

BBA Report

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Preparation of immunologically responsive liposomes with phosphonyl and phosphinyl analogs of lecithinS.C. KINSKY^a, P.P.M. BONSEN^b, C.B. KINSKY^a, L.L.M. VAN DEENEN^b and A.F. ROSENTHAL^c^a*Departments of Pharmacology and Microbiology, Washington University School of Medicine, St. Louis, Mo. 63110 (U.S.A.);*^b*Biochemistry Laboratory, State University, Utrecht (The Netherlands); and*^c*Long Island Jewish Medical Center, New Hyde Park, N.Y. 11040 (U.S.A.)*

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SUMMARY

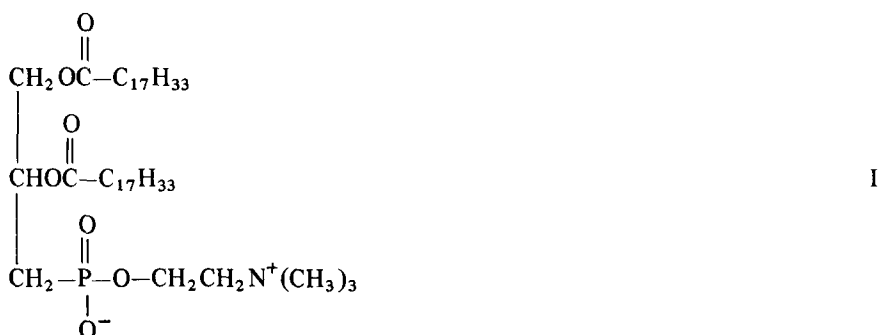
Liposomes, which release trapped glucose marker upon incubation with an appropriate antiserum and complement source, have been prepared from analogs of lecithin that cannot serve as substrates for phospholipases A, B, C, or D. These results confirm and extend previous studies with radioactive liposomes which have suggested that direct attack on membrane phospholipids by the above enzymatic activities may not be responsible for complement-dependent damage.

Previous reports have described the preparation and properties of liposomal model membranes which may be useful objects for the study of the molecular basis of immune lysis¹⁻⁴. These liposomes release trapped glucose marker when incubated with both an appropriate antiserum as a source of antibodies, and fresh guinea pig or human serum (or purified components isolated from the latter) as a source of complement.

The possibility that functional impairment of these model membranes occurs as a consequence of phospholipid degradation has been recently investigated using liposomes prepared with ³²P-labelled lecithin or sphingomyelin⁵. Chromatographic analysis of reaction mixtures containing liposomes that had released 50–80% of their trapped glucose did not reveal the appearance of any radioactive phosphatidic acid, phosphorylcholine, lysolecithin, glyceryl-phosphorylcholine, or sphingosyl-phosphorylcholine, and at least 98% of the recovered radioactivity was in the form in which it was originally incorporated into the liposomes, *i.e.* as either lecithin or sphingomyelin. These observations have led to the tentative conclusion that activation of the complement sequence does not result in the generation of any known degradative enzymes acting directly on membrane phospholipids⁵. This conclusion, is, however, based on negative findings (*i.e.* the failure to detect any new radioactive spot(s) on thin-layer plates) and therefore is subject to the sensitivity of the

assay methods employed; these would have been able to pick up a 1% degradation of phospholipid had the action of complement resulted in the formation of compounds with chromatographic properties similar to those listed above.

To circumvent the limitations of this approach, the present studies were undertaken to determine whether immunologically responsive liposomes could be made with analogs of lecithin that cannot serve as substrates for enzymes involved in the catabolism of this phospholipid. Initial experiments were carried out with DL-1,2-dioleoyloxypropyl-3-(choline) phosphonate (Compound I), hereafter referred to as the phosphonyl analog; details of its synthesis will be described elsewhere⁶. Table I shows that liposomes made with the



phosphonyl analog share the following properties with liposomes prepared with lecithin.

(a) Essentially the same percentage of trapped glucose marker is released upon incubation with antiserum and guinea pig serum. (b) Heating of the guinea pig serum (56° for 30 min) to destroy its hemolytic complement activity has a similar effect on its ability to promote loss of marker from liposomes in the presence of antiserum. (c) Positively charged liposomes, prepared with stearylamine as the charged amphiphile, are as responsive as negatively charged liposomes, containing dicetyl phosphate. (d) Sensitization of liposomes to the action of antibody and complement can be conferred by amphipathic antigens of widely different structure, *e.g.* ceramide derivatives (Forssman) or bacterial lipopolysaccharides (alkali-treated mR5 LPS derived from a rough form of *Salmonella minnesota*). Additional results (not reported) have demonstrated that glucose is released at comparable rates from Forssman-sensitized liposomes made with either the phosphonyl analog or lecithin, and that there is no appreciable difference in the amount of antigen required for half-maximal sensitization, *i.e.* half-maximal glucose release.

Liposomes made with the phosphonyl analog do, however, differ from those prepared with lecithin in one important respect. Table I shows that, under the experimental conditions employed, phospholipase C releases a significant amount of glucose from lecithin liposomes but not from those prepared with the phosphonyl analog; this is of course consistent with the fact that the latter compound could not be a substrate for the enzyme because it lacks the susceptible C—O—P bond.

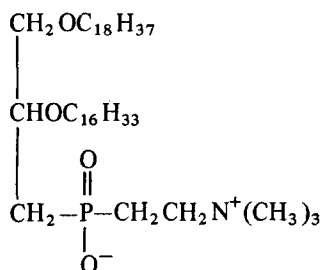
Similar experiments (although less extensive due to the scarcity of material) have been performed with DL-1-octadecoxy-2-hexadecoxy-propyl-[2'-trimethylammonium]ethyl phosphinate (Compound II), hereafter referred to as the phosphinyl analog; the synthesis of this compound has been described⁷.

TABLE I

COMPARATIVE PROPERTIES OF LIPOSOMES PREPARED WITH LECITHIN OR THE PHOSPHONYL ANALOG

Complete experimental details regarding preparation of liposomes and the spectrophotometric assay for following glucose release have been previously described in refs. 4 and 5. Liposomes were generated from mixtures containing phospholipid, sterol, and charged amphiphile, in molar ratios of 2:1.5:0.22, respectively. Liposomes were sensitized with either 10 μg of Forssman antigen or 6 μg of alkali-treated mR5 lipopolysaccharide per μmole of phospholipid. The phospholipids used were egg lecithin (PC) or the phosphonyl analog (PNYL); the charged amphiphiles were dicetyl phosphate (DCP) or stearylamine (SA); cholesterol (CHOL) was the sterol. The trapped glucose marker content of the liposomes employed in these experiments ranged from 97 to 143 nmoles/5 μl (the volume of liposome preparation used in the assay). Phospholipase C dependent release was determined in the presence of 0.45 μg of *Clostridium welchii* enzyme. Immune release was determined in the presence of 113 μl of guinea pig serum (GPS) plus either 30 μl of a 1:10 dilution of rabbit antiserum erythrocyte serum in the case of Forssman-sensitized liposomes, or 13 μl of undiluted rabbit anti-mR5 serum in the case of lipopolysaccharide-sensitized liposomes.

Liposome composition	Antigen incorporated	% Trapped glucose released in 30 min by:			
		No enzyme	Phospholipase C	Antiserum, native GPS	Antiserum, heated GPS
PC/CHOL/DCP	Forssman	3.5	67.5	70.2	17.2
		3.9	65.5	67.2	11.4
PNYL/CHOL/DCP	Forssman	3.7	6.0	73.4	12.4
		3.9	4.1	70.2	15.8
PC/CHOL/SA	Forssman	1.0	98.8	87.4	4.9
		1.0	72.2	70.9	2.9
PNYL/CHOL/SA	Forssman	1.3	3.6	65.4	6.8
		5.9	7.5	96.9	12.0



II

Table II shows that the phosphinyl analog can be used to prepare immunologically responsive liposomes which require the prior incorporation of antigen, as well as the presence of antiserum and native (*i.e.* unheated) guinea pig serum for maximum glucose release. The phosphinyl analog also cannot be attacked by phospholipase C and has been shown to be a potent competitive inhibitor of this enzyme⁷.

