Acta virol. **36**, 129-144, 1992

QUANTITATIVE RECONSTITUTION OF ISOLATED INFLUENZA HAEMAGGLUTININ INTO LIPOSOMES BY THE DETERGENT METHOD AND THE IMMUNOGENICITY OF HAEMAGGLUTININ LIPOSOMES

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Received January 15, 1991

Summary. - The reconstitution of influenza virus haemagglutinin into liposomes from lipid/protein/detergent mixtures by detergent removal provides vesicles that are similar in structure to viral particles. The dissociation properties of haemagglutinin aggregates and the molar ratio of lipid to protein in the starting mixture are the key factors for the individual and total yield of protein incorporation into liposomes. Structural properties of the detergent used as well as special reconstitution conditions are of minor importance for the formation of haemagglutinin liposomes. As determined by radial immunodiffusion-, haemolysis- and fusion experiments, specific properties of haemagglutinin were maintained to a large extent on liposomal incorporation, but its immunogenicity is increased, if the antigen is incorporated into the lipid bilayer of liposomes.

Key words: haemagglutinin; immunogenicity; influenza virus; liposomes; reconstitution

Introduction

Haemagglutinin (HA) is the dominant glycoprotein of influenza viruses. It bears properties, that are responsible for a virus infection as well as for the induction of an immune response. The reconstitution of functionally intact HA into liposomes provides a convenient model, that may be used to study these specific protein functions and related processes. We were particularly concerned with the preparation and characterization of HA-liposomes for the purpose of investigating their immunogenicity. To obtain unambiguous results we were most interested in producing well defined and homogeneous vesicle preparations. With respect to an economical use of HA and to avoid

expensive free/bound separation steps, a quantitative insertion of HA into liposomes should be achieved.

Since 1975, several authors have described a reconstitution of HA into liposomes. In principle, 3 various methods were used: application of ultrasound on mixtures of preformed liposomes and protein (Almeida *et al.*, 1975; Oxford *et al.*, 1981); insertion of the protein into preformed liposomes by means of detergents (Thibodeau *et al.*, 1981; Boudreault *et al.*, 1985); and dialysis of lipid/protein/detergent mixtures (Huang *et al.*, 1980; Kawasaki *et al.*, 1983; Nussbaum *et al.*, 1987; Stegmann *et al.*, 1987; Sizer *et al.*, 1987).

With the exception of Sizer and coworkers none of them were able to incorporate the protein into liposomes quantitatively. Their electron micrographs show vesicles with variable protein density on their surfaces, but, in addition, also rosettes of free protein. In our opinion the use of detergent methods seems to be particularly advantageous, because of their low risk of protein denaturation compared with other methods. Furthermore, detergents are frequently used also for protein isolation from membranes. However, reproducing literature data, we were unable to incorporate HA into liposomes by detergent removal on a quantitative scale. Moreover, the proportion of HA incorporation varied for different protein preparations. Previously performed experiments on dissociation of HA aggregates by detergents revealed that only incomplete dissociation of the rosettes takes place (Stahn *et al.*, 1991) which may affect the reconstitution result.

In this paper we report on the quantitative incorporation of isolated HA. Using radiolabelled tracers of lipid and protein, respectively, we could actually show that at least a partial dissociation of HA-rosettes is an undispensable precondition for their incorporation into liposomes. The preparation of differently covered HA-liposomes is possible by using definite ratios of lipid and dissociable protein in the starting mixture. As determined by immunodiffusion, haemolysis and fusion experiments, essential properties of HA, were not altered during the reconstitution procedure. In addition, it has been demonstrated with a variety of bacterial and viral antigens that liposomes have adjuvant properties (Gregoriadis *et al.*, 1989). However, up to now, the mode of adjuvant action of the liposomes is largely unknown. Probably, an antigen incorporation into liposomes, rendering the antigen presentation similar to natural immunogenic structures, could be advantageous for induction of a strong immune response. Alternatively, the special way of processing liposomally bound antigen by the immunocompetent cells may be decisive and, finally, some kind of depot effect of the liposomes may be operative.

Several authors have already reported on the immunogenicity of HA-liposomes (Oxford *et al.*, 1981; Thibodeau *et al.*, 1981; Boudreault *et al.*, 1985; Tan *et al.*, 1989; El Guink *et al.*, 1989). Their results, although obtained with liposomes differing in structure and composition, show unambiguously an adjuvant effect of the liposomal incorporation of HA. Its quantification, however, is very difficult for the different preparations, because they always contained

both, HA-liposomes and free HA in unknown quantitive ratio. Accordingly, the immunopotentiating effects observed had been induced by several immunogens.

