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Incorporation of the Major Outer Membrane Protein of *Neisseria gonorrhoeae* in Saponin-Lipid Complexes (Iscoms): Chemical Analysis, Some Structural Features, and Comparison of Their Immunogenicity with Three Other Antigen Delivery Systems

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We incorporated the major outer membrane protein (PI) of *Neisseria gonorrhoeae* into immunostimulating complexes (iscoms) and examined some analytical, physicochemical, and immunological properties of these structures. The immunogenicity was compared with that of three other PI-containing structures, i.e., liposomes, outer membrane complexes produced by the bacterium, and protein-detergent-adjuvant complexes. $AlPO_4$ and dioctadecyldimethylammonium bromide were used as adjuvants. Our results show that iscoms are much more immunogenic than liposomes and protein-detergent complexes but are also much more toxic. The localization of PI in iscoms was investigated. Therefore, the chymotrypsin susceptibility of PI in iscoms was tested, and the incorporation of fragments of PI was determined. Amphiphilic fragments of PI were incorporated in iscoms, but hydrophilic and hydrophobic fragments were not. Chymotrypsin degradation of PI in iscoms indicated that the protein is exposed to the environment in a similar manner as PI in outer membrane complexes, i.e., with both termini anchored in the iscom.

The morphological effects of saponin on biological membranes and lipid monolayers have been known for almost a quarter of a century (1, 10, 12). Lucy and Glauert (16) proposed a model for these structures with hexagonal symmetry, appearing after saponin treatment of cholesterol (Chol) or Chol-phosphatidylcholine (PC) dispersions. This model is based on PC-Chol and saponin-Chol micelles and micelle-micelle interactions, possibly by means of hydrogen bonding.

Similar structures were observed when viruses were treated with saponin in an attempt to isolate viral subunits (13, 19). Morein et al. (17) tried to develop saponin-containing complexes for use as vaccines and developed a method for the incorporation of viral membrane proteins into structures with a well-defined size and a high immunogenicity. In this procedure these so called iscoms (immunostimulating complexes) are formed by solubilizing the viral membrane with a detergent followed by exchanging the detergent for saponin by ultracentrifugation on a saponin-containing sucrose gradient. Morein et al. complexed membrane components of parainfluenza 3, measles, and rabies viruses with the saponin Quil A (QA). The resulting iscoms induced antibodies directed against these viruses in mice or rats. Osterhaus et al. (18) showed that iscoms containing gp70/85 from feline leukemia virus could protect cats against a feline leukemia virus infection. Iscoms carrying cytomegalovirus antigen enhanced the cellular immune response in monkeys (22).

Other than the immunogenicity, little information is available concerning the structure of these iscoms. In this report we provide morphological and chemical data for iscom structures containing an amphiphilic protein. Protein IB of *Neisseria gonorrhoeae* was used as an example of such a protein because it is easy to isolate in substantial amounts (5, 20) and because data were available regarding the location of this pore protein in the bacterial membrane (2, 4, 6): both N and C termini are anchored in the membrane, and the middle part is exposed to the aqueous environment. PI was also fragmentated with cyanogen bromide (CNBr), and the positions of the fragments relative to the membrane were determined (21) (Fig. 1). Not only the intact PI but also these and other fragments of the protein were incorporated in iscoms to gain insight into the molecular requirements for uptake of proteins into iscoms.

The immunogenicity of PI-containing iscoms was compared with that of three other systems containing PI, i.e., liposomes, outer membrane complexes (OMC), and protein-detergent-adjuvant complexes. $AlPO_4$ and dioctadecyldimethylammonium bromide (DDA) were used as adjuvants.

MATERIALS AND METHODS

PI isolation. *N. gonorrhoeae* C3 was cultivated, PI was purified, and OMC were prepared as described by Teerlink et al. (20).

PI fragmentation. PI was fragmentated with CNBr (Pierce Chemical Co., Rockford, Ill.) to three peptides (CB 1, 2, and 3) or with chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) to two peptides (CT 1 and 2) as described by Teerlink et al. (21).

