Interactions between cationic liposomes and an antigenic protein: the physical chemistry of the immunoadjuvant action

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Abstract The 18 kDa antigenic protein from *Mycobacterium leprae* (P) or its N-acyl derivative (AP) was incorporated in dioctadecyldimethylammonium bromide (DODAB) liposomes in water or in phosphate-buffered saline (PBS). In water, 100% P incorporation in liposomes contrasts with 65% in PBS. There is 75–80% AP incorporation to liposomes in water against 55–65% in PBS, showing that attachment of hydrophobic residues to the protein, instead of increasing, further decreases incorporation to the liposomes. From protein adsorption on latex, P affinity is larger than AP affinity for the latex surface whereas limiting adsorption for AP is much larger than that obtained for P, possibly due to AP aggregation in solution. P-induced rupture of liposomes containing [^{14}C]sucrose was evaluated from dialysis of protein/liposomes mixtures. In water, P incorporation to the liposomes causes leakage of radioactive contents contrasting with the absence of leakage for P incorporation in PBS. Immunization tests for delayed type hypersensitivity indicate a enhancement of cell-mediated immunological response towards P/DODAB complexes that is not obtained for the isolated protein. Absence of leakage for P in PBS is associated with a P "lying-over" on the liposome and optimization of protein presentation to the immunological system.—**Tsuruta, L. R., W. Quintilio, M. H. B, Costa, and A. M. Carmona-Ribeiro.** Interactions between cationic liposomes and an antigenic protein: the physical chemistry of the immunoadjuvant action. *J. Lipid Res.* 1997. **38:** 2003–2011.

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Synthetic quaternary ammonium compounds that form liposomes and vesicles in aqueous medium such as dioctadecyldimethylammonium chloride (DODAC) or bromide (DODAB) (1) have outstanding properties as antigen-specific immunostimulators (2). In general, DODAB has proven to be effective in inducing humoral antibodies, resistance to challenge with virulent viruses, and delayed-type hypersensitivity (a marker for cell mediated-immunity) (3–5). Induction of delayed type hypersensitivity (DH) against proteins with extreme differences in isoelectric points was demonstrated in mice (2). Nevertheless, neither physico-chemical aspects of the antigen/liposome interaction nor physiological mechanism(s) by which DODAB increases the production of DH are well understood, all the past evidence seeming to support the hypothesis that DODAB binds electrostatically to oppositely charged antigens in general and, as an antigen carrier particle, promotes the localization of antigen in the paracortex of the lymph nodes thereby leading to the cell-mediated immunoresponse characteristics of DH (6, 7).

On the other hand, conserved protein families of pronounced immunogenicity are those of the mycobacteria heat shock proteins including hsp-18, the 18 kDa heat shock protein with an isoelectric point equal to 5.5 (8, 9). These proteins are considered as important targets of the immune response to mycobacteria and, as such, relevant to subunit vaccine design. Peripheral blood mononuclear cells and T-cell lines from *Mycobacterium leprae*-vaccinated subjects proliferated in response to the 18-kDa HSP of *M. leprae*, P (10). Recently, overexpression and scaling-up of P production in *Saccharomyces cerevisae* was achieved (11, 12).

In this work, P presentation using DODAB liposomes as effective inducers of DH towards P is described after characterizing physico-chemical aspects of the protein/liposome interaction.

Abbreviations: P, 18 kDa heat shock protein from *Mycobacterium leprae;* AP, N-acyl derivative of P; DODAB, dioctadecyldimethylammonium bromide; DODAC, dioctadecyldimethylammonium chloride; PBS, phosphate-buffered saline; DOC, sodium deoxycholate; DH, delayed type hypersensitivity.

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MATERIALS AND METHODS

Chemicals

Dioctadecyldimethylammonium bromide 99.9% pure (DODAB) was obtained from Fluka Chemie AG (Switzerland) and used as such without further purification. All other reagents were analytical grade and were used without further purification. Water was Milli-Q quality.

Liposomes preparation

A DODAB dispersion containing 1–5 mg/mL DODAB was prepared by heating the DODAB powder in water or in phosphate-buffered saline (PBS) for 30 min at 56°C as previously described (2). Analytical DODAB concentration was determined by microtitration (13).