We report on the humoral immune response of mice to HA-liposomes that were free of unbound antigen. It is demonstrated that the adjuvant action of liposomes is coupled to the insertion of HA into the lipid bilayer in an analogous way as in the virus membrane. However, only slightly increased antibody titres could be measured compared to those induced by same doses of HA-subunits only.

Materials and Methods

Haemagglutinin (HA) and ^{125}I-haemagglutinin (^{125}I-HA). The preparation of HA and ^{125}I-HA was performed as described previously. Briefly, influenza X73 viruses (H3N2) were grown in embryonated eggs, purified by zonal sucrose gradient centrifugation, disrupted and the components separated using a 0 to 50 % sucrose gradient, containing Na-deoxycholate. Sucrose and sometimes detergent were removed by dialysis. HA was stored in phosphate buffered saline (PBS), pH 7.2 at 4 °C in the presence or absence of detergent. Radioiodination of HA was carried out applying the chloramine T method, ^{125}I-HA was purified by gelchromatography on Sephadex G-50. The specific activities of the tracers used ranged from 0.03 to 0.3 MBq/μg HA; ^{125}I-HA was characterized by SDS-polyacrylamide gel electrophoresis, density gradient centrifugation and radial immunodiffusion (Stahn *et al.,* 1991).

Lipids and ^{14}C-egg yolk phosphatidylcholine (^{14}C-PC). Phosphatidylcholine (PC) was extracted from egg yolk according to the method of Singleton (Singleton *et al.,* 1965). Phosphatidic acid (PA) was prepared from PC by hydrolysis with phospholipase D (SERVA), as described by Eibl (Eibl *et al.,* 1981). The preparation of dipalmitoyl phosphatidyl choline (DPPC) was also performed from PC using the procedures of Brockerhoff and Gupta, respectively (Brockerhoff *et al.,* 1965; Gupta *et al.,* 1977). Cholesterol was purchased from SERVA. ^{14}C-PC was prepared by the reaction of phosphatidyl ethanolamine and ^{14}C-methyloiodide (Zentralinstitut für Kernforschung, Rossendorf) according to Smith (Smith *et al.* 1978). The specific activity amounted to 820 MBq/nmol.

Lipopolysaccharide (LPS). LPS was extracted from *E. coli* bacteria (0139:K 82 (B)) and purified as described by Leive *et al.* (1972), Westphal *et al.* (1965) and Galanos *et al.* (1975).

Detergents. Na-cholate and Na-deoxycholate were products of SERVA and used without further purification. Octylglucoside (OG) was prepared according to Keana *et al.* (1978).

Reconstitution. The starting mixture of lipid/HA/detergent in PBS was produced by adding to a dried lipid film either

- a solution, being 0.06...6.2 nmol/l in HA and 50 mmol/l in OG or 23 mmol/l in Na-cholate, or
- successively, a solution of detergent and a solution of HA, both in appropriate concentrations to reach the above mentioned values, or
- successively too, a solution of detergent and HA/detergent, both of the same detergent concentration.

The lipid concentration amounted from 0.66 to 2.62 μmol/l, including traces of ^{14}C-PC. HA was labelled by ^{125}I.

The mixtures were equilibrated for at least 24 hr in a N_2 atmosphere at room temperature. The removal of detergent was accomplished by three different methods: (1) Dialysis was performed for 72 hr (Na-cholate) or 48 hr (OG) with 12-hr changes of 1000 ml PBS buffers, (2) gel chromatography on Sephadex G 50 was performed by using a column of 1.5 x 28 cm and PBS as an eluant, and (3) rapid dilution to a detergent concentration below the critical concentration of micelle

formation (CMC) followed by dialysis as described above. Sometimes, samples had to be concentrated, which was accomplished by dialysis against 50 % polyethylene glycol in PBS or sucking out the solvent through a collodium tube (Sartorius).

Density gradient centrifugation. All reconstitution products were characterized by this method. 200 μl samples were applied to the top of a 10 to 50 % continuous sucrose gradient and centrifuged in a SW 50.1 Beckmann rotor for a period of 16 hr at 150 000 g, 10 °C. The gradients were fractionated and measured for ^{14}C and ^{125}I.

Electron microscopy. HA-liposome samples were adsorbed on carbon coated films and stained with 2 % phosphotungstic acid, pH 6.5, for 3 min.

Degradation of HA-liposomes by pronase. According to Hoyle and Almeida (Hoyle *et al.*, 1971) pronase E (SERVA) in a final concentration of 10 % (w/w) was added to samples of HA-liposomes (Free of unbound HA), incubated at 37 °C for at least 48 hr and fractionated on a Sepharose 4 B column (8 x 50 cm).

Immunodiffusion-, haemolysis- and fusion assays.

