied to a Sephacryl S-300 column (80 by 3.6 cm) previously equilibrated with 50 mM Tris–200 mM NaCl–10 mM EDTA–0.05% (wt/vol) Z3-14 (pH 8.0). The column was eluted at a flow rate of 50 ml/h. PI-containing fractions were pooled (purified PI) and stored at 4°C with 0.02% (wt/vol) sodium azide as a preservative. Purified PI was precipitated from the Zwittergent Z3-14 solution by adding 96% ethanol to a final ethanol concentration of 80% (vol/vol). After centrifugation (15 min, 2,000 × *g*), precipitated PI was resolubilized in 150 mM octyl glucoside in either 10 mM Tris–0.9% (wt/vol) NaCl (pH 7.4) (buffer A) or 10 mM Tris–0.9% NaCl adjusted to pH 3.0 with dilute HCl (buffer B), depending on whether the protein would be incorporated into neutral or positively charged liposomes.

SDS-PAGE. Acrylamide gels (11%, wt/vol) were used with the Laemmli system (20). Samples were heated for 5 min at 100°C in 2% (wt/vol) SDS–20% (vol/vol) glycerol–5% (wt/vol) 2-mercaptoethanol–0.062 M Tris hydrochloride (pH 6.8). The gels were silver stained as described by Marshall (21).

Preparation of liposomes. (i) Stage i: solubilization of lipids. PC, cholesterol, and DCP or one of the lipoidal adjuvants (if used), molar ratio 7:2(:1), were rotary evaporated in a 50-ml flask from a chloroform-methanol mixture to dryness. The lipid film obtained was left under low pressure (below 3 kPa) for 2 to 3 h. Next, a solution of 150 mM octyl glucoside was added. The neutral mixed micelles were prepared in buffer A, whereas the positively charged mixed micelles (containing DDA or Avridine) were prepared in buffer B. Solubilization was performed by gently shaking until an absolutely clear solution was obtained. The desired amount of PI was added to the mixed micelles, yielding a final PC concentration of 10 mM.

(ii) Stage ii, technique i. As the initial ratio of PI to PC, a value of about 40 μg of protein per μmol of PC was chosen. Two milliliters of the micellar dispersion was dialyzed at 4°C in a shaking bath (120 strokes per min) against 260 ml of buffer A. Dialysis lasted for 48 h. After 24 h the dialysate was replaced by buffer A. The exchange area was about 10 cm^2; high-permeability cellulose membranes (molecular weight [MW] cutoff, 10,000; Diachema, Ruschlikon, Zurich, Switzerland) were used. After dialysis the dispersion was sequentially filtered under nitrogen at pressures up to 0.6 MPa through polycarbonate membrane filters with 1,000-, 600-, and 200-nm pores (Nuclepore Corp., Pleasanton, Calif.). Filtration and subsequent manipulation of the liposome dispersion were performed under aseptic conditions.

(iii) Stage ii, technique ii. Two milliliters of mixed micelles was transferred to a beaker of 50 ml. If neutral mixed micelles were used, the sample was diluted with 20 ml of buffer A for 16 s at a constant rate with a Multi-Dosimat titration unit (Metrohm, Switzerland). If positively charged mixed micelles were used, the samples were diluted with buffer B for 30 min at a constant rate with Perfusor ED 1-300 (B. Braun, Melsungen AG, West Germany). During the dilution step the sample was magnetically stirred as fast as possible without splashing. After dilution, the pH of the liposome dispersions prepared in buffer B as readjusted to 7.4 with dilute NaOH. Eighteen milliliters of the resulting liposome dispersion was concentrated to about 1 ml by ultrafiltration through cellulose triacetate filters (MW cutoff, 20,000; Sartorius GmbH, Göttingen, West Germany) under 0.4-MPa nitrogen pressure. The sample was magnetically stirred to prevent obstruction of the filter pores. Dilution and concentration of the dispersions were carried out at room temperature. For each type of liposome, three batches were prepared as described above. After concentration, the batches were pooled and buffer A was added, yielding a final