Recombinant hsp-18 kDa (P) and its N-acyl-derivative (AP)

Overexpression of the P antigen in *S. cerevisiae* (11) and scaling-up of this procedure (12) were previously described, allowing us to obtain a pure protein, as determined from Western blot (12). N-acyl P was obtained from N-hydroxysuccinimide ester of palmitic acid following a procedure previously described to incorporate antibodies in liposomes (reference 14, p. 84). Fatty acid chains were attached to P via amide linkages to various sites on the protein so that the hydrophobicity of the P molecule was increased. Typically, 20 µg of N-hydroxysuccinimidyl–palmitic acid ester was dissolved in chloroform, dried under nitrogen, and 0.5 mL sodium deoxycholate (DOC) 4% in PBS or in water was added and the mixture was gently shaken to dissolve the ester. Thereafter, ca. 10 mg P in 0.5 mL water or PBS was added to the previous mixture and incubated overnight in a rotating mixer at 37°C (14). Without a subsequent dialysis step to remove the bile salt, the mixture containing the N-acyl derivative of P will also contain a micellar solution of the bile salt that is responsible for solubilizing AP.

P and AP quantitative determination

There is one tryptophan and one tyrosine residue in the primary structure of P (15) so that the Lowry method or determination of absorbances at 280 nm does not allow enough analytical sensitivity for quantitative P analysis. However, P presents an absorption maximum at 230 nm and there is a linear relationship between absorbance at 230 nm and P amount in aqueous solution (0–0.5 mg/mL) that covers the 0 to 1 range of absorbances (not shown). Thus, in order to quantify P (or AP) incorporation in DODAB liposomes, P (or AP) was determined from absorbance at 230 nm. One should notice that sodium deoxycholate has an absorption maximum at 200 nm that does not interfere with determination of absorbance at 230 nm for AP analysis.

Determination of P or AP incorporation in DODAB liposomes

Two different procedures were used to promote the interaction protein/liposome: either the protein was added to the liposomes dispersion previously prepared or the liposomes were prepared in the protein aqueous solution. Thereafter, the mixture was incubated (1 h, 40°C) and centrifuged (15,800 *g* for 1 h at 4°C) to separate liposomes from free protein in solution. The supernatant was filtered through polycarbonate membranes (0.4 µm cut-off). Liposomes retention by the filtering membranes was evaluated from determination of turbidity at 400 nm in the filtered solution. Protein incorporation into liposomes was obtained by determining absorbance at 230 nm in the filtered solutions where liposomes were completely absent.

Determination of P or AP adsorption onto sulfate polystyrene microspheres

P (0.5 mL) or AP solutions (0–1 mg/mL) and 0.5 mL of a 10^{12} particles/mL dispersion of sulfate polystyrene microspheres with 249 nm mean external diameter (Interfacial Dynamics Corporation, Portland, OR) were incubated at 40°C for 1 h. Thereafter, microspheres were separated from free protein by centrifugation (15,800 *g* for 1 h at 4°C) and protein concentration was determined in the supernatant. Protein adsorption on the microspheres was expressed as mg adsorbed protein per m^2 polystyrene. Total surface area on the particles was calculated from the specific surface area and the particle number density. Adsorption isotherms were linearized using the Langmuir model in order to obtain an affinity constant K for the protein adsorption on the hydrophobic surface and the limiting adsorption $(x/m)_{max}$ (16).

Determination of liposome rupture upon interaction with proteins

Entrapment efficiency for DODAB liposomes was obtained from dialysis and radioactive labeling of the intraliposomal aqueous compartment (17). Equal volumes of liposomes prepared in water or in PBS solutions both containing [^{14}C]sucrose (LS) and a dialysis control of water containing [^{14}C]sucrose (S) were dialyzed in two separate bags against 2 L of water or PBS (changed three times), respectively, overnight with vigorous stirring. Before dialysis, aliquots of LS and S were reserved for the determination of [^{14}C]sucrose entrapment efficiency of the liposomes (ENT). After dialysis, the radioactivity, in counts per minute (cpm), was

determined for the two dialyzates and for the reserved aliquots. Entrapment can be taken as (17):

$$ENT = (1/C)(cpm_2/cpm_1 - cpm_{2c}/cpm_{1c})$$

where 1 and 2 subscripts refer to counts of LS before and after dialysis, respectively; 1c and 2c subscripts are counts of S before and after dialysis, respectively; and C is the molar DODAB or DODAC concentration. Thus, ENT is expressed in M^{-1}.