The single radial immunodiffusion test (SRD) was performed as described by Schild (Schild *et al.*, 1975). The degree of haemolysis, induced by HA, was determined by incubating various amounts of HA-liposomes (containing 2...20 μg bound HA) with 0.5 % (v/v) human erythrocytes for 5 min at 0 °C, in a final volume of 1 ml at pH 5. The suspension was then incubated for 1 hr at 37 °C, centrifuged at 3000 rev/min for 5 min and an 200 μl aliquot was separated from the supernatant. 1 ml 0.5 % (g/g) NH_4OH was added and the absorbance measured at 410 nm. The value of 100 % haemolysis was determined by mixing up 200 μl erythrocytes with NH_4OH.

The fusion of HA-liposomes and unsealed human erythrocyte ghosts, prepared according to Dodge (Dodge *et al.*, 1963), at pH 5.0 was measured according to Pritzen and Herrmann (Pritzen *et al.*, 1988). Briefly, HA-liposomes were labelled with the amphiphilic fluorescence marker octadecyl rhodamin B chloride (R 18) as described by Hoekstra (Hoekstra *et al.*, 1984). 160 μl acetate buffer pH 5.0 were mixed with 20 μl ghost suspension and 20 μl of fluorescence labelled liposomes, containing 430 μg bound HA/ml, and kept at 0 °C for 5 min. Then 1.8 ml warmed buffer (37 °C) were added, and the fluorescence intenssity monitored continuously. For quantification it was related to the maximum intensity as obtained by addition of 50 μl 20 % (g/v) Triton X 100.

Vaccination of mice. Groups of 8 mice (ICR) at least were injected twice in intervals of 28 days intraperitoneally with 0.5 ml antigen suspensions, containing 0.008...10 μg HA/ml. By 10 days after the second immunization, the mice were challenged intranasally with an infection dose of 1000 MID_{50}. 3 days later the humoral immune response of vaccinated mice was tested by the haemagglutination inhibition test (Mayr *et al.*, 1977). The significance of the titre differences was determined by the double t-test, at the $p < 0.05$ level (Storm, 1976).

Virus growth in hens eggs was tested after injection of 0.1 ml lung suspension (dilution 1:10) of challenged mice.

Results

Reconstitution experiments

The preparation of HA-liposomes from lipid/protein/detergent mixtures was followed quantitatively by density gradient centrifugation using radio-labelled protein- and lipid tracers. Depending on the special conditions applied, up to three fractions were observed, representing different ratios of lipid and protein.

Fig. 1 shows a typical centrifugation profile of a reconstitution product, prepared by dialysis of a PC/HA/Na-cholate mixture. Corresponding to the ratio of PC and HA, peak A represents protein only, peak E protein rich HA-li-

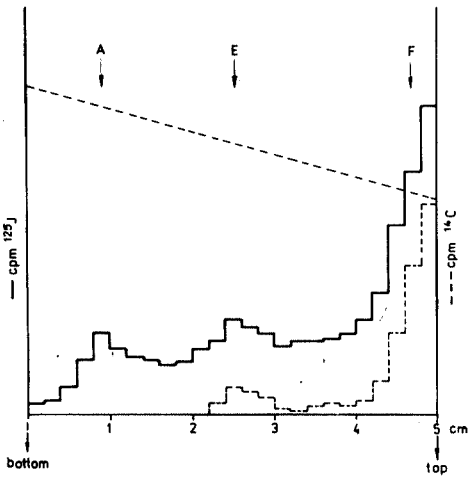

Fig. 1
Density gradient centrifugation of a HA-liposome preparation, obtained by dialysis of a PC/HA/Na-cholate mixture (10 %... 50 % sucrose gradient; 16 hrs; 150 000 g; cpm ^{125}I:^{125}I-HA/HA, cpm ^{14}C: ^{14}C-PC/PC; peak A: unbound HA; peak E: protein rich HA-liposomes; peak F: protein poor HA-liposomes)

posomes and peak F lipid rich HA-liposomes. Peak A was detected always in reconstitution products made of detergent free stored HA. In principle, neither the composition of the lipid/protein/detergent starting mixture, nor the time of equilibration of this mixture, nor the rate of detergent removal, had any influence on the proportion of protein insertion into liposomes (for detail information see under Materials and Methods).

If HA was stored in detergent solution, e.g. in 23 mmol/l Na-cholate or 10 mmol/l CTAB, it could be inserted quantitatively even after a storage period of one year. Table 1 lists a choice of HA incorporation yields for different starting

Table 1. Dependence on the HA storage medium of the quantity of protein insertion into liposomes

HA-storage medium	Starting mixture ratio PC/HA [mol/mol]	Reconstitution product HA^1_{free} [%]	Reconstitution product HA^2_{bound} [%]
PBS	150	19	52
PBS/Na-cholate	150	-	72
PBS	300	22	54
PBS/Na-cholate	300	-	83
PBS	3000	19	59
PBS/Na-cholate	3000	-	91

1: amount of peak A, Fig. 1
2: amount of peak E and peak F, Fig. 1
(reconstitution mixture: PC = 0.66...2.62 μmol/ml, HA = 0.06...6.2 nmol/ml, Na-cholate = 10 μmol/l PC)

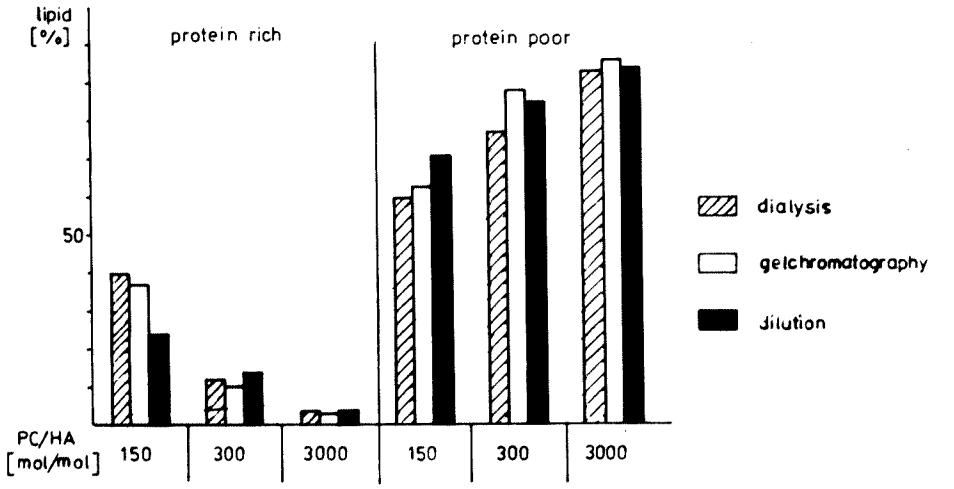

Fig. 2
Influence of the molar ratio lipid/protein and the rate of detergent removal, respectively, on the formation of protein rich- and protein poor HA-liposomes from PC/HA/Na-cholate mixtures

mixtures. The quantities of protein rich and lipid rich vesicles, respectively, depend on the molar-ratio of lipid and protein in the starting mixture. A ratio corresponding to that of the influenza virus provides protein rich vesicles predominantly, whereas an excess of lipid favours the formation of lipid rich vesicles. However, also under the last mentioned conditions a small amount of protein rich vesicles is formed, which could not be modified by rates of detergent removal (Fig. 2). These results were confirmed by electron microscopic investigations. The micrographs showed vesicles with protein spikes on their surfaces, representing HA-trimers. Their length and thickness corresponded exactly to the virus spikes.

In accordance with the interpretation of the centrifugation results the micrographs show protein rich and protein poor vesicles and sometimes HA rosettes (Fig. 3). The high importance of the state of HA-aggregation for the reconstitution efficiency was demonstrated once again by using isolated HA-fractions prepared by density gradient centrifugation of HA/detergent solutions: rosettes, parts of rosettes or single trimers. We found that HA-rosettes could not be incorporated into liposomes, whereas parts of HA-rosettes could. With the latter, only protein rich liposomes were formed, irrespective of the lipid to protein ratio of the starting mixture. Only single HA-trimers proved to be suitable for the formation of protein rich or protein poor liposomes, depending on the composition of the reconstitution mixture.

Protein orientation in HA-liposomes

The inside/outside distribution of HA in the bilayer of the vesicles may be important for their immunogenicity. Moreover, conclusions concerning the mechanism of protein incorporation into liposomes as well as the best reconstitution conditions could be drawn from that distribution. We determined the protein orientation of HA-liposomes by pronase cleavage of the outside portion of HA followed by gel chromatographic separation of the digested fragments.

A comparison of the experimental inside/outside ratios to the ratio that is to be expected for statistical inside/outside orientation, yielded a small asymmetry in favour of the outside surface (Table 2). Only very small sized vesicles were free of inside protein, presumably for the reason of the HA size. Furthermore, an incubation of preformed liposomes - containing sometimes cholesterol - with HA in the presence of octylglucoside provided to incorporation yields of 1 % or 12 %, after periods of 1 or 24 days, respectively. This is in a good agreement with results of Oxford and Trudel (Oxford *et al.*, 1981; Trudel *et al.*, 1981) but contradictory to the data of Thibodeau (Thibodeau *et al.*, 1981). The