volume of 6.0 ml. This sample was dialyzed at 4°C against 260 ml of buffer A for 66 h. The buffer was replaced two times. The exchange area of the high-permeability cellulose membranes, MW cutoff 10,000 (Diachema), was 10 cm^2. Dialysis was carried out in a shaking bath (120 strokes per min; Grant Instruments Ltd., Cambridge, U.K.) protected from light. After dialysis, the liposome dispersion was sequentially filtered through polycarbonate membrane filters with 600- and 200-nm pores (Nuclepore Corp.). Filtration and subsequent manipulation of the liposome dispersions were performed under aseptic conditions.

Preparation of protein-detergent and protein-adjuvant complexes. Protein-detergent complexes were prepared by lowering the octyl glucoside concentration of purified PI in the mixed micellar condition to a value below the critical micellar concentration of the detergent. This was achieved by dilution with 0.15 M NaCl to a protein concentration of 5 μg/ml. For preparation of the protein-adjuvant complexes, the desired adjuvant was suspended in 0.15 M NaCl. Enough adjuvant suspension was added to PI (500 μg/ml in 150 mM octyl glucoside in buffer A) to obtain an adjuvant-protein ratio equal to the final ratio in the liposomes. This ratio (wt/wt) was 3.3 for DDA, 3.4 for Avridine, and 3.7 for MDP-C13. For $AlPO_4$ a ratio of 100 was used. Next, the mixture was diluted with 0.15 M NaCl to a protein concentration of 5 μg/ml.

Analytical methods. Phospholipid concentration was determined by phosphate analysis (2) in a modification of the method of Bottcher et al. (7).

Protein concentration was determined by the method of Peterson (25), in a modification proposed by Peterson (26), by washing the trichloroacetic acid-deoxycholate precipitate with diethyl ether-ethanol (3:1, vol/vol) to remove interfering substances. The washing procedure was carried out by adding 1 ml of washing fluid to the precipitate, vortexing, and centrifugation (45 min, 9,000 × *g*).

Octyl glucoside content was determined by gas chromatography as described previously (W. Jiskoot, T. Teerlink, E. C. Beuvery, and D. J. A. Crommelin, Pharm. Weekbl. [Sci.], in press).

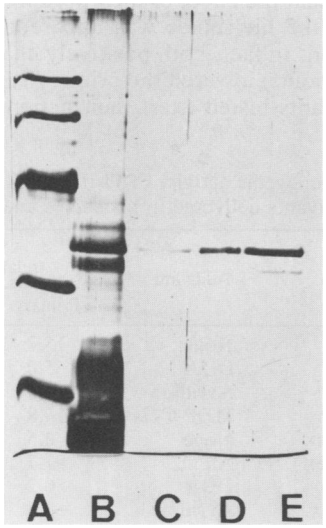

FIG. 1. SDS-PAGE analysis. Lane A, MW markers (from the top): phosphorylase B (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100); lane B, OMC from strain B2 (500 ng); lanes C to E: purified PI from strain B2 (20, 80, and 320 ng, respectively).

TABLE 1. Characteristics of PI-containing liposomes prepared by the dialysis-filtration technique

Molar composition	Final ratio, PI/PC (μg/μmol)[a]	% PI incorporated	ζ Potential (mV)	Mean diam (nm)[b]
PC-cholesterol, 7:2	34	83	—[c]	296 (5)
PC-cholesterol-DCP, 7:2:1	34	79	−10	227 (3)

[a] Initial ratio (before dialysis) was about 40 μg of PI to 1 μmol of PC.
[b] The polydispersity index is shown in parentheses (0 = monodisperse, 9 = extremely heterodisperse).
[c] —, Below detection limit (between +3 and −3 mV).

Particle size was determined by dynamic light scattering with a Nanosizer (Coulter Electronics Ltd., Luton, U.K.). Microelectrophoresis was carried out with a Mark II microelectrophoresis apparatus (Ranks Brothers, Bottisham, U.K.) as described previously (10). The microelectrophoresis apparatus was equipped with a helium-neon laser (Spectra-Physics Inc., Eugene, Oreg.) to detect the individual liposomes.

