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CONTAINING LIPOSOMES OF VARYING LIPID COM-
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STUDY OF PROTECTIVE PROPERTIES OF ANTIGEN-CONTAINING LIPOSOMES OF VARYING LIPID COMPOSITION IN PLAGUE

V. I. Zakrevskiy and N. G. Plekhanova*

Inclusion of antigens in liposomes in many cases makes it possible to produce an immune response of a higher level [8]. This approach is very promising in terms of creating highly immunogenic preparations to prevent infectious illnesses [6]. There has been very little study of the mechanism for the stimulating effect of liposomes. However, there is no doubt that immunogenicity of the preparation is determined a great deal by the nature of interaction between the antigen-containing liposomes and the immunocompetent cells. The physicochemical properties of the liposome membrane depending on its phospholipid composition play a significant role in this case [15]. We previously demonstrated the higher protective properties of the capsule antigen of the plague pathogen when it is included in liposomes [4].

This work has studied the impact of the lipid composition of liposome membranes, as well as inclusion in them of lipopolysaccharide protective properties of the antigen-containing liposomes during plague.

Materials and Methods

The vaccine strain *Yersinia pestis* EV served as the antigen source. The bacteria were cultivated at 37°C for 48 h on agarized Hottinger medium with pH 7.2. The capsule antigen,

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fraction 1 (F1) was isolated from the sterile acetone-dried bacterial mass by method [11], the main somatic antigen (MSA) and the lipopolysaccharide (LPS) by method [2]. The serological activity of the antigens was assessed in reactions by indirect hemagglutination and double immunodiffusion in gel.

The work used egg lecithin (EL), "lecithin standard" and phospholipids (PL) of egg yoke ("alcohol solution of phospholipids") produced by the Kharkov plant of bacterial preparations. The PL preparation contained phosphatidilcholine, phosphatidilethanolamine, sphingomyelin (SM) and lysophosphatidilcholine in weight correlations 40:50:5:5. In addition, 1 α -dipalmitoilphosphatidilcholine (DPPC; Calbiochem), dicetylphosphate (DCP; Serva), stearylamine (SA; Sigma) and cholesterol (Chol; Reakhim, USSR) were used.

SM from cattle brain was isolated by method [7]. Liposomes were prepared by the method of phase inversion in phosphate-salt buffer (137 mM NaCl+2.7 mM KCl+1.5 mM KH₂PO₄ + 1 mM Na₂HPO₄ + 0.15% of sodium desoxycholate) with the addition of palmitoylated F1, MSA and LPS [14]. F1 was modified by palmitoylchloride (Fluka Ag) as we have previously described [5]. The lipid content in the antigens is indicated in the description of the experiment. The nonincluded material was separated from the liposomes by centrifuging at 100,000 g for 1 h. The effectiveness of antigen inclusion into the liposomes was assessed by serological reactions and by the content of marked ¹²⁵I F1 [5].

The animals were immunized by single injections of preparations in the femur area in a dose of 0.5 ml (guinea pigs) and 0.2 ml (white mice). Within 21 days the animals were infected by subcutaneous injection of a virulent strain of

I. pestis 231. Immunization effectiveness was expressed in magnitudes of LD₅₀ (number of microbe cells, m.c.). Ten animals were taken for each infection dose. In a number of experiments the antigens were injected with complete Freund adjuvant (Difco) in a volumetric correlation of 1:1.

Results and Their Discussion

The literature has described different compositions of lipids used to prepare lecithin and liposomes. EL, Chol and DCP were used most often in molar ratios of 7:2:1. in this /42 composition the "contribution" of Chol to stabilization of the membrane is low insofar as the maximum effect is observed when its content is 50% by mole.

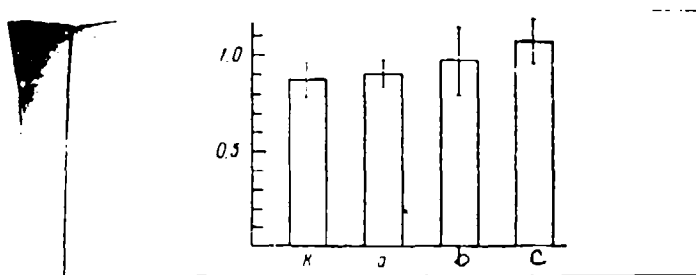


Figure. Lifetime of Guinea Pigs After Infection by 100 m.c. of Plague Pathogen

Key:

On y axis--mean geometric magnitudes of lifetime (in lgN day)

k. free Fl

a. Fl in liposomes from EL:Chol:DCP in 7:2:1 correlation

(Key continued on next page)