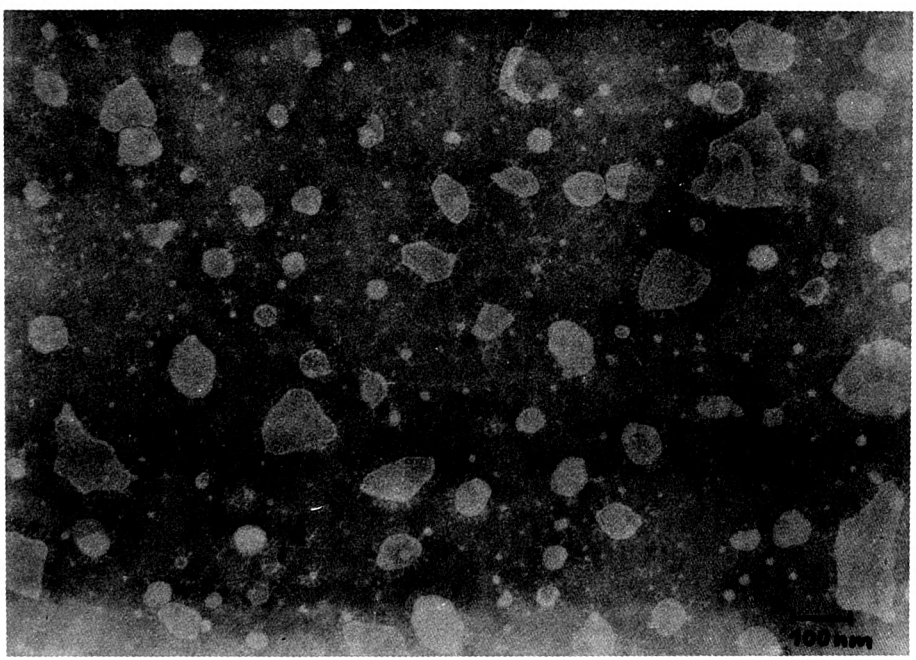

Fig. 3

Electron micrograph of HA-liposomes, prepared by dialysis of a PC/HA/Na-cholate mixture (negatively stained with phosphotungstic acid, pH 6.5; PC/HA of the reconstitution mixture: 300 [mol/mol])

Table 2. Inside/outside distribution of HA in liposomes

Ratio lipid/protein [mol/mol]	Vesicle diameter[1] [nm]	HA-distribution [%] experiment inside	experiment outside	calculation[2] inside	calculation[2] outside
50	26± 6	-	87±3	-	100
300	47±15	2	72±4	12	88
3000	86±23	11±3	75±6	26	73

1: determined by electron microscopy
2: for vesicle diameters of 25 nm, 50 nm and 80 nm, resp.; size of a HA-trimer protruding inside or outside: 13.5 nm lenghth, 14.5 nm thickness (Wilson *et al.*, 1981)

latter authors quote to have incorporated HA with high yield into preformed liposomes in the presence of octylglucoside applying slow detergent removal by gradient dialysis.

Functional properties of haemagglutinin

We were particularly interested in the reconstitution of functionally intact HA into liposomes. In this respect, contradictory data are reported in the literature using different detergents (Stegmann *et al.*, 1987*a*; Sizer *et al.*, 1987; Huang *et al.*, 1980; Lapidot *et al.*, 1987). For example, Stegmann and coworkers report on the loss of fusion activity of HA by application of Na-cholate, whereas the preparations of Sizer and coworkers, who used cholate too, maintained their fusogenic properties.

Our HA-liposomes, prepared by dialysis of a PC/HA/Na-cholate mixture, proved to be reactive in haemolytic and fusion experiments. According to Fig. 4, haemolysis of human erythrocytes by HA-liposomes as well as viruses increased sharply below pH 5.4, reaching an optimum at pH 5.0. The fusion activity was determined by a membrane mixing assay, monitoring the fusion of

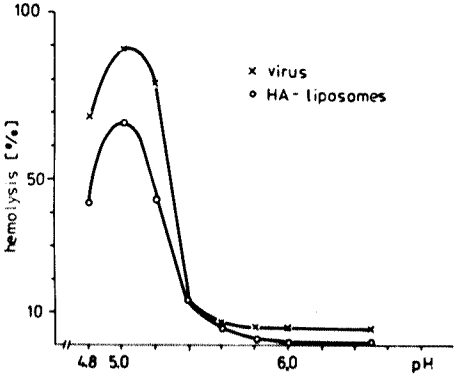

Fig. 4
pH dependence of the haemolytic activity of HA-liposomes or X-73 viruses

Table 3. Haemolytic and fusogenic activities of HA-liposomes

HA-liposomes[1] PC/HA [mol/mol]	Spike density	Haemolysis [%]	Fusion (max. fluorescence intensity) [%]
50	↑	99	35
150	increasing	53	20
3000	↓	40	no signal
virus particles[1]		100	39
'blank' liposomes		10	no signal

1: [HA] = const = 430 μg/ml

fluorescence labelled HA-liposomes or viruses with human erythrocyte ghosts by fluorescence dequenching (Fig. 5). The graphs obtained with both preparations were comparable. The haemolytic and fusogenic activities of HA-liposomes were raising with increasing spike density on the liposome surface (Table 3).