Liposome preparation. Liposomes were prepared by the dilution-dialysis-filtration technique (F. van Dalen, G. F. A. Kersten, T. Teerlink, E. C. Beuvery, and D. J. A. Cromme-

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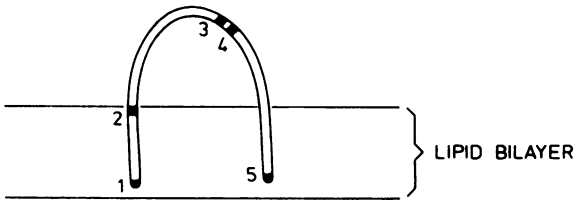

FIG. 1. Schematic representation of PI in the outer membrane of *N. gonorrhoeae*. CNBr cleaves near positions 2 and 4, generating CB 1 (fragment 1-2), CB 2 (fragment 2-4), and CB 3 (fragment 4-5). Chymotrypsin cleaves near position 3, generating CT 1 (fragment 1-3) and CT 2 (fragment 3-5).

lin, J. Controlled Release, in press). PC and Chol (Sigma) (molar ratio, 7:2) in chloroform-methanol (1:1) were dried under nitrogen, and the lipid film was resolubilized in TN buffer (10 mM Tris hydrochloride, 140 mM NaCl [pH 7.4]) with 150 mM octyl glucoside (OG) (Sigma). PI stock solution (PI in 50 mM Tris hydrochloride, 200 mM NaCl, 10 mM EDTA, 0.05% Zwittergent 3-14 [Calbiochem-Behring, La Jolla, Calif.], and 0.02% sodium azide [E. Merck AG, Darmstadt, Federal Republic of Germany] [pH 8.0]) was precipitated with cold ethanol and resolubilized in TN buffer with 150 mM OG. Lipid and protein were mixed in a ratio of 20 to 1 (wt/wt). The mixed micelles were diluted 11-fold in 16 s with TN buffer at 25°C. The liposomes were dialyzed for at least 48 h against three changes of 1 liter of TN buffer at 4°C, followed by filtration through polycarbonate membrane filters (pore sizes, 600 and 200 nm; Nuclepore Corp., Pleasanton, Calif.). DDA-containing liposomes were prepared with and without PI. DDA (Eastman Kodak Co., Rochester, N.Y.) was dissolved in chloroform and added to the lipid solution in chloroform. The PC/Chol/DDA molar ratio was 7:2:1. The procedure was done as described above for PI-containing liposomes. For the incorporation of PI and DDA in the same particle, it was necessary to lower the pH of the mixed micelles and dilution buffer to 3.0 because at pH 7.4 a precipitate appeared, probably consisting of DDA and PI (14). After dilution of the micelles the pH was adjusted to 7.4. Empty liposomes (i.e., without protein or adjuvant) were prepared as described above for PI-containing liposomes except that TN buffer with 150 mM OG was used instead of PI stock solution.

Iscom preparation. Iscoms were prepared by a procedure slightly modified from one described previously (P. de Vries, A. L. van Wezel, and E. C. Beuvery, European patent application EP 0231039 A1, 1987). For the incorporation of PI and both CT peptides, a mixture of phosphatidylethanolamine type III-A (PE) (Sigma) and Chol in chloroform (weight ratio, 1:1) was dried under nitrogen and the lipid film was resolubilized in TN buffer with 136 mM OG. PI or CT peptides in TN buffer with 136 mM OG were added. The ratio of lipid (PE plus Chol) to protein was 10 to 1 (wt/wt). QA (Iscotec AB, Lulea, Sweden) was added as a 10% solution (wt/vol) in water. The ratio of lipid to QA was 1 to 2 (wt/wt). The lipid concentration was typically 1 mg/ml. Iscoms were formed by dialysis against two changes of 1 liter of TN buffer during at least 24 h at 4°C. The iscoms were separated from nonincorporated components by centrifugation through a 10 to 60% sucrose gradient in TN buffer (18 h at 50,000 × *g*). The gradient was fractionated, starting at the top, with an Auto Densi-Flow II C gradient fractionator (Haake Buchler Instruments, Inc., Saddle Brook, N.J.). The protein-containing fractions were dialyzed against TN buffer. For iscoms without protein or adjuvant (empty iscoms), iscoms with DDA, and routinely prepared PI-iscoms, the turbid iscom band was directly removed and dialyzed against TN buffer. For the incorporation of the CB peptides of PI, 8 M urea was added to prevent precipitation. The procedure was done as described for PI-containing iscoms except that after lipid resolubilization, solid urea was added. As a control, intact PI was incorporated in the presence of 8 M urea. Empty iscoms were prepared as described for PI-iscoms except that TN buffer with 136 mM OG was added instead of the protein solution. DDA-containing iscoms were prepared as described for PI-iscoms. DDA was added as a chloroform solution to the lipid solution in chloroform and dried under nitrogen (PE/Chol/DDA ratio, 20:20:9 [wt/wt/wt]).