Furthermore, because the phosphinyl analog is a completely nonhydrolyzable phospholipid, it cannot function as a substrate for phospholipases A, B, or D. We have previously considered it unlikely that the latter activity could be the ultimate cause of complement-dependent liposomal damage because the product of phospholipase D acting

TABLE II
REQUIREMENTS FOR GLUCOSE RELEASE FROM
PHOSPHINYL ANALOG LIPOSOMES

Liposomes were generated from mixtures containing the phosphinyl analog, cholesterol, and dicetyl phosphate, in molar ratios of 2:1.5:0.22, respectively, plus 8 μg of Forssman antigen per μmole of analog as indicated. Liposomes prepared without and with the antigen contained 212 and 103 nmoles of trapped glucose per 7 μl (the volume of liposome preparation used in the assay). Immune release (30 min incubation at room temperature) was determined in the presence of 30 μl of a 1:10 dilution of rabbit antiserum erythrocyte serum and 113 μl of guinea pig serum (GPS); in the control experiment, antiserum was replaced by normal rabbit serum.

Addition			% Trapped glucose released Antigen incorporated:	
Anti-serum	Native GPS	Heated GPS	None	Forssman
+	+	—	6.6	50.5
+	—	+	1.2	3.5
—	+	—	4.8	4.1

on lecithin is phosphatidic acid, and very high amounts of phosphatidic acid can be employed as the negatively charged amphiphile in the preparation of immunologically responsive liposomes (unpublished experiments). Similarly, an association of phospholipase A activity with activation of the complement system did not seem plausible because immunologically responsive liposomes could be prepared with sphingomyelin^{4,5} and this phospholipid can be regarded as an analog of lecithin which, because it lacks any fatty acid ester bonds, cannot be cleaved by phospholipases A or B. These earlier findings did not, however, eliminate the possibility that activation of the terminal complement components resulted in the generation of an enzymatic activity that could remove fatty acids in both ester and amide linkage. This alternative can now be excluded because the aliphatic side chains are attached *via* ether linkages in the phosphinyl analog. Finally, it should be noted that the phosphinyl analog contains only saturated aliphatic residues. Thus, it seems unlikely that activation of the complement sequence produces an enzyme which alters liposomal permeability by catalyzing the (per)oxidation of unsaturated bonds.

The present results therefore support the previous suggestion^{4,5} that complement-dependent damage to liposomes (and, by implication, natural cell membranes) occurs as a consequence of noncovalent bond rupture and thus may be similar to the action of a detergent.

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REFERENCES

- 1 J.A. Haxby, C.B. Kinsky and S.C. Kinsky, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 300.
- 2 C.R. Alving, S.C. Kinsky, J.A. Haxby and C.B. Kinsky, *Biochemistry*, 8 (1969) 1582.
- 3 J.A. Haxby, O. Götze, H.J. Müller-Eberhard and S.C. Kinsky, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 290.
- 4 S.C. Kinsky, J.A. Haxby, D.A. Zopf, C.R. Alving and C.B. Kinsky, *Biochemistry*, 8 (1969) 4149.
- 5 K. Inoue and S.C. Kinsky, *Biochemistry*, 9 (1970) 4767.
- 6 P.P.M. Bensen, G.H. De Haas, G.J. Burbach-Westerhuis and L.L.M. Van Deenen, manuscript in preparation.
- 7 A.F. Rosenthal, S.V. Chodsky and S.C.H. Han, *Biochim. Biophys. Acta*, 187 (1969) 385.

Biochim. Biophys. Acta, 233 (1971) 815–819