INFECTION AND IMMUNITY, June 1988, p. 1661–1664
0019-9567/88/061661-04$02.00/0

Vol. 56, No. 6

Immunogenicity of Liposomes and Iscoms Containing the Major Outer Membrane Protein of *Neisseria gonorrhoeae*: Influence of Protein Content and Liposomal Bilayer Composition

GIDEON F. A. KERSTEN,[1]* ANNE-MARY VAN DE PUT,[2] TOM TEERLINK,[1] E. COEN BEUVERY,[1] AND DAAN J. A. CROMMELIN[2]

Departments of Inactivated Viral Vaccines and Bacterial Vaccines, National Institute for Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven,[1] and Department of Pharmaceutics, University of Utrecht, 3522 AD Utrecht,[2] The Netherlands

Received 5 November 1987/Accepted 16 March 1988

The influences of *Neisseria gonorrhoeae* protein IB content and bilayer composition of liposomes and protein content of iscoms on immunogenicity were investigated. Changes in the protein content of liposomes did not influence the immunoglobulin G response, whereas the response was lowered when the amount of protein in iscoms was increased. Bilayer composition only influenced the primary immunoglobulin G response; immunological memory was not affected.

Liposomes are possible vehicles for the delivery of antigens to the immune system. Some parameters, such as lipid composition and epitope density, influence the immunogenicity of haptenated liposomes (6, 16, 28, 32) and antigen-containing liposomes (1, 7, 8, 11, 25). From these data, it becomes clear that there seem to be some general principles that determine the immunogenicity of an antigen-containing liposome but that these principles cannot be blindly transferred to other liposome antigen systems. In this report, we present results of a study concerning the influence of epitope density, cholesterol content, and phase transition temperature of unilamellar vesicles containing the gonococcal protein IB (PI) on the humoral immune response in mice. The results were compared with those of PI in iscoms with different protein-to-Quil A (QA) ratios.

Gonococcal PI was purified by the method of Teerlink et al. (29). Liposomes were prepared as described elsewhere (F. van Dalen, G. F. A. Kersten, T. Teerlink, E. C. Beuvery, and D. J. A. Crommelin, J. Controlled Release, in press). In brief, lipids (Sigma Chemical Co., St. Louis, Mo.) in chloroform were dried and resolubilized in TN buffer (10 mM Tris hydrochloride, 140 mM NaCl [pH 7.4]) with 150 mM octylglucoside (OG). PI, also in TN buffer with OG, was added (lipid-to-protein ratio was 20:1 by weight for standard preparations). The mixed micelles were diluted 11-fold in 16 s with TN buffer. After dialysis against TN buffer, the liposomes were extruded through 600- and 200-nm (pore size) polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.).

Iscoms were prepared by dialysis of phosphatidylethanolamine-cholesterol-QA-PI-OG micelles at a ratio of 5:5:20:1:200 by weight for standard preparations (OG concentration was 2%) (15). Iscoms were purified by ultracentrifugation on sucrose gradients (18 h, 50,000 × *g*).

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

The membrane fluidity of liposome preparations was determined by measuring steady-state fluorescence polarization in a fluorescence spectrophotometer (Elscint MV-1a; Elscint Ltd., Haifa, Israel), as described by van Blitterswijk et al. (30). 1,6-Diphenyl-1,3,5-hexatriene (Sigma) was used as the fluorescent probe. To correct for light scattering, each solution was diluted until a plateau value of the fluorescence polarization (*P*) was reached.

Cholesterol was determined by gas chromatography, with the standard addition method as described by van Dalen et al. (in press). Phospholipid was determined by the phosphorus assay described by Bartlett (2). QA was determined by reversed-phase high-pressure liquid chromatography as described earlier (15). Protein composition was determined by polyacrylamide gel electrophoresis according to Laemmli (18). Protein was visualized by Coomassie brilliant blue staining or silver staining (31). Quantitive protein determination was done according to Bradford (3).

Groups of eight mice (strain Cpb:SE, random bred) were immunized subcutaneously 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 per group, and antibody levels were measured relative to a reference serum with an enzyme-linked immunosorbent assay with PI as antigen coat.

The initial molar lipid composition of the liposomes in which protein content was varied was egg phosphatidylcholine-cholesterol-egg phosphatidylglycerol at a ratio of 7:2:1. The liposomes were prepared with three different protein concentrations. The lipid-to-protein ratio and the particle size were established. There was a slight increase in hydrodynamic diameter when protein concentration was increased (Table 1).