The fusion activity of influenza viruses is irreversibly lost by their preincuba-

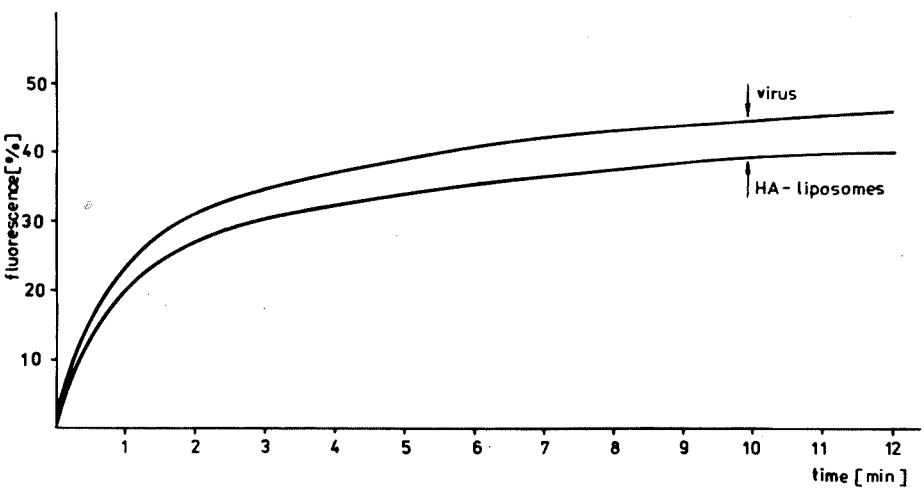

Fig. 5
Fusion of fluorescent labelled HA-liposomes or X-73 viruses with unsealed human erythrocyte ghosts at pH 4.8

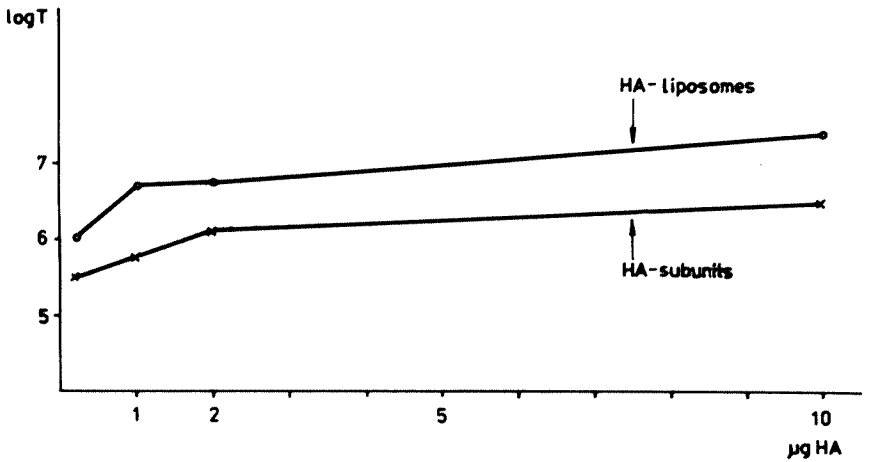

Fig. 6
Comparison of humoral antibody levels to HA induced by HA-liposomes or HA-subunits (double immunization of mice; ^{2}log T: haemagglutination inhibition titre)

tion at pH 5.0 and 37 °C in the absence of the target membrane (Stegmann *et al.*, 1987*b*). Alternatively viruses as well as HA-liposomes, preincubated without ghost membranes, or with dithiothreitol, or with nonlytic concentrations of Na-cholate, exhibited a drastic decrease of the fluorescence intensity. This shows that the observed increase of fluorescence intensity was the result of the protein controlled fusion between HA-liposomes and ghost membranes.

Immunogenicity of HA-liposomes

Another specific property of HA is its antigenicity. As determined by radial immunodiffusion it differed only within the margin of error of the test for both, HA before and after incorporation into liposomes. Groups of mice were immunized twice with same doses of HA-liposomes and HA-subunits, respectively. The humoral antibody titres induced after injection of 1 to 10 μg HA per mouse differed significantly for HA-liposomes and HA-subunits. Independent of the antigen dose injected in this range, higher responses by one ^{2}log T step approximately were induced by HA-liposomes throughout (Fig. 6).

After immunization with an antigen dose lower than 1 μg, however, the antibody titres for HA-liposomes and HA-subunits did not differ significantly. Moreower, it is remarkable, that lowering the antigen dose by factor 1000, approximately results in a decrease of the antibody titre only by one ^{2}log step (Table 4). In addition to the determination of humoral antibodies, the efficacy of various antigen suspensions was checked by estimating the virus concentration in the lungs of the test animals after the high dose challenge infection (1000 MID_{50}). Hence it followed, that an antigen dose as low as 8 ng HA per mouse, about one thousandth of the usually applied dose, is sufficient to

Table 4. Humoral antibody titres of mice after double immunization with HA-liposomes and HA-subunits, respectively

Series	Antigen dose [μg]	HA-liposomes* ^{2}log T**	HA-subunits ^{2}log T**	Significance $p < 0.05$
A	10	7.38±1.06	6.50±1.31	+
	2	6.75±1.49	6.12±1.25	+
B	1	6.70±0.82	5.75±0.71	+
	0.2	6.00±1.41	5.50±1.19	-
C	0.04	6.89±0.77	6.67±1.48	-
	0.008	6.56±1.57	6.75±1.04	-

* molar ratio PC/HA: 3000 mol/mol
** groups of ≧ 8 mice; ^{2}log T: haemagglutination inhibition titre

induce a complete protection against influenza infection irrespective of the HA preparation with or without lipid.The lipid composition, affecting the surface charge and fluidity of the vesicles, their size and the kind of association between antigen and liposome may be critical in stimulating an optimal immune response (Shek, 1984).

Therefore, we prepared antigen liposomes with different properties, hoping to potentiate the antibody formation obtained by egg yolk PC/HA-liposomes. However, modification of the surface charge and of the fluidity of the vesicles, using mixtures of PC/PA and PC/Chol, respectively, or DPPC, remained without any effect (Fig. 7). Another attempt to enhance the immune response against HA was to incorporate the immunomodulating agent LPS into HA-liposomes. LPS is known to increase both, the humoral and the cell mediated response to antigens (Warren *et al.*, 1986). As demonstrated in Fig. 7 only a small increase of the humoral antibody titre could be observed for HA-liposomes containing as much as 10 % (w/w) LPS. Application of lower LPS quantities remained without effect.

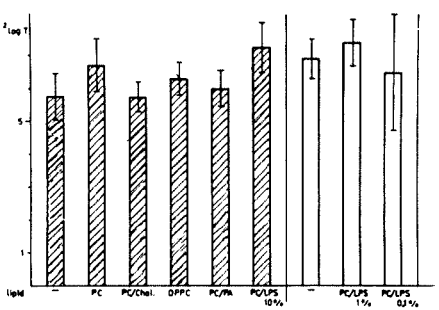

Fig. 7
Influence of the lipid matrix of HA-liposomes on the antibody formation in mice (double immunization; 8 mice per group; dose: 1 μg HA per mouse; ^{2}log T: haemagglutination inhibition titre)

Table 5. Humoral antibody titres of mice to HA-liposomes of different antigen surface density

HA-liposomes PC/HA [mol/mol]	Protein surface density	^{2}log T*
30	decreasing	6.00±2.07
300		6.00±1.51
3000		6.42±1.62

* haemagglutination inhibition titre; groups of 8 mice; double immunization; dose: 1 μg per mouse

A further series of tests should demonstrate the importance for the immunogenicity of the kind of association of HA to liposomes. As described above, HA anchored in the lipid bilayer of vesicles, thus mimicking virus particles, induces a stronger immune response compared with the isolated antigen. However, no influence of the protein density on the surface of HA-liposomes could be detected (Table 5). On the other hand, HA entrapped in the inner volume of liposomes, as well as mixtures of HA and empty liposomes are equally or less efficient than HA-subunits in promoting an immune response (Fig. 8).

Discussion

The results of the reconstitution experiments indicate that the quantity of HA incorporated into liposomes as well as the special variety of HA-liposomes formed is a function of the protein aggregation state in the starting mixture. If

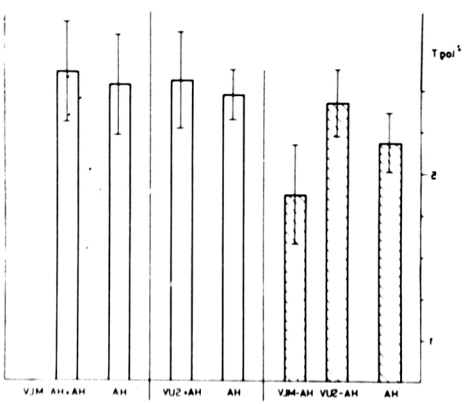

Fig. 8
Humoral antibody titres of mice after immunization with various types of associates of liposomes and HA (double immunization; groups of 8 mice; dose: 1 μg HA per mouse; ^{2}log T: haemagglutination inhibition titre; SUV: monolayer liposomes; MLV: multilayer liposomes; HA-SUV: HA-liposomes mimicking virus particles; HA-MLV: HA-liposomes with entrapped antigen)

HA is stored in detergent solution after its isolation, it can be inserted quantitatively into liposomes even following a storage period of one year at 4 °C. Depending on the state of HA aggregation and on the molar ratio of lipid and HA in the starting mixture, protein rich and lipid rich vesicles are formed in different quantities.

The aggregation state of protein and the degree of their dissociation, respectively, seems to be a key factor for the reconstitution of protein into liposomes (Helenius *et al.*, 1981; Mimms *et al.*, 1981). As recently reported, HA aggregates of high kinetic stability, differing in the number of HA trimer, coexist in protein preparations irrespective of the presence or absence of the detergent. The portion of such different aggregates as well as their size strongly depends on the storage medium of HA (Stahn *et al.*, 1991). As a result of our reconstitution experiments, using isolated HA fractions of different aggregation state, an at least partial dissociation of protein aggregates is necessary. Actually, HA rosettes, that are not dissociated by detergent, cannot be incorporated into liposomes. Only if at least a dissociation to parts of HA rosettes has taken place a liposomal incorporation can be observed. However, only protein rich liposomes were formed. If HA rosettes dissociate into single HA trimers, a formation of protein rich or protein poor vesicles, depending on the molar ratio of lipid and protein, takes place. These observations are believed to be of some general importance for the incorporation of membrane proteins like HA into liposomes: if proteins of unknown aggregation state are used, the prefractionation may be helpful in order to prepare special types of protein liposomes.

Electron microscopic investigations confirmed the results of HA reconstitution into liposomes. Additionally the micrographs show, that the way of HA incorporation into the lipid bilayer largely resembles that in the virus. Concerning the mechanism of protein insertion into liposomes two ways are discussed: (1) The protein is directly involved in the formation process of the vesicle bilayer, resulting in statistical inside/outside orientation of the protein; (2) Liposomes are formed first, followed by the protein incorporation which is mediated by detergent. The latter should result in the formation of vesicles exclusively with outside orientation of the protein (Eyton, 1982).

According to our results, regarding the inside/outside distribution of HA in the vesicle bilayer as well as the small incorporation yield of HA observed with preformed liposomes (Table 2), mechanism (1) seems to be operative for HA reconstitution. However, one reason for the preferential outside orientation of HA in liposomes could be the local dimension and shape of the protein molecule. For example, an increased outside orientation was observed for the reconstituted funnel shaped acetyl choline receptor too (Jones *et al.*, 1987).

The results of haemolysis, fusion and immunodiffusion experiments confirm the functional reconstitution of HA into liposomes and the preservation of specific HA properties during the reconstitution procedure. It should be emphasized, however, that the haemolytic and fusogenic activities observed do

not prove that all HA molecules incorporated into liposomes have maintained their functional properties. But many of them have indeed, as may be concluded from the comparison of quantitative data to virus activities (Table 3). Some few experiments pointed to a decreased haemolytic and fusogenic activity of HA-liposomes, if the detergent is incompletely removed from the preparation. This could be the reason for the contradictory results of some authors for the functional reconstitution of HA by the detergent method (Stegmann *et al.*, 1987*a*; Sizer *et al.*, 1987).

A small immunoadjuvant activity of liposomes for HA in mice could be observed at antigen doses from 1 to 10 μg, if HA was incorporated into the lipid bilayer of the vesicles. Injections of the same doses of HA together with empty liposomes were less effective and it appears to be true also for HA, that is included into the inner volume of liposomes, as realised with multilamellar vesicles. However, for HA doses below 1 μg no difference between humoral antibody titres raised to different antigen presentations were found (Table 5, Fig. 8).

This result is surprising, but there may be a correlation between this effect and the very low dose of carrier liposomes in the low antigen dose range because the ratio of antigen/lipid was held constant throughout. Apparently, it makes no difference whether an antigen is administered free or in the presence of adjuvant, if the amount of adjuvant is below a certain limit. But of course, observations like this may be become evident only if the immunogenicity of the antigen is fairly high. It is remarkable, furthermore, that an antigen dose as low as 8 ng is sufficient to induce a whole protection against an influenza infection in mice, irrespective of the type of antigen preparation administered.

Though HA-liposomes preparations were definetely free of unbound antigen and preparations with different physicochemical properties were used, the humoral antibody titres detected did not increase beyond those already observed by other authors (Oxford *et al.*, 1981; Thibodeau *et al.*, 1981; Boudreault *et al.*, 1985; Tan *et al.*, 1989; ElGuink *et al.*, 1989). It can be concluded that liposomes did not prove suitable for the effective action of the influenza subunit vaccine.

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