Immunization of mice. Groups of eight random-bred mice (strain Cpb:SE) were injected intravenously or intraperitoneally with PI in liposomes or in complexes. The preparations were diluted with 0.15 M NaCl to a protein concentration of 5 μg/ml. The amount injected was 1.0 μg of protein (experiment 1) or 2.5 μg of protein (experiment 2). A second dose was given after 6 weeks. Blood samples were obtained 4 weeks after the primary immunization and 1 week after the booster dose.

ELISA procedure. The immunoglobulin G antibody levels of pooled sera were determined by an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated overnight at room temperature with OMC diluted with 0.15 M NaCl to a protein concentration of 5 μg/ml. The subsequent steps of the ELISA were performed as described before (3). The antibody levels are expressed as percentages of the level of an anti-PI reference serum.

RESULTS

Purification of PI. The purification procedure described gave a typical yield of 25 mg of PI from 2.5 g (dry weight) of bacteria. The protein profile on SDS-PAGE showed that the preparation contained only minor amounts of contaminating proteins or degradation products (Fig. 1). The OMC contained a large amount of silver-staining material running near the front of the gel, which probably represents lipopolysaccharide. The purified PI preparation contained no detectable amount of this material.

Liposome characterization. (i) Liposomes prepared via the dialysis-filtration technique. The characteristics of the liposome preparations after dialysis and filtration are presented in Table 1. The data indicate that 34 μg of PI was incorporated per μmol of PC. The presence of DCP in the bilayer did not influence the incorporation of protein. A control experiment with only protein-octyl glucoside-mixed micelles (without any lipid) showed that less than 3% of the initial amount of PI was detected in the filtrate after dialysis and filtration through membrane filters with 200-nm pores. After removing the octyl glucoside by dialysis, PI aggregates were formed that could not pass through the 200-nm pores. It is therefore likely that PI found in the filtrate when lipids were

TABLE 2. Characteristics of PI-containing liposomes prepared by the dilution-concentration-dialysis-filtration technique

Molar lipid composition	Ratio, PI/PC (μg/μmol)		% PI incorporated[c]	ζ Potential (mV)	Mean diam (nm)[d]
	Initial[a]	Final[b]			
PC-cholesterol-DDA, 7:2:1	29.8	27.2	91	+13	165 (3–4)
PC-cholesterol-Avridine, 7:2:1	28.0	28.4	101	+24	170 (4)
PC-cholesterol-MDP-C13, 7:2:1	27.0	26.3	97	—[e]	138 (4)
PC-cholesterol, 7:2	32.0	28.2	88	—	138 (3)

[a] After the dilution step.
[b] After the filtration steps.
[c] (Final PI/PC ratio)/(initial PI/PC ratio).
[d] The polydispersity index is shown in parentheses (0 = monodisperse, 9 = extremely heterodisperse).
[e] —, Below detection limit (±3 mV).

present in the mixed micellar system indeed was associated with the lipid structures in the filtrate after filtration through 200-nm pores. Negatively charged liposomes were smaller in diameter than the neutral liposomes and had a narrower size distribution. The incorporation protein did not induce a detectable zeta-potential on the liposomes composed of PC and cholesterol. Under our experimental conditions it was impossible to prepare stable liposomes, well defined in size, with the positively charged lipoidal adjuvants in the bilayer. Therefore an alternative method was developed: the dilution-concentration-dialysis-filtration technique.

(ii) Liposomes prepared with the dilution-concentration-dialysis-filtration technique. Table 2 shows the characteristics of the four types of PI-containing liposomes. From the initial and final ratios of PI to PC, it follows that PI was nearly quantitatively incorporated (ranging from 88 to 101%) in the four types of liposomes. For the positively charged liposomes it was necessary to dilute the mixed micelles at pH 3. At higher pH values flocculated precipitates were formed after the dilution step, possibly resulting from electrostatic interaction of PI (ζ potential of precipitated PI aggregate in buffer A amounted to −9 mV) and the positively charged amphiphile, DDA or Avridine. For the neutral PI-containing liposomes, no electrophoretic response was observed. The ζ-potential of the positive PI-containing liposomes was +13 mV (Table 2). This was somewhat lower than for positively charged liposomes without PI (Jiskoot et al., in press), indicating that at least part of the negatively charged groups of the protein was exposed to the outer surface of the liposomes. The particle sizes of the different liposomes were of the same order of magnitude, with mean diameters ranging from 138 to 170 nm and a polydispersity index of 3 to 4 (Table 2). Residual octyl glucoside levels were very low in the four types of liposomes, i.e., the molar ratio of octyl glucoside to PC was below 0.001.

Immunogenic activity of various PI preparations. Mice were immunized with PI incorporated into various types of liposomes and with PI-detergent complexes. For the first immunization experiment, liposomes were prepared by the detergent-dialysis technique, whereas for the second experiment liposomes were prepared by the detergent-dilution technique. In addition, the influence of the route of immunization was studied in the first experiment.

Table 3 shows the relative antibody levels after one and after two intraperitoneal injections with neutral and negative liposomes in comparison with the protein-detergent complexes. The liposomes induced a higher primary response

TABLE 3. Immunogenic activity of PI delivered in liposomes and in complexes[a]

Presentation form	Charge	Response (relative IgG level)	
		Primary[b]	Secondary[c]
Liposome	Neutral	3.1	6.0 (1.9)
Liposome	Negative	2.9	9.0 (3.1)
Detergent complex	ND[d]	1.0	38.0 (38)
Detergent complex plus $AlPO_4$	ND	39.4	806.0 (20)
Control		<0.1	<0.1

[a] Liposomes were prepared by the detergent-dialysis technique. Immunogens were injected intraperitoneally.
[b] Standard deviation in the decimal logarithmic scale amounted to approximately 0.20; this implies in the levels presented a 95% confidence factor of about 2.
[c] Booster effect is shown in parentheses: ratio of antibody level after the second dose to the antibody level after the first dose.
[d] ND, Not determined.

than the PI complexes, but the complexes caused a more pronounced booster effect. The charge of the liposomes had no observable effect. Essentially the same results were obtained after intravenous immunization (data not shown). $AlPO_4$ potentiated the response to PI in the complexes. The results of the second experiment are presented in Table 4. Without adjuvants, the liposomes induced a sixfold-higher primary response than the protein-detergent complexes. However, after a booster dose, the protein-detergent complexes caused a ninefold increase in antibody level, whereas the liposomes induced only a small increase in antibody level. This resulted in a comparable secondary response for both preparations. Incorporation of the lipoidal adjuvants into PI-containing liposomes influenced the antibody response. The effect on the primary response was small, but the secondary response was increased by a factor of 1.5 (MDP-C13) to 4 (Avridine) compared with liposomes without adjuvant. Complexing of the adjuvants DDA, Avridine, and MDP-C13 with PI hardly influenced the primary response, whereas after a booster dose the antibody levels were approximately three- to fivefold higher than without added adjuvant.

It is striking that the effect of incorporation of the lipoidal adjuvants into the liposomes was apparent only after the booster injection. In fact, both positively charged adjuvants (DDA and Aviridine) lowered the primary response. Apparently the adjuvants tested exert their action by stimulating

TABLE 4. Immunogenic activity of PI in combination with three amphipatic adjuvants delivered in liposomes and in complexes[a]

Presentation form	Adjuvant	Response (relative IgG level)[b]	
		Primary	Secondary
Liposome	None	15.7	18 (1.1)
Liposome	DDA	5.0	62 (12.4)
Liposome	Avridine	4.4	68 (15.4)
Liposome	MDP-C13	14.8	27 (1.8)
Detergent complex	None	2.5	23 (9.2)
Detergent complex	$AlPO_4$	97.7	604 (6.2)
Adjuvant complex	DDA	6.2	63 (10.2)
Adjuvant complex	Avridine	3.3	112 (34)
Adjuvant complex	MDP-C13	1.2	67 (56)
Control	None	<0.1	<1

[a] Liposomes were prepared by the detergent dilution technique. Immunogens were injected intraperitoneally.
[b] See footnotes *b* and *c* of Table 3.

the immunological memory. This is different from the adjuvant effect of $AlPO_4$. When the PI-detergent complex was adsorbed to this adjuvant, the primary response was enhanced approximately 40-fold.

DISCUSSION

PI is the major outer membrane protein of the gonococcus. It is present in this membrane as channel-forming trimers that span the bilayer. There is evidence that the main part of PI is embedded in the membrane, with only a small portion being exposed to the environment (1, 6). To prepare an effective vaccine from PI, it is necessary to administer the protein in a form that will induce the production of antibodies directed against the exposed part of the protein. Only these antibodies will be able to react with the intact gonococcus and thus contribute to protection. One way to achieve this is to incorporate PI into liposomes. In this condition it is likely that PI will adopt a conformation resembling the native one. Successful attempts have been made to incorporate *Escherichia coli* porins into planar bilayers (31) and membrane vesicles (11). The gonococcal porin has also been incorporated into lipid membranes (9, 36).

The techniques described in this paper allowed the rapid and efficient incorporation of PI into liposomes composed of PC and cholesterol. The experiments were performed with a PIA preparation. However, we could also incorporate PIB, which has a different structure and surface exposure, into liposomes (results not shown). We were able to incorporate DCP and the three lipoidal adjuvants in the same liposome. Optimal adjuvating effect can be expected under these conditions (33). We did not investigate the orientation of PI in the liposomal membrane. However, it has been shown that many plasma membrane proteins are incorporated in an orientation comparable to the native one (12). This has been observed for the spike protein of Semliki Forest virus (17). Therefore, we assumed that a significant part of the incorporated PI has the same orientation as in the outer membrane of the bacterium.

We compared the immunogenic activity of the PI-containing liposomes with that of preparations of PI-detergent complexes. The results showed that the liposomes were more immunogenic than the PI complexes. However, in contrast to the complexes, the liposomes induced only a very small booster effect. Apparently the liposome preparation is not capable of inducing adequate immunological memory. The same conclusion was drawn by Morein and co-workers in the case of viral proteins (22–24). Monomeric forms of these proteins were shown to be poorly immunogenic, whereas protein-detergent complexes and proteins reconstituted into lipid vesicles (virosomes) were highly immunogenic. It might be that in liposomes the phospholipid environment changes the processing of the antigen by the macrophage, stimulating a different subset of lymphocytes involved in antibody production (14).

When mice are immunized with OMC, a pronounced booster effect is observed (3). The main difference between these OMC and liposomes is the presence of substantial amounts of lipopolysaccharide in the former. Lipopolysaccharide has strong adjuvating activity and may therefore be responsible for the induction of immunological memory. This led us to investigate the influence of the incorporation of adjuvants into liposomes on their immunostimulating properties.

Both positively charged adjuvants, DDA and Avridine, significantly stimulated the induction of memory. With MDP-C13 a much smaller effect was observed. Surprisingly, the primary response seemed to be reduced somewhat by the positively charged adjuvants. It may be that the short treatment at pH 3.0 during the preparation of these liposomes caused a partial denaturation of PI, resulting in a lower effective dose.

It has been shown that the antibody response to encephalomyocarditis virus and Semliki Forest virus is enhanced by addition of either negatively or positively charged liposomes, demonstrating that charge alone can influence the immune response (19). In our experiments, the adjuvating effect of DDA and Avridine was apparent only after the administration of a booster dose of either liposomes or complexes. This is in agreement with experiments performed with cholera toxin and procholeragenoid. Avridine-containing liposomes were shown to have no significant effect on the primary response, but memory was enhanced five- to sevenfold (27).

In conclusion, our experiments show that incorporation of PI together with an adjuvant into liposomes or complexes yields a product capable of eliciting significant antibody response. However, the PI-detergent complex adsorbed to $AlPO_4$ induced higher antibody levels than the most immunogenic liposome preparation. Possibly the immunogenic activity of PI in the liposome preparations can be further improved by optimizing parameters such as protein density, lipid composition, membrane fluidity (35), amount of adjuvant, etc. Furthermore, liposomal preparations are perhaps better suited to induce a local mucosal response (28), which is probably necessary to obtain immunity against gonorrhea. We are currently investigating these possibilities.

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LITERATURE CITED

1. **Barrera, O., and J. Swanson.** 1984. Proteins IA and IB exhibit different surface exposures and orientations in the outer membranes of *Neisseria gonorrhoeae*. Infect. Immun. **44**:565–568.
2. **Bartlett, J. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
3. **Beuvery, E. C., F. Miedema, R. W. van Delft, J. Haverkamp, A. B. Leussink, B. J. te Pas, K. S. Teppema, and R. H. Tiesjema.** 1983. Preparation and physicochemical and immunological characterization of polysaccharide-outer membrane protein complexes of *Neisseria meningitidis*. Infect. Immun. **40**: 369–380.
4. **Blake, M. S., and E. C. Gotschlich.** 1983. Gonococcal membrane proteins: speculation on their role in pathogenesis. Prog. Allergy **33**:298–313.
5. **Blake, M. S., and E. C. Gotschlich.** 1984. Purification and partial characterization of the opacity-associated protein of *Neisseria gonorrhoeae*. J. Exp. Med. **159**:452–462.
6. **Blake, M. S., E. C. Gotschlich, and J. Swanson.** 1981. Effects of proteolytic enzymes on the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. **33**:212–222.
7. **Bottcher, C. J. F., C. M. van Gent, and C. Pries.** 1961. A rapid and sensitive sub-microphosphorous determination. Anal. Chim. Acta **24**:203–204.
8. **Buchanan, T. M., and J. F. Hildebrandt.** 1981. Antigen-specific serotyping of *Neisseria gonorrhoeae*: characterization based upon principle outer membrane protein. Infect. Immun. **32**:985–994.
9. **Committee on Issues and Priorities for New Vaccine Development.** 1985. Prospects for immunizing against *Neisseria gonor-*

rhoeae, p. 365–384. *In* New Vaccine Development, vol. I, appendix L. National Academy Press, Washington, D.C.
10. **Crommelin, D. J. A.** 1984. Influence of lipid composition and ionic strength on the physical stability of liposomes. J. Pharm. Sci. **73**:1559–1563.
11. **Engel, A., A. Massalski, H. Schindler, D. L. Dorset, and J. P. Rosenbuch.** 1985. Porin channel triplets merge into single outlets in *Escherichia coli* outer membranes. Nature (London) **317**:643–645.
12. **Eytan, G. D.** 1982. Use of liposomes for reconstitution of biological functions. Biochim. Biophys. Acta **694**:185–202.
13. **Frantz, I. D., Jr.** 1942. Growth requirements of the meningococcus. J. Bacteriol. **43**:757–761.
14. **Gerlier, D., O. Bakouche, and J. F. Dore.** 1983. Liposomes as a tool to study the role of membrane presentation in the immunogenicity of a MuLV-related tumor antigen. J. Immunol. **131**:485–490.
15. **Gotschlich, E. C.** 1984. Development of a gonorrhoea vaccine: prospects, strategies and tactics. Bull. WHO **62**:671–680.
16. **Gotschlich, E. C.** 1984. Gonorrhea, p. 353–371. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
17. **Helenius, A., E. Fries, and J. Kartenbeck.** 1977. Reconstitution of Semliki Forest virus membrane. J. Cell Biol. **75**:866–880.
18. **Hildebrandt, J. F., L. W. Mayer, S. P. Wang, and T. M. Buchanan.** 1978. *Neisseria gonorrhoeae* acquire a new principal outer membrane protein when transformed to resistance to serum bactericidal activity. Infect. Immun. **20**:267–273.
19. **Kraaijeveld, C. A., M. Schilham, J. Jansen, B. Benaissa-Trouw, M. Harmsen, A. J. van Houte, and H. Snippe.** 1984. The effect of liposomal charge on the neutralizing antibody response against inactivated encephalomyocarditis and Semliki Forest viruses. Clin. Exp. Immunol. **56**:509–514.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Marshall, T.** 1984. Detection of protein in polyacrylamide gels using an improved silver stain. Anal. Biochem. **136**:340–346.
22. **Morein, B., M. Sharp, B. Sundquist, and K. Simons.** 1983. Protein subunit vaccines of parainfluenza type 3 virus: immunogenic effect in lambs and mice. J. Gen. Virol. **64**:1557–1569.
23. **Morein, B., and K. Simons.** 1985. Subunit vaccines against enveloped viruses: virosomes, micelles and other protein complexes. Vaccine **3**:83–93.
24. **Morein, B., B. Sundquist, S. Hoglund, K. Dalsgaard, and A. Osterhaus.** 1984. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature (London) **308**:457–460.
25. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**:346–356.
26. **Peterson, G. L.** 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal. Biochem. **100**:201–220.
27. **Pierce, N. F., and J. B. Sacci, Jr.** 1984. Enhanced mucosal priming by cholera toxin and procholeragenoid with a lipoidal amine adjuvant (Avridine) delivered in liposomes. Infect. Immun. **44**:469–473.
28. **Pierce, N. F., J. B. Sacci, Jr., C. R. Alving, and E. C. Richardson.** 1984. Enhancement by lipid A of mucosal immunogenicity of liposome-associated cholera toxin. Rev. Infect. Dis. **6**:563–566.
29. **Sandstrom, E. G., K. C. S. Chen, and T. M. Buchanan.** 1982. Serology of *Neisseria* gonorrhoeae: coagglutination serogroups WI and WI/III correspond to different outer membrane protein I molecules. Infect. Immun. **38**:462–470.
30. **Sandstrom, E. G., J. S. Knapp, and T. M. Buchanan.** 1982. Serology of *Neisseria gonorrhoeae*: W-antigen serogrouping by coagglutination and protein I serotyping by enzyme-linked immunosorbent assay both detect protein I antigens. Infect. Immun. **35**:229–239.
31. **Schindler, H., and J. P. Rosenbusch.** 1978. Matrix protein from *Escherichia coli* outer membranes forms voltage-controlled channels in lipid bilayers. Proc. Natl. Acad. Sci. USA **75**: 3751–3755.
32. **Swanson, J.** 1979. Studies on gonococcus infection. XVIII. ^{125}I-labeled peptide mapping of the major protein of the gonococcal cell wall outer membrane. Infect. Immun. **23**:799–810.
33. **Tamauchi, H., T. Tadakuma, T. Yasuda, and K. Saito.** 1983. Enhancement of immunogenicity by incorporation of lipid A into liposomal model membranes and its application to membrane-associated antigens. Immunology **50**:605–612.
34. **Teerlink, T., R. Breas, R. van Eijk, R. H. Tiesjema, and E. C. Beuvery.** 1985. Isolation and immunological characterization of gonococcal porin protein, p. 259–264. *In* G. K. Schoolnik, G. F. Brooks, S. Falkow, C. E. Frasch, J. S. Knapp, J. A. McCutchan, and S. A. Morse (ed.), The pathogenic neisseriae. Proceedings of the Fourth International Symposium on the Pathogenic Neisseriae. American Society for Microbiology, Washington, D.C.
35. **Yasuda, T., G. F. Dancey, and S. C. Kinsky.** 1977. Immunogenicity of liposomal model membranes in mice: dependence on phospholipid composition. Proc. Natl. Acad. Sci. USA **74**:1234–1236.
36. **Young, J. D.-E., M. Blake, A. Mauro, and Z. A. Cohn.** 1983. Properties of the major outer membrane protein from *Neisseria gonorrhoeae* incorporated into model lipid membranes. Proc. Natl. Acad. Sci. USA **80**:3831–3835.