The effect of protein concentration in iscoms was also investigated. We already reported the high toxicity of iscoms compared with other antigen delivery systems (15). The amount of the toxic component QA to be administered was reduced by the incorporation of more protein into the iscoms, compared with the standard conditions. However, as a consequence, the particle size, as well as the polydispersity (data not shown), increased dramatically (Table 1).

Mice were immunized with the liposomes and iscoms, and the humoral response was measured in an enzyme-linked immunosorbent assay. All liposome preparations induced

* Corresponding author.

TABLE 1. Influence of protein content of PI-containing liposomes and iscoms on size and immunogenicity

PI concn (μg/ml)	Proportion of protein[a]	Hydrodynamic size (nm)	Relative IgG response (%)[b]	
			Primer	Booster
Liposomes[c]				
35	22.4	110	3	29
84	9.5	132	2	24
121	7.5	150	2	25
Iscoms[d]				
18	12.2	80	34	703
27	5.9	208	24	289
54	2.8	3,700	42	269

[a] Values are ratios of lipid to protein for liposomes and ratios of QA to protein for iscoms.

[b] IgG, Immunoglobulin G. Percentages are with respect to the value for 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 in the levels presented.

[c] Initial molar composition was 7:2:1 egg phosphatidylethanolamine-cholesterol-egg phosphatidylglycerol.

[d] Initial composition was 1:1:4 (by weight) egg phosphatidylethanolamine-cholesterol-QA.

similar immune responses, whereas the response to iscoms tended to decrease when the protein-to-QA ratio was increased (Table 1), except after the primer with the iscom having a QA-to-PI ratio of 2.8.

The liposomal lipid variables investigated were the amount of cholesterol present in the bilayer and the chain length and degree of saturation of the phospholipid. Phospholipid content, cholesterol content, particle size, membrane fluidity, and immunogenicity were determined (Table 2). It was not possible to prepare distearoylphosphatidylcholine liposomes containing no cholesterol or a low amount of cholesterol or dipalmitoylphosphatidylcholine liposomes without cholesterol. After dilution, aggregation occurred, and almost no phosphate was present after extrusion through the 200-nm (pore size) filter. The membrane fluidity of the stable liposomes was determined by fluorescence polarization measurements. High polarization values (Table 2) mean low membrane fluidity. A trend can be observed in the immunoglobulin responses; immunoglobulin G response increased as membrane fluidity decreased. After the booster, this effect disappeared.

In this report, we discuss the effect of three parameters on the immunogenicity of PI-containing liposome preparations, namely, protein content, cholesterol content, and chain length of the phospholipid. In addition an attempt was made to correlate the immune response with a parameter describing the membrane fluidity.

We did not find a different immune response by increasing the protein-to-lipid ratio, whereas for haptens, such a difference has been reported. Haptenated phospholipids are, up to a certain concentration, homogeneously distributed over the bilayers (13). This means that the epitope density indeed increases with increasing hapten concentration. However, several investigators have reported a nonrandom protein distribution in liposome dispersions (4, 5, 14, 17, 23). Protein-rich liposomes might be coexisting with protein-free liposomes. Therefore, it is very well possible that our liposome preparations are also not homogeneous. There are several explanations for this phenomenon. In the mixed micelle stage of the liposome preparation, there exist probably at least two types of micelles: detergent-protein-lipid micelles and detergent-lipid micelles. Differences in the stability of the two types of micelles could cause a nonhomogeneous distribution of protein (14). Another explanation is the following. Vesicle formation occurs via fusion of small bilayer fragments (19, 24). These fragments must have the proper orientation to fuse with the fast-growing bilayer. Fragments without protein will orientate faster than protein-containing fragments will. If we neglect differences in the strength of the hydrophobic interactions, these differences in the velocity of the orientation of "empty" and protein-containing bilayer fragments mean that in the beginning liposomes are formed that contain no or few protein molecules, whereas at the end of the dilution process, more protein is incorporated. This process could be amplified if

TABLE 2. Influence of bilayer composition on size, membrane fluidity, and immunogenicity of PI-containing liposomal preparations[a]

Initial molar composition	Molar phospholipid/chol ratio of final product	Hydrodynamic size (nm)	Fluorescence polarization (P)[b]	Relative IgG response (%)[c]	
				Primer	Booster
PC-chol-PG					
7:0:1	8:0	89	0.142	1	35
7:2:1	8:1.9	118	0.191[d]	3	29
7:6:1	8:5.2	126	0.289	4	28
7:10:1	8:7.2	115	0.330	4	23
DPPC-chol-DPPG					
7:0:1	NS				
7:2:1	8:2.1	285	0.421[e]	8	29
7:6:1	8:6.1	189	0.408	10	20
7:10:1	8:10.1	186	0.398	5	23
DSPC-chol-DSPG					
7:0:1	NS				
7:2:1	NS				
7:6:1	8:8.5	223	0.414	5	18

[a] Abbreviations: PC, egg phosphatidylethanolamine; PG, egg phosphatidylglycerol; chol, cholesterol; DPPC, dipalmitolyphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPC, distearoylphosphatidylcholine; DSPG, distearoylphosphatidylglycerol; IgG, immunoglobulin G; NS, not stable.

[b] $P = I_{11} - I/I_{11} + I$, where I_{11} is the intensity of the emitted light parallel to the direction of the polarized excitation light and I is the intensity of the part of the emitted light perpendicular to the excitation light. The maximal value of P is 0.5 (27).

[c] Percentages are with respect to the value for 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 in the levels presented.

[d] P for this preparation without protein, 0.183.

[e] P for this preparation without protein, 0.420.

the micellar solution has not reached equilibrium at the start of the dilution process. This dilution was generally started less than 10 min after mixing the lipid-OG micelles with the protein-OG micelles. Although the OG concentration is relatively high, 10 min might be too short for reaching equilibrium (20, 21). In conclusion, increasing the protein-to-lipid ratio does not necessarily mean that the epitope density (i.e., the number of epitopes per surface unit per liposome) indeed increases. Maybe only the number of empty liposomes decreases. We were not able to separate empty and PI-containing liposomes on Percoll gradients, probably because the internal volume was not equilibrated with the Percoll solution (data not shown). For a proper separation of liposomes with different protein contents, it is necessary for the gradient component to equilibrate with the internal volume (17). In sucrose or metrizamide gradients, fluid liposomes were not stable.

With iscoms, the protein itself strongly influenced the size of the complexes. In this case, there are two other variables, in addition to protein density differences: the size of the particles and the concentration of QA, which is an adjuvant itself (9, 10, 26). Therefore, we can not draw a definite conclusion about the influence of epitope density on the immunogenicity of iscoms, as was done by Lovgren et al. for haptenated preformed (i.e., protein-containing) iscoms (22). However, we succeeded in reducing the relative amount of toxic QA more than 4-fold, causing a 2.6-fold-lower humoral response.

We did find a small influence of lipid composition on immunogenicity without the use of peritoneal exudate cells or Freund complete adjuvant (1). More cholesterol induced a stronger primary response if egg phosphatidylethanolamine was used, and for dipalmitoylphosphatidylcholine, there was an optimum for the liposomes with the intermediate cholesterol content. A low lipid fluidity enhanced the primary response more than fluid liposomes did. These effects were absent in the booster reaction. In our system, the stimulation of memory cells was not influenced by the bilayer composition of the liposomes. We do not have an explanation for this phenomenon. Probably, the answer can only be found by unraveling the interactions of the vesicles with the immune system. These interactions seem to be complicated and diverse. Liposomes are not only processed by macrophages, but they also directly affect B and T lymphocytes. The magnitude of this effect also depends on bilayer composition (12).

We thank W. J. van Blitterswijk (Antonie van Leeuwenhoekhuis, Amsterdam, The Netherlands) for permission to use the fluorescence spectrophotometer and assistance with the measurements, A. K. Hagen-Fast for performing the QA determinations, and Marijke van de Nadort for preparing the manuscript.