Preparation of protein-detergent-adjuvant complexes. Protein–Zwittergent 3-14–$AlPO_4$ complexes were produced by simple dilution of the PI stock solution (PI/Zwittergent ratio, 1:2 [wt/wt]) to the desired concentration and addition of an $AlPO_4$ suspension (PI/$AlPO_4$ ratio, 1:100 [wt/wt]). The Zwittergent concentration after dilution was 0.0003%. Protein-Zwittergent-DDA complexes were prepared by drying 75 μg of DDA in chloroform, resolubilizing it in 200 μl of 0.05% Zwittergent in TN buffer, adding 25 μg of PI stock solution, and diluting the solution to the desired concentration. The Zwittergent concentration after dilution was about 0.002%.

Dynamic light scattering. Mean hydrodynamic diameters were determined by dynamic light scattering with a size analyzer (System 4600; Malvern Instruments, Ltd., Worcestershire, United Kingdom).

Electron microscopy. For electron microscopy two techniques were used, the negative-staining procedure (two-step procedure with 2% phosphotungstic acid [pH 5.2] or 2% ammonium molybdate [pH 5.1]) and the freeze-etching technique, essentially as described by Branton (8).

Protein determination. Protein composition was determined by polyacrylamide gel electrophoresis by the method of Laemmli (15); 16% acrylamide gels were used. Protein was visualized by Coomassie brilliant blue staining or, if a low detection limit was necessary, by silver staining as described by Wray et al. (23). Quantitative protein determination was done by the method of Bradford (7), with minor modifications; the reaction volume was reduced to make it suitable for absorbance reading in a microdilution plate reader (Titertek Multiskan; Flow Laboratories, Herts, United Kingdom).

Chol determination. Chol was determined by gas chromatography. To a sample of 20 μl, 100 μl of internal standard (epicoprostanol [Sigma], 100 mg/ml in ethanol) was added. The samples were dried under nitrogen at 60°C. The residues were treated with a trimethylsilylation reagent, and the reaction products were analyzed as described by Derks et al. (9).

DDA determination. DDA was determined with an ion pair extraction method as described by Van Dalen et al. (J. Controlled Release, in press).

Phospholipid determination. Phospholipid was determined by the phosphorus assay of Bartlett (3).

QA determination. QA was determined by reversed-phase high-pressure liquid chromatography. Column material was Hypersil ODS 5 μ (Shandon, Runcorn, United Kingdom). The mobile phase was an acetonitrile-in-water gradient, using 80% (solution A) and 20% (solution B) acetonitrile (wt/vol) in 0.01 M Tris buffer (pH 6.0). The starting ratio was 20% solution A in solution B, and the ending value was 27% solution A in solution B, reached after 67 min. Peaks were detected with a UV spectrophotometer operating at 208 nm. Quantification was based on the automatic integration of the

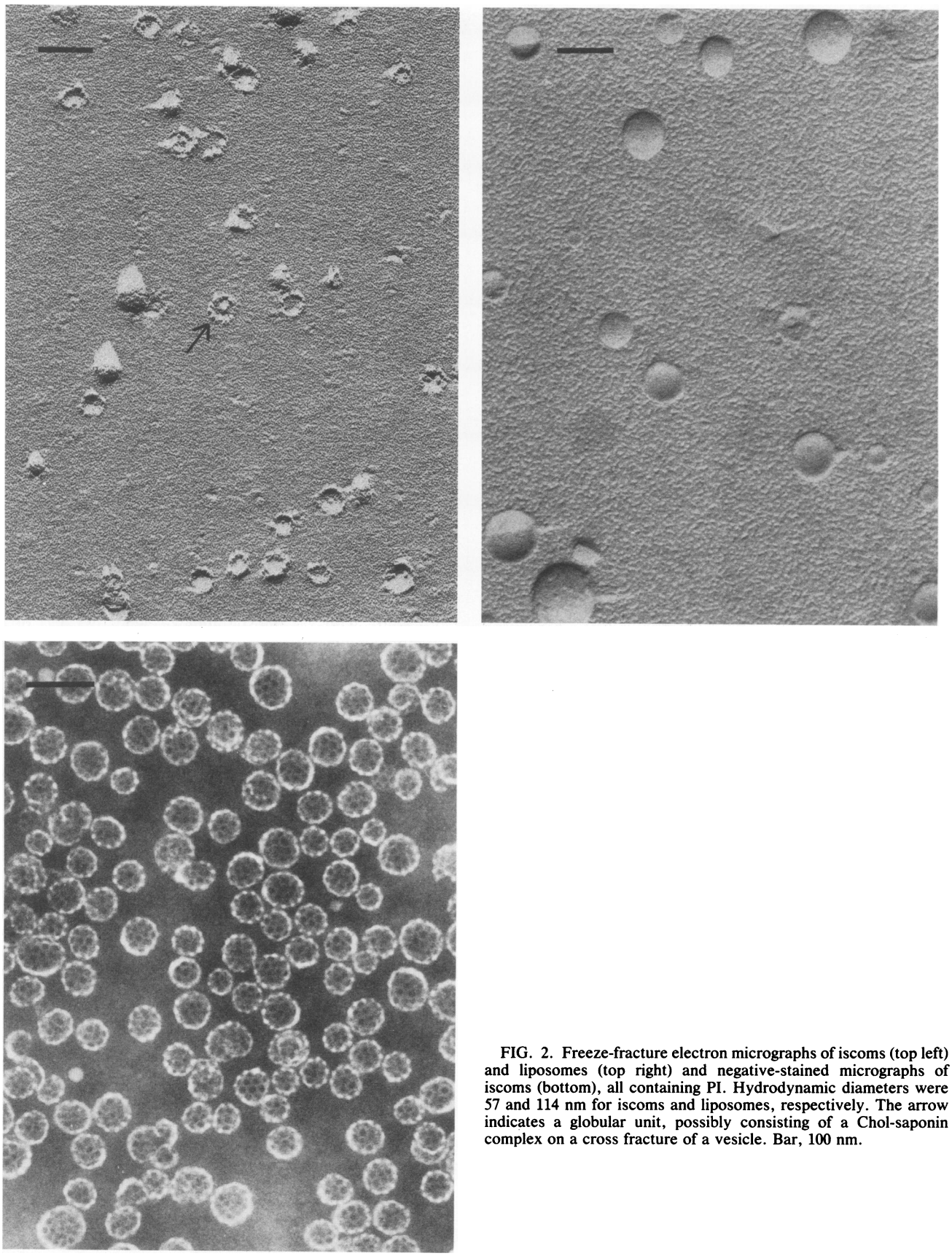

FIG. 2. Freeze-fracture electron micrographs of iscoms (top left) and liposomes (top right) and negative-stained micrographs of iscoms (bottom), all containing PI. Hydrodynamic diameters were 57 and 114 nm for iscoms and liposomes, respectively. The arrow indicates a globular unit, possibly consisting of a Chol-saponin complex on a cross fracture of a vesicle. Bar, 100 nm.

TABLE 1. Chemical composition of iscoms

Prepn	Concn (μg/ml) of:			
	Chol	QA	Phospholipid	PI
Iscoms with PI	128	696	150	35
Empty iscoms	132	577	124	ND[a]
PI stock solution	<5	ND	<30[b]	950

[a] ND, Not determined.
[b] Total phosphate was 30 μg/ml, originating from lipopolysaccharide and possibly phospholipids.

peaks that were proven to contribute mainly to the hemolytic activity of QA.

Effect of $AlPO_4$ on liposomes and iscoms. An $AlPO_4$ suspension was added to solutions of PI-iscoms (35 μg of protein per ml), PI-liposomes (45 μg of protein per ml), empty iscoms, and empty liposomes. The protein/$AlPO_4$ ratio was 0.01 (wt/wt). The samples were incubated for 90 min at 4°C and centrifuged for 10 min at 9,000 × *g*. The particle size of the samples was measured by dynamic light scattering. The pellets were suspended in TN buffer until the original volume was reached. The $AlPO_4$ was dissolved by adding 25 mg of sodium citrate (E. Merck) per mg of $AlPO_4$, followed by incubation overnight at 37°C. The samples, which possibly contained protein, were analyzed by polyacrylamide gel electrophoresis.

Orientation of PI in iscoms. Chymotrypsin degradation was performed as described by Van Dalen et al. (J. Controlled Release, in press). Briefly, PI-containing iscoms and OMC were treated with chymotrypsin (PI/chymotrypsin ratio, 1:25 [wt/wt]), and samples were taken at various times. The peptide mixtures were separated by polyacrylamide gel electrophoresis, and PI degradation was measured by scanning the gel with a laser densitometer (Ultrascan XL; LKB, Bromma, Sweden). Chymotrypsin-treated iscoms were analyzed on a 10 to 60% sucrose gradient as described above.

Immunization and antibody production. Groups of eight mice (strain Cpb:SE, random bred) were immunized subcutaneously (s.c.) with 2.5 μg of protein and boosted 4 weeks after the first injection with an identical preparation. Blood was collected 4 weeks after the first immunization and 2 weeks after the second immunization. The sera were pooled by group, and antibody levels were measured relative to that in a reference serum with an enzyme-linked immunosorbent assay. Purified PI (diluted stock solution) or OMC were used as the antigen coat. Bound antibodies were detected with peroxidase-labeled goat anti-Fc mouse antibodies (Organon Teknika, Malvern, Pa.). Absorbance was measured in a microdilution plate reader with a 450-nm filter.

RESULTS

Analytical data. To characterize the iscoms, a chemical analysis was done and the mean hydrodynamic diameter was determined. Two electron microscopic techniques were applied for visual analysis, negative staining and freeze-fracture. The results are given in Table 1 and Fig. 2. The micrographs obtained by the negative-staining technique show round structures which can be interpreted as spherical or disk shaped. From the freeze-etching micrographs, however, it can be concluded that iscoms are spherical vesicles. Moreover, it is of interest that at cross fractures of the vesicles, the membrane is built up of globular units with a diameter of about 10 nm (Fig. 2, arrow).

The DDA concentration was about 100 μg/ml for liposomes and iscoms, indicating that DDA recovery was complete. It was not possible to prepare iscoms containing both PI and DDA. At pH 3.0, QA precipitated. At pH 7.4, a precipitate of PI and DDA was formed.

The presence of Chol in iscoms was confirmed by gas chromatography-mass spectrometry. A Chol standard and empty iscoms were pretreated and chromatographed as described above. Mass spectra were determined for both presumed trimethylsilyl-Chol peaks. The standard and sample both showed the characteristic ions of Chol-trimethylsilylether (*m/z* 458, 443, 368, 353, 329, 301, 275, 255, 247, 213, 163, 145, 129, 107, 95, and 81) with similar relative intensities.

Effect of $AlPO_4$ on integrity of iscoms and liposomes. Because $AlPO_4$ was chosen as adjuvant, possible interactions between antigen carriers and $AlPO_4$ were investigated. Neither iscoms nor liposomes were affected by the $AlPO_4$ suspension (Table 2); particle sizes did not change much, and PI in liposomes and iscoms was not absorbed by $AlPO_4$.

Orientation of PI in iscoms. PI in Zwittergent micelles, OMC, and liposomes has one chymotrypsin-sensitive site, and more than 90% of the PI is degraded within 1 h (PI/chymotrypsin ratio, 25; 25°C) (Van Dalen et al., J. Controlled Release, in press). Similar effects were observed for PI in iscoms. After 2.5 min, 78% of PI was degraded relative to that in nonincubated iscoms, and after 60 min, the degraded fraction was 93%.

Chymotrypsin-treated iscoms were analyzed on a sucrose gradient. Iscoms with intact PI were used as a control. After centrifugation, the two PI fragments, CT 1 and 2, were located at exactly the same position as PI in the control gradient. The iscoms were also found in this protein-containing fraction (data not shown). This indicated that both termini of PI interact with iscoms.

Equimolar mixtures of chymotrypsin-generated and CNBr-generated peptides (the latter [CB 1, 2, and 3] in 8 M urea) were used for incorporation in iscoms. As a control the same procedure was done without the addition of Chol and PE. The protein distribution in the sucrose gradients is shown in Fig. 3. Without lipids, no iscoms were formed and all peptides were on top of the gradients. When lipids were added, iscoms were formed. CB 3, CT 1, and CT 2 migrated further into the gradients, whereas CB 1 and CB 2 did not show a different sedimentation behavior. Iscoms were in the same fractions as CB 3, CT 1, and CT 2 (electron microscopic observation and dynamic light scattering measurements). Their mean diameters were 52 nm for the CB 3-iscoms and 49 nm for the iscoms with the chymotrypsin-induced peptides.

If PI was incorporated in iscoms in the presence of 8 M urea, no differences were observed in the sedimentation behavior and structure compared with those of normal

TABLE 2. Effect of $AlPO_4$ on size and protein content of iscoms and liposomes[a]

Prepn	Hydrodynamic diam (nm)		Protein[b]	
	Pre-incubation with $AlPO_4$	Post-incubation with $AlPO_4$	Pellet	Supernatant
PI-iscom	78	81	−	+
Empty iscom	84	89		
PI-liposome	138	130	−	+
Empty liposome	135	131		

[a] Experimental details are given in the text.
[b] −, No detectable protein; +, protein detected.

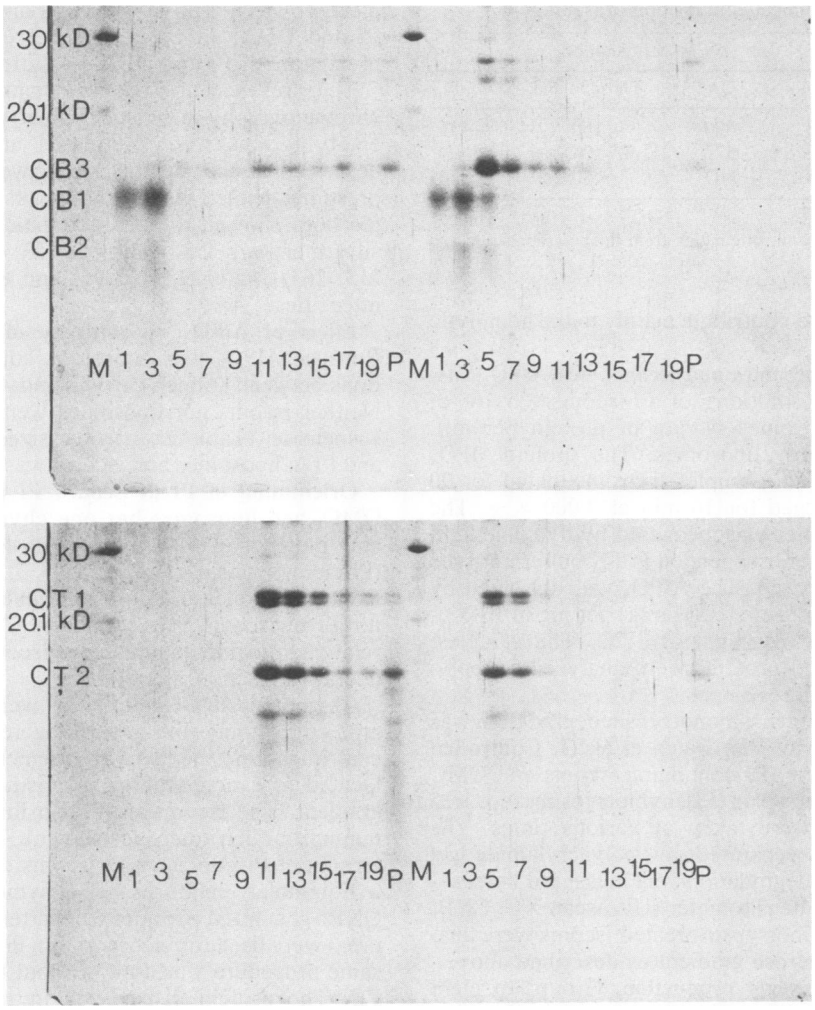

FIG. 3. (Top) Distribution of peptides CB 1, 2, and 3 in a sucrose gradient after iscom preparation (left) and after an identical procedure but without the addition of lipids (right). Iscoms are in fractions 11 to 13 of the gradient at the left. (Bottom) Distribution of peptides CT 1 and 2 in a sucrose gradient after iscom preparation (left) and after an identical procedure but without the addition of lipids (right). Iscoms are in fractions 11 to 13 of the gradient at the left. Lanes: 1, top fraction; 19, bottom fraction; P, solubilized pellet; M, markers.

iscoms. PI comigrated with iscoms on a sucrose gradient, and only iscoms were seen with the negative-staining technique, indicating that PI was incorporated.

Immunogenic activity of PI-containing iscoms. Immunization was not performed via the regularly used intraperitoneal (i.p.) route because in preliminary experiments it was found that i.p. injections of iscoms sometimes caused death within 24 h. To establish the cause of death, two groups of three mice were given 2.5 and 5 μg of PI in iscoms (i.p.). This corresponds to about 50 and 100 μg of QA, respectively. After the mice died, autopsies were done. The main cause of death was liver degeneration. The control group (receiving TN buffer) showed no pathological deviations. Therefore, the s.c. route (less direct contact with the blood compartment) was chosen for further experiments. However, even after s.c. administration one mouse of the group injected with PI-iscoms and two mice of the group injected with PI-iscoms plus DDA-iscoms died after the first immunization. The booster injection caused the death of three more mice of the group injected with PI-iscoms plus DDA-iscoms.

The immunization results are given in Table 3. Two antigen coats were used for the enzyme-linked immunosorbent assay, purified PI and OMC. Because OMC also carry other proteins and lipopolysaccharide, the response is not comparable with that of the other preparations: the PI content was less than 2.5 μg per dose because of the protein impurities, but on the other hand, the lipopolysaccharide acted as an adjuvant. The reason for using OMC in the immunization experiment was because they constitute the most native presentation form and the serum against OMC were subsequently used as a reference to establish which parts of PI antibodies are formed against (T. Teerlink, G. F. A. Kersten, and E. C. Beuvery, manuscript in preparation). Except for OMC, there were no quantitative differences in the enzyme-linked immunosorbent assay signals between purified PI and OMC as the antigen coat. Iscoms gave the highest response, after both the first and second immunizations, followed by protein-Zwittergent-$AlPO_4$ complexes. Liposomes were the least immunogenic.

Under the experimental conditions used, DDA was not a better adjuvant than $AlPO_4$. DDA present in the same liposome as PI did not influence the humoral response

TABLE 3. Relative IgG response of mice against PI after immunization with several PI-containing preparations

Presentation form[a]	Relative IgG response (%) against[b]:			
	OMC		PI	
	Primary	Secondary	Primary	Secondary
PI-iscom + $AlPO_4$	84[c]	595	111	621
PI-liposome + $AlPO_4$	12	108	23	91
PI-Zwittergent-$AlPO_4$ complex	17	194	38	162
OMC + $AlPO_4$	228[d]	1,312[d]	187	366
PI-iscom + DDA-iscom	24[e]	640[f]	57	726
PI-liposome + DDA-lipsome	13	67	14	59
(PI + DDA)-liposome	3	68	6	57
PI-Zwittergent-DDA complex	8	44	9	40
Control (TN buffer)	<1	<1	<1	<1

[a] The immunization route was s.c. The dose was 2.5 μg of protein and 250 μg of $AlPO_4$ or 7.5 μg of DDA.
[b] Eight pooled serum samples per group except when indicated otherwise. IgG levels are expressed as a percentage of the IgG level of an anti-PI reference serum. The standard deviation in the decimal logarithmic scale was 0.20; this implies a 95% confidence factor of about 2 for the levels shown.
[c] Seven pooled serum samples.
[d] Response against all antigens in OMC, not only PI.
[e] Six pooled serum samples.
[f] Three pooled serum samples.

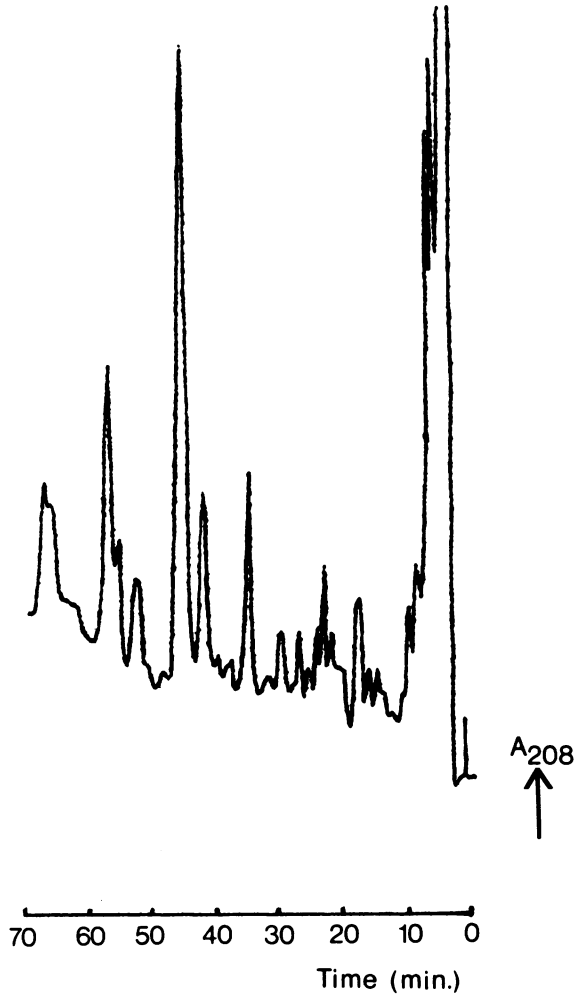

FIG. 4. Chromatogram of QA. A 25-μg sample was injected in 50 μl. Experimental details are given in the text. The chromatographic pattern of QA in iscoms was approximately the same (data not shown).

compared with the response against a mixture of DDA- and PI-containing liposomes.

DISCUSSION

In this report some nonimmunological properties of iscoms, such as chemical composition and protein-iscom interaction, are presented.

Iscoms prepared by the dialysis method and purified by ultracentrifugation on a sucrose gradient contained not only QA and antigen but also Chol and phospholipid (QA and Chol were not contaminated with phosphorus). This is not in agreement with previous findings that iscoms consist of glycoside and antigen (B. Morein, European patent EP-A-0 180 564, October 1985).

The ratio of the components QA, Chol, and PE in the initial mixed micelles was roughly that found in iscoms. This is in agreement with the observation that it is necessary not to diverge much from the starting ratio if iscoms are to be formed, that is, colloidal particles with a hydrodynamic diameter of 40 to 80 nm. If the starting mixture was equal portions of Chol and QA and no PE was present, then a precipitate of extended lamellar structures consisting of units with the same hexagonal arrangement as iscoms was formed after removal of the OG (A. Spiekstra, National Institute for Public Health and Environmental Hygiene, The Netherlands, personal communication). These structures looked similar to those described by several investigators who treated Chol and biological membranes with saponin (1, 10, 12). PE might prevent "clotting" of the iscoms to precipitating aggregates. If the QA/lipid ratio was low, amorphous liposomelike structures were formed; the hexagonal units had disappeared.

The toxicity of QA is not diminished by incorporation in iscoms. Flebbe and Braley-Mullen (11) reported that 25 μg of free QA administered i.p. was the maximal amount without dramatic toxic effects, whereas 50 μg of QA in iscoms was fatal. Further purification of QA (Fig. 4) and mass spectrometric analysis for structural characterization is under way, as are attempts to incorporate more protein in iscoms.

In this study, experimental support was provided for the hypothesis that the orientation of PI in iscoms and the way PI is incorporated in OMC are rather similar. First, the velocity of PI degradation and the total amount of PI that

was susceptible to chymotrypsin were the same as for the OMC. Thus, PI is probably situated with the chymotrypsin-sensitive site exposed to the aqueous environment. The newly formed fragments were still associated with the iscoms, which supports the presumption that both PI termini interact with the iscom.

Three peptides were formed after CNBr treatment of PI: CB 1 (13 kilodaltons), CB 2 (8 kilodaltons), and CB 3 (15 kilodaltons). The CNBr-sensitive spot between CB 1 and 2 is located on a part of PI which is normally embedded in the membrane, and the spot between CB 2 and 3 is located in a more hydrophilic (i.e., exposed) region. Therefore, we could use a hydrophobic (CB 1), a hydrophilic (CB 2), and two amphiphilic peptides (CB 3 and intact PI). In addition, the two amphiphilic fragments CT1 and 2, which are formed after chymotrypsin treatment of PI, were used (6). The chymotrypsin-sensitive spot is very close to the CNBr-sensitive spot between CB 2 and 3 (21) (Fig. 1). CB 3 was incorporated in iscoms, but CB 2 was not. More surprisingly, the normally membrane-buried CB 1 was not incorporated either. But if CB 1 was extended with the hydrophilic CB 2 (which approximates CT 1), this peptide was incorporated. Obviously, a criterion for incorporation is amphiphilicity; the molecule to be incorporated has to be directed into the polar-nonpolar interface.

The protein-detergent-$AlPO_4$ complexes were less immunogenic than expected. Jiskoot et al. (14) reported that these complexes were about 10 times more immunogenic than liposomes containing DDA and protein. We found that the PI-Zwittergent-$AlPO_4$ complexes were almost three times more immunogenic than the PI-DDA-liposomes. There are three possible causes for this difference: the immunization route was different (s.c. instead of i.p.), protein IB was used instead of protein IA, and the detergent in the complex was Zwittergent instead of OG.

The immunoglobulin G level after the booster was equal for PI-DDA-liposomes and PI-liposomes plus DDA-liposomes. The adjuvant activity, if present, was effective in both antigen delivery systems. However, it is possible that the low pH at which the PI-DDA-liposomes were made induced conformational changes resulting in a lower or other, i.e., against epitopes which do not exist in native PI, immunoglobulin G response against PI.

Again, iscoms appear to be very potent immunogens, but their toxic properties are a drawback. The PI-detergent-$AlPO_4$ complexes do not have this disadvantage but are less immunogenic. There is a need for further research to find the optimal balance between toxicity and immunogenicity. This research will encompass the further purification of QA, the use of other glycosides, and the exploration of other mixed-micelle systems.

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