Figure. Key continued:

- b. Fl in liposomes from EL:Chol:DCP in correlation 5:4:1
- c. Fl in liposomes from EL:Chol:DCP in 5:4:1 correlation

TABLE L. PROTECTIVE PROPERTIES OF PLAGUE MICROBE ANTIGENS IN FREE AND LIPOSOMAL FORM

Preparation	lg LD ₅₀	$\begin{matrix} g+ \\ g- \end{matrix}$	LD ₅₀ , m.c.
Capsule antigen (Fl)	2.6	-	397
Liposomes with Fl	3.34	0.4	2183
Fl + MSA	3.17	-0.6	1476
		0.4	
Liposomes with Fl + MSA	3.44	-0.7	2748
		0.4	
Fl + LPS	2.51	-	323
Liposomes with Fl + LPS	3.74	± 0.5	5482
Fl + MSA + LPS	2.99	0.3	975
		-0.8	
Liposomes with Fl + MSA + LPS	4.16	0.7	14365
		-0.4	
Control	1.06	0.6	10
		-0.4	

Studies were made of the protective properties of liposomes of the following composition: a) liposomes from EL, Chol-"poor", EL:Chol:DCP and molar correlations 7:2:1; b) liposomes from EL, Chol-"rich", EL:Chol:DCP 5:4:1; c) liposomes from total (PL) eggs, Chol-"rich", PL:Chol:DCP, 5:4:1.

Liposomes containing palmitoylated Fl (palm-Fl) were injected once subcutaneously into guinea pigs from a calculation of 15 μ g of antigen and 30 μ g mole of lipids per animal. The control was immunization of pure palm-Fl in a dose of 15

µg per animal. Thirty animals were immunized by each preparation. Within 21 days the guinea pigs were infected subcutaneously by suspension of plague pathogen I. pestis 231 in doses 10^2 , 10^3 and 10^4 m.c. Ten animals were infected with each dose. As a result of infection all the animals died, however the periods of their death differed depending on the preparation injected and the infection dose (see Figure). Animals immunized by liposomes of group c have the longest lifetime. However there were significant differences compared to the control. There were no differences in effectiveness of liposomes of groups a and b. They yielded a somewhat better effect than free antigen, however they were inferior to liposomes of group c. One can hypothesize that the immunogenicity of the antigen of liposomes of this group is influenced by the presence in their composition of lysophosphatidilcholine, which could cause changes in the structure and functions of the cellular membranes, including immunocompetence. Increase in membrane rigidity of the liposomes because of Chol has an influence on immunogenicity of the preparations to a significantly lower degree. At the same time the duration of existence of the liposomes in the body, as well as their bonding with cells depends significantly on the rigidity of the membrane [9]. It could be improved by using phosphatidilcholine with high temperature of phase transition or by introduction into the SM bilayer. The latter is distributed uniformly in the bilayer with EL if its content does not exceed 33% by mole [9].

We studied the impact of adding SM on the protective properties of antigen-containing liposomes. White mice were immunized for this purpose with a preparation of liposomes consisting of PL:Chol:DCP (7:2:1) and PL:SM:Chol:DCP (3:3:3:1). The liposomes also contained palm-Fl, MSA and LPS. The dose

of antigens and lipids per mouse was 10 μ g Fl, 100 μ g MSA, 100 μ g LPS and 12 μ mole of lipids. Infection by the plague pathogen was done in doses of 250, 2500, 25,000 and 250,000 m.c. per mouse. The protective effect was expressed as LD₅₀ magnitude. For the nonimmune animals it was 10 m.c. Protectiveness of the liposomal preparations with SM was 5 times higher (LD₅₀ equalled 14365) than without it (LD₅₀ equalled 3960). This indicates the impact of "solid" PL on the immunobiological properties of liposomes. At the same time the nature of the antigens and their exposure on the outer surface of the vesicles are very important.

Fl and MSA yield a protective effect when they are in free form [3]. Their inclusion in the liposomes could influence the protective properties of the preparations, intensifying, or on the contrary, weakening protection because of screening of the antigen determinants.

We immunized white mice by free antigens and antigens immobilized in liposomes. LPS of the plague microbe has antigen and immunostimulating properties, therefore we used it in combination with other antigens in free and liposomal form. The antigens were injected from a calculation of 10 μ g Fl, 100 μ g MSA, 100 μ g LPS per mouse. In order for the antigen doses to be the same, we did not separate the liposomes from the nonincluded material. The technique of assessing the protective effect of preparations was similar to that described above. The results presented in Table 1 indicate that when the Fl antigen is included or its combination with MSA and LPS into the liposomes, their protective effect was no lower than during immunization by the antigen in free form. On the contrary, the liposomal preparation containing all three antigens has a more pronounced protective effect. The

magnitude of LD₅₀ of this preparation differed reliably from that of the mixture Fl, MSA and LPS and was higher compared to the LD₅₀ of the remaining preparations. The protectiveness /43 of preparations Fl and LPS in the free and liposomal form differed reliably. Evidently the increase in immunogenicity of liposomes containing Fl and LPS is due to the impact of lipid A on the immunogenic properties of liposomes [10]. S. Kinski [12] in experiments with liposomes containing synthetic haptene N-(2,4-dinitrophenyl-6-aminocaproil)-phosphatidil-ethanolamine as the antigen determinants reveal a significant increase in immunogenicity when lipid A was introduced in them. The author established that this occurs because of change in the critical threshold of the epitope density of haptene necessary to produce an immune response. It is quite possible that in our case as well a similar mechanism occurs. It is precisely the hydrophobic groups of lipid A and their accessibility to the surface of the membrane that are responsible for the mitogenic activity of lipid A in liposomes [10]. Additional injection into the liposomal preparation of MSA which is polysaccharide determinants of the O-antigen increases even more the protection of the immunized animals from plague infection. The presence of all three antigens on the liposomal surface promotes their single-moment supply and interaction with the immunocompetent cells. In the absence of liposomes, the likelihood of this is low and joint injection of Fl, MSA and LPS does not yield a significant protective effect.

Assessment of the impact of phase transition temperature of PL on immunobiological properties of liposomes could be more precise if only PL is used with known transition temperature. For this purpose we used synthetic DPPC with $T_m = 41^\circ\text{C}$. The animals were immunized by preparations of liposomes

infected positively (SA) and negatively (DCP) with the addition of or without the addition of Chol. For comparison of the protective properties, groups of animals were taken that were immunized by the same antigens (Fl, MSA, LPS) in liposomes of composition PL:SM:Chol:DCP (3:3:3:1) and in a mixture with complete Freund adjuvant. Each mouse was injected with 12 micro-mole of lipids: 7 g of Fl and about 100 g of MSA in LPS. The animals were infected with a suspension of plague pathogen in doses 2500 and 25,000 m.c. The results are presented in Table 2.

TABLE 2. PROTECTIVE PROPERTIES OF ANTIGEN-CONTAINING LIPOSOMES OF VARYING LIPID COMPOSITION

Composition of Lipids and Their Correlation	lg LD ₅₀	g+ g-	LD ₅₀ , m.c.
DPPC:SA, 7:2	5.33	-	21275
DPPC:Chol:SA, 7:2:2	4.76	0.6 -0.4	57544
DPPC:DCP, 7:2	4.62	0.6 -0.4	39622
DPPC:Chol:DCP, 7:2:2	4.59	0.6 -0.4	42456
Total PL of egg: SM:Chol:DCP, 3:3:3:1	5.0	0.8	100,000
Complete Freund adjuvant	-	-	Over 250,000
Control (immune animals)	-	-	5

Note. All preparations contained Fl, MSA and LPS.

Among the tested liposomal preparations, positively infected liposomes with "rigid" membrane yielded the greatest protective effect. DPPC even in the body of animals remained "hard," which apparently also fostered the interaction of

liposomes and cells [9]. Addition of Chol reduces the protective properties of liposomes. It is known that Chol "dilutes" the membrane consisting of solid PL, as it were reducing the temperature of phase transition [9]. The impact of infected amphophils was very pronounced. The presence of SA improved protective properties of liposomal preparations. A similar effect of SA has been described by N. Latif and V. Bachhawt; in this case they noted its stimulating effect on the tissue [13]. It is possible that stimulation of tissues at the site of injection intensifies the adjuvant properties of liposomes. In conclusion we should note that all liposomal preparations in immunostimulating properties are inferior to the complete Freund adjuvant. When it is used, only single cases of animal death with maximum infection dose were noted.

Conclusions

1. Improvement in the "rigidity" of the liposomal membranes because of injection of PL with high phase transition temperature intensifies the immunogenic properties of the preparations.
2. Inclusion of the LPS immunostimulator together with capsule and main somatic antigens increased the protective effect, which could be due to the single-moment supply and their interaction with the same immunocompetent cell.
3. Increase in immunogenicity of the antigen-containing liposomes promotes introduction into their membranes of positively infected amphophil, SA.

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