LITERATURE CITED

1. **Bakouche, O., and D. Gerlier.** 1986. Enhancement of immunogenicity of tumour virus antigen by liposomes: the effect of lipid composition. Immunology **58**:507–513.
2. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Carroll, C. R., and E. Racker.** 1977. Preparation and characterization of cytochrome C oxidase vesicles with high respiratory control. J. Biol. Chem. **252**:6981–6990.
5. **Curman, B., L. Klareskog, and P. A. Peterson.** 1980. On the mode of incorporation of human transplantation antigens into lipid vesicles. J. Biol. Chem. **255**:7820–7826.
6. **Dancey, G. F., T. Yasuda, and S. C. Kinsky.** 1978. Effect of liposomal model membrane composition on immunogenicity. J. Immunol. **120**:1109–1113.
7. **Davis, D., A. Davies, and G. Gregoriadis.** 1986/1987. Liposomes as adjuvants with immunopurified tetanus toxoid: the immune response. Immunol. Lett. **14**:341–348.
8. **Davis, D., and G. Gregoriadis.** 1987. Liposomes as adjuvants with immunopurified tetanus toxoid: influence of liposomal characteristics. Immunology **61**:229–234.
9. **Flebbe, L. M., and H. Braley-Mullen.** 1986. Immunopotentiating effects of the adjuvants SGP and Quil A. I. Antibody responses to T-dependent and T-independent antigens. Cell. Immunol. **99**:119–127.
10. **Flebbe, L. M., and H. Braley-Mullen.** 1986. Immunopotentiation by SGP and Quil A. II. Identification of responding cell populations. Cell. Immunol. **99**:128–139.
11. **Gregoriadis, G., D. Davis, and A. Davies.** 1987. Liposomes as immunological adjuvants: antigen incorporation studies. Vaccine **5**:145–151.
12. **Grover, A., and G. Sundharadas.** 1986. Effect of liposomes on lymphocytes: induction of proliferation of B lymphocytes and potentiation of the cytotoxic response of T lymphocytes to allo-antigens. Eur. J. Immunol. **16**:665–670.
13. **Humphries, G. M. K., and H. M. McConnell.** 1977. Membrane-controlled depletion of complement activity by spin label-specific IgM. Proc. Natl. Acad. Sci. USA **74**:3537–3541.
14. **Jackson, M. L., and B. J. Litman.** 1982. Rhodopsin-phospholipid reconstitution by dialysis removal of octyl glucoside. Biochemistry **21**:5601–5608.
15. **Kersten, G. F. A., T. Teerlink, H. J. G. M. Derks, A. J. Verkleij, T. L. van Wezel, D. J. A. Crommelin, and E. C. Beuvery.** 1988. 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. Infect. Immun. **56**:432–438.
16. **Kinsky, S. C., J. E. Loader, and A. L. Benson.** 1983. An alternative procedure for the preparation of immunogenic liposomal model membranes. J. Immunol. Methods **65**:295–306.
17. **Konigsberg, P. J.** 1982. Resistance of Ca^{2+}-ATPase to dilution by excess phospholipid in reconstituted vesicles. Biochim. Biophys. Acta **685**:355–366.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lasic, D. D.** 1982. A molecular model for vesicle formation. Biochim. Biophys. Acta **692**:501–502.
20. **Lichtenberg, D., R. J. Robson, and E. A. Dennis.** 1983. Solubilization of phospholipids by detergents. Structural and kinetic aspects. Biochim. Biophys. Acta **737**:285–304.
21. **Lichtenberg, D., Y. Zilberman, P. Greenzaid, and S. Zamir.** 1979. Structural and kinetic studies on the solubilization of lecithin by sodium deoxycholate. Biochemistry **18**:3517–3525.
22. **Lovgren, K., J. Lindmark, R. Pipkorn, and B. Morein.** 1987. Antigenic presentation of small molecules and peptides conjugated to a preformed iscom as carrier. J. Immunol. Methods **98**:137–143.
23. **Madden, T. D., M. J. Hope, and P. R. Cullis.** 1983. Lipid requirements for coupled cytochrome oxidase vesicles. Biochemistry **22**:1970–1974.
24. **Mazer, N. A., R. F. Kwasnick, M. C. Casey, and G. B. Benedek.** 1977. Quasielastic light scattering spectroscopic studies of aqueous bile salt, bile salt-lecithin and bile salt-lecithin-cholesterol solutions, p. 383–402. *In* K. L. Mittal (ed.), Micellization, solubilization, and microemulsions, vol. 1. Plenum Publishing Corp., New York.
25. **Raphael, L., and B. H. Tom.** 1984. Liposome facilitated xenogeneic approach for studying human colon cancer immunity: carrier and adjuvant effect of liposomes. Clin. Exp. Immunol. **55**:1–13.
26. **Scott, M. T., M. Goss-Sampson, and R. Bomford.** 1985. Adjuvant activity of saponin:antigen localization studies. Int. Arch. Allergy Appl. Immunol. **77**:409–412.

27. **Shinitzky, M., and Y. Barenholz.** 1978. Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim. Biophys. Acta **515:**367–394.
28. **Tadakuma, T., T. Yasuda, S. C. Kinsky, and C. W. Pierce.** 1980. The effect of epitope density on the in vitro immunogenicity of hapten-sensitized liposomal model membranes. J. Immunol. **124:**2175–2179.
29. **Teerlink, T., R. Breas, R. van Eijk, R. 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. American Society for Microbiology, Washington, D.C.
30. **van Blitterswijk, W. J., R. P. van Hoeven, and B. W. van der Meer.** 1981. Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. Biochim. Biophys. Acta **644:** 323–332.
31. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118:**197–203.
32. **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.