Protein-induced vesicle disruption was evaluated using two different assays. *1*) The LS dialyzate containing a total radioactivity equal to cpm_{total} was added to P or AP inside hemichamber a of an equilibrium dialysis chamber and leakage was estimated from total radioactivity in hemichamber b (cpm_b) once equilibrium was attained between a and b compartments, which were separated by a cellulose dialysis membrane. *2*) The LS dialyzate was added to P or AP and the mixture was placed inside a dialysis bag, submitted to 6 h dialysis against water or PBS (2 L, 3×), radioactivity being counted before (cpm_1) and after the dialysis procedure (cpm_2).

The percentiles of liposome rupture from the equilibrium and the conventional dialysis procedures described above were calculated respectively as:

$$\%R_e = 100\ [cpm_b/(cpm_{total}/2)]$$
$$\%R_d = 100\ [(cpm_1 - cpm_2)/cpm_1]$$

Animals and immunization

BALB/c female mice were obtained from the animal facilities in the Department of Biochemistry, Institute of Chemistry, University of S. Paulo, S. Paulo, SP, Brazil and were used at an age of about 10 weeks. For each experiment, groups containing six to eight mice were used. Fifteen μg P dissolved in 0.1 mL saline (PBS) or in 0.1 mL of a 1 mg/mL DODAB liposomes dispersed in PBS was injected intracutaneously (i.c.) in the abdomen, at two separate sites, so that the total immunological priming dose was 30 μg P. As a control, a group of animals was injected with the DODAB dispersion alone. In this case, 0.1 mL of the same 1 mg/mL DODAB dispersion in PBS was administered at two separate sites yielding a total dose of 0.2 mg.

Assay for delayed-type hypersensitivity (DH)

The footpad swelling test was carried out essentially as described previously (18, 19). On the fifth day post-immunization, mice pretreated with P alone, P + DODAB, or DODAB alone (see the subsection above) were challenged in the left-hind footpad with a total elicitation dose of 3 or 30 μg P in 0.05 mL PBS. Footpad swelling was measured 24 h later with a Mitutoyo engineering micrometer. Depending on the age of the animals, the thickness of uninjected hind footpads varied from 1.60 to 1.70 mm. Percentage footpad swelling (%fs) is calculated according to the formula below with results expressed as %fs ± mean standard deviation for six to eight mice.

$$\%fs = \frac{100[(\text{left hind footpad thickness}) - (\text{right hind footpad thickness})]}{(\text{mean thickness uninfected left hind footpads})}.$$

RESULTS AND DISCUSSION

Incorporation of an antigenic protein in DODAB liposomes

In order to detect the occurrence of the protein/liposome interaction, its incorporation in the liposomes was measured over a range of experimental conditions, namely, for the interaction in water or in PBS both for P and its N-acyl derivative AP (**Table 1**). This was done for three different experiments. In A, protein was added to a previously prepared DODAB dispersion. In B, liposomes were prepared in a protein solution. In C, DOC (used to acylate P and obtain AP) was removed by dialysis of the liposome/AP/DOC mixture. Table 1 shows protein incorporation measured in water (94–100%) against incorporation in PBS (67–74%). The larger figures for incorporation in water suggest that a substantial contribution of the electrostatic attraction between protein and liposome that drives incorporation in water is absent in PBS. One should recall that the isoelectric point for P is 5.5 so that it is negatively charged both in water (pH 6.4) and in PBS (pH 7.4). However, in PBS, the high ionic strength screens the electrostatic attraction between P and DODAB although, in PBS, hydrophobically driven incorporation is still very large: 67–74%.

The comparison between experiments A and B allows us to establish to which extent entrapment of the hydrosoluble protein in the internal aqueous compartment of the liposome is important. Incorporation obtained when liposomes are prepared in the protein solution (experiment B) is practically equal to that obtained by adding protein to previously prepared liposomes (experiment A), showing that once presented to the liposome, the protein definitely prefers to interact with its bilayer membrane and is not trapped by the internal aqueous compartment of the liposome. The case of the N-acyl protein AP is special because of its preparation at 2% (46 mM) DOC, i.e., in the presence of DOC micelles. As to the liposomes themselves, DOC also acts as

TABLE 1. Protein incorporation (INC %) in DODAB liposomes as a function of the medium, absence (P) or occurrence of acylation in the protein (AP), and experiment type (A,B, or C)

	INC %					
	In Water			In PBS		
Protein	A	B	C	A	B	C
P	94	100	—	67	74	—
AP	82	87	88	56	63	79

Protein/liposome mixtures contained 0.45 mg protein and 2.6 mg DODAB in 1.5 mL water or PBS. Final DODAB concentration in the mixtures was 2.6 mM. Final DOC concentration for mixtures containing AP was 1.0 mM. In A, protein was added to the previously prepared liposomes. In B, liposomes were prepared in a protein solution. In C, AP was added to the previously prepared DODAB dispersion and the mixture was dialyzed to eliminate DOC.

a solubilizer for the protein. AP incorporation is smaller than P incorporation (Table 1). The final DOC concentration in the DODAB/AP/DOC mixture is about 1.0 mM against 2.6 mM DODAB. Possibly, at this submicellar DOC concentration, AP molecules aggregate with each other. This aggregation in solution would decrease the hydrophobic attraction between AP and the liposome. Thereby, AP results are, smaller than P incorporation (Table 1). When DOC is washed out from the mixture by dialysis in experiment C, in water, AP incorporation does not change but, in PBS, significantly increases as compared to figures for experiments A and B (Table 1). Possibly, AP aggregates in water do not include DOC molecules because both AP and DOC are negatively charged and the charges are not screened. DOC elimination by dialysis would not affect AP affinity for the liposome. On the other hand, in PBS, charge neutralization on DOC and AP would allow DOC inclusion and stabilization of AP aggregates. This could reduce aggregate affinity for the liposome bilayer (recall that charges on the bilayer and on the aggregate are screened in PBS). Upon dialysis and DOC removal, destabilization of AP aggregates would increase its transfer to the bilayer.

At a fixed DODAB amount in each mixture, P incorporation linearly increases as a function of added protein (**Fig. 1**). One should notice that DODAB concentration in the mixture is not a limiting factor towards protein adsorption. Excess DODAB in the mixtures cause the linear increase of adsorbed protein as a function of the total amount added. By increasing ionic strength, P incorporation decreases as depicted from the comparison between incorporation obtained in water (squares) and in PBS (circles).

Figure 2 shows a similar experiment for AP. One should recall that the protein acylation procedure is done in a medium that contains sodium deoxycholate at a concentration above its critical micellar concentration, namely 46 mM DOC. However, these micelles are absent in the AP/liposomes mixtures where DOC concentrations range from 0.31 up to 3.1 mM due to dilution of the original AP solution in the mixtures. DODAB concentration was fixed in the mixtures at 2.6 mM. Therefore, the DOC/DODAB ratio in each mixture in Fig. 2 varies from 0.13 (for the smallest AP mass added) up to 1.2 (for the largest AP mass added), meaning that ratios are low. At low DOC/lipid ratios DOC will almost completely partition into the liposome bilayer, DODAB/DOC micelles being practically absent and liposomes being retained by the filtering membrane. This validates the liposome retention assay used. If DODAB/DOC micelles were present, they would not be retained by the filtering membrane and AP incorporation measured would be underestimated.

An increase in protein hydrophobicity by attaching acyl residues to the protein backbone would apparently be expected to increase protein insertion in the liposomal bilayer membrane thereby increasing the amount of AP adsorbed onto the liposome. This was not observed (Table 1 and Figs. 1 and 2). On the contrary, AP adsorption measured was always smaller than P adsorption under similar experimental conditions. Possibly the reason for this unexpected behavior of the acylated protein is to be found in its aggregative properties in aqueous solution. Acylation might lead to formation of protein aggregates with a lower affinity for the bilayer. Aiming at a better investigation of this possibility, we measured P and AP affinity for a typically hydrophobic surface, namely, the surface of highly homodisperse polystyrene microspheres electrostatically stabilized by sulfate functional groups.

P and AP adsorption isotherms on sulfate polystyrene microspheres in water are shown in **Fig. 3.** The linearization of these Langmuirian curves allows us to determine P or AP affinity for the surface (K) and the respective adsorption maxima, $(x/m)_{max}$. The inserts in Fig. 3 show the linearized curves. **Table 2** shows K and $(x/m)_{max}$ for both proteins. In fact, AP has a lower affinity than the affinity exhibited by P for the latex. However, the adsorption maximum for AP is more than twice the

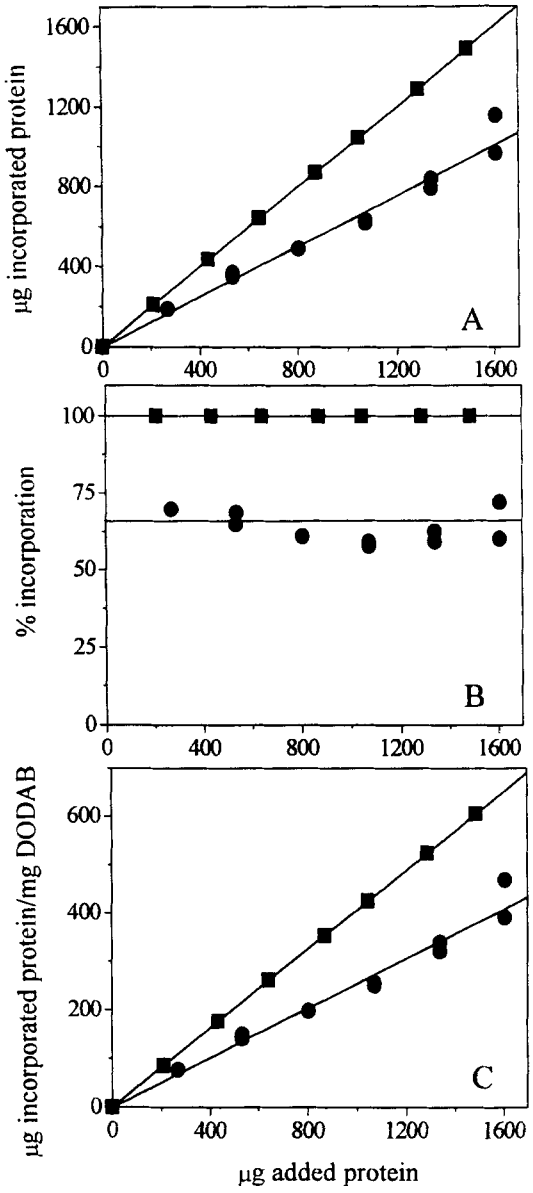

Fig. 1. P incorporation as a function of P mass (µg) added to the DODAB liposome/protein mixture in water (■) or in PBS (●). There is 2.47 mg DODAB per 1.5 mL of total volume in each mixture (2.6 mM DODAB). For these experiments, P was added to previously prepared DODAB liposomes.

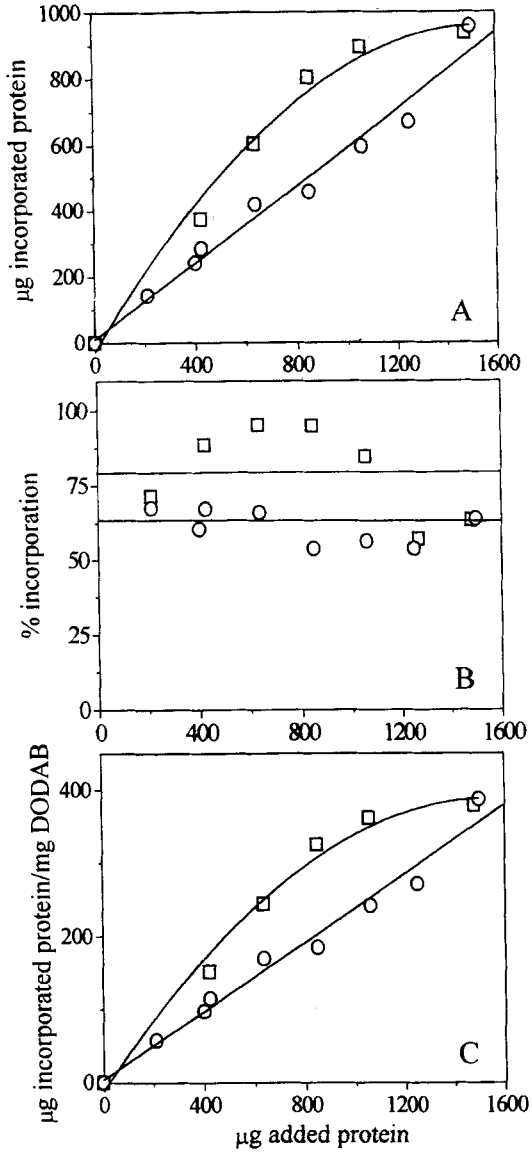

Fig. 2. AP incorporation as a function of AP mass (µg) added to the DODAB liposome/protein mixture in water (□) or in PBS (○). There is 2.47 mg DODAB per 1.5 mL of total volume in each mixture (2.6 mM DODAB). DOC final concentration increases as a function of total AP mass in the mixtures (from 0.61 up to 3.1 mM DOC). For these experiments, AP was added to previously prepared DODAB liposomes.

one obtained for P. This confirms AP aggregation not only in solution but also at the latex surface, supporting the possibility that acylation can indeed decrease protein affinity for an hydrophobic surface or for the hydrophobic region of a bilayer membrane in a liposome. At this point, the interaction DOC/sulfate latex has to be addressed. Although a hydrophobically driven DOC adsorption onto the latex is possible, there is an electrostatic repulsion between DOC and the negatively charged latex (the experiment was performed in water) acting against DOC adsorption. From previous work with negatively charged monosialoganglioside (GM1) and oppositely charged latex, there is no adsorption of such an hydrophilic biomolecule onto hydrophobic latex surface latex in spite of the electrostatic attraction between GM1 and latex and in spite of GM1 ceramide moiety, which is hydrophobic (20, 21). From these considerations we would not expect DOC binding to the latex.

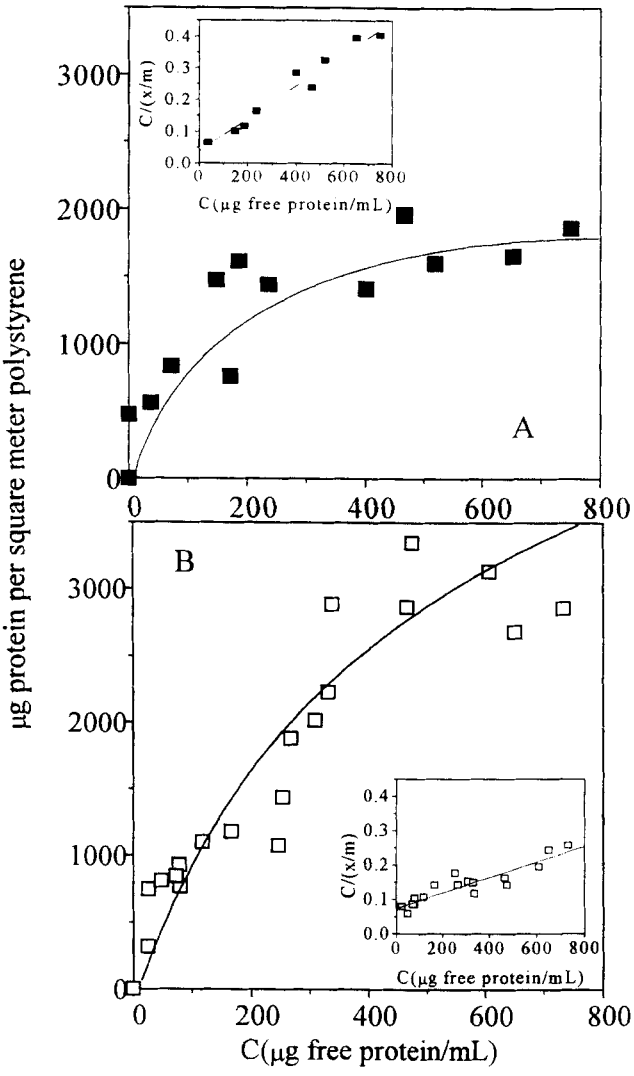

Fig. 3. P (■) and AP (□) adsorption onto sulfate polystyrene microspheres in water at 0.5×10^{12} particles. The inserts show linearizations obtained assuming the Langmuir model for protein adsorption onto latex. From the intercept and the slope it is possible to obtain affinity constants and maximal adsorption values for both proteins as presented in Table 2.

TABLE 2. Affinity constant (K) and maximal adsorption [$(x/m)_{max}$] for P and AP adsorption onto polystyrene microspheres in water as obtained from the linearized adsorption isotherms shown in Fig. 3

Protein	K	$(x/m)_{max}$
	10^{-2} L/mg	mg/m^2
P	1.264	1.947
AP	0.297	4.484

Mode of protein adsorption onto DODAB liposomes

Protein insertion in model bilayer membranes can often cause leakage of intravesicular contents. This leakage would be associated with an apparent liposome rupture. Data on the effect of P or AP in water or in PBS on apparent liposome rupture are shown in **Table 3.** DODAB liposomes, besides containing radioactively labeled sucrose in their internal aqueous compartment, also adsorb this sugar at the bilayer surface (22). Thereby, upon increasing the total DODAB bilayer surface offered to radioactive sucrose, there is a corresponding overestimation of the entrapment efficiency related to the increase in the total surface area available for sucrose adsorption (**Table 4**). Fortuitously, this sucrose adsorption on the DODAB liposome was useful to discriminate between protein insertion or lying-over modes for interaction with the bilayer.

Apparent liposome rupture calculated from dialysis (%R_d) or equilibrium dialysis experiments (%R_e) are shown in Table 3. Upon dialysis, there is an apparent rupture of DODAB liposomes alone, probably due to desorption of the radioactive sucrose that was attached to the external liposome surface. There was no significant difference for sucrose desorption for liposomes in water or in PBS. As expected, by increasing DODAB amount in the dispersion, the apparent liposome rupture increases (Table 3). The interaction between DODAB liposomes and the 18 kDa protein in water significantly increased the apparent liposome rupture for both types of dialysis. However, in PBS, the protein decreased the apparent liposome rupture. This unexpected result can be understood from a simple assumption. In PBS, the protein somehow hampered sucrose desorption from the liposome external surface. This would suggest, that, in PBS, a certain proportion of the protein adsorbed is lying over the liposomal external surface. Thereby, adsorbed sucrose molecules would be "sandwiched" between the external liposome surface and the "lying over" protein and, in this case, sucrose desorption would not occur. In PBS, liposome rupture due to insertion was absent so that P insertion did not occur. This led us to another interesting conclusion: in water, the electrostatic attraction between liposome and protein does seem to be important to orient protein insertion. When electrostatic attraction is absent, so is P insertion or, at least, so is a complete insertion capable of causing leakage of internal contents of the liposome.

It was shown previously that DODAC vesicles do not leak sucrose so that this solute is useful to measure the entrapment efficiency (ENT) of the vesicles population (1, 22). Sucrose retention at two different DODAB concentrations for vesicles that were prepared in radioactively labeled sucrose are in Table 4. ENT, a property that should depend uniquely on vesicle size and lamel-

TABLE 3. Apparent liposome rupture (% R) calculated from leakage of radioactive sucrose from the liposomal internal compartment upon interaction with P or AP

Sample (1 mL)	% R_d Exp. 1/Water	% R_e Exp. 2/Water	% R_d Exp. 3/PBS
DODAB, 1.7 mg	15.5	—	20.4
DODAB, 2.5 mg	—	19.5	—
DODAB, 1.7 mg/P, 0.3 mg	37.8	79.2	4.1
DODAB, 1.7 mg/DOC, 0.5 mg	91.8	67.5	85.1
DODAB, 1.7 mg/AP, 0.3 mg/DOC, 0.5 mg	92.0	71.5	92.6

DOC is present in mixtures containing the derivatized protein AP. The raw data that allowed calculation of % R are in Table 4. DODAB final concentration is 2.7 mM. Mixtures with DOC have 1.1 mM DOC.

arity, doubles by doubling DODAB concentration (Table 4). The most probable interpretation is occurrence of sucrose adsorption which is indeed expected to increase with DODAB concentration. Quantitation of sucrose retention for sucrose that was added to preformed large DODAC vesicles was previously described (22) showing sucrose adsorption to DODAC vesicles. As DODAC differs from DODAB only by the chloride counterion, a certain analogy of behavior towards sucrose adsorption can be expected for both systems. Given the occurrence of sucrose adsorption, the protein "lying over" hypothesis not only reasonably explains the decrease in apparent liposome rupture (Table 3, experiment in PBS) but also explains the very large immunoadjuvant effect of DODAB liposomes in PBS regarding P presentation (see next section). In PBS, the P "lying over" position on the liposome facilitates recognition by an improvement in the response of the immunological system.

Regarding liposome lamelarity, although direct examination by electron microscopy for DODAB as prepared from the Snippe's formula is not available from the literature, the analogy with large unilamellar DODAC vesicles (22) allows one to expect unilamellarity for DODAB liposomes. A mean zeta–average diameter of 400 nm was determined for DODAB liposomes in water. Calculation of the entrapment efficiency for an unilamellar vesicle with 400 nm mean diameter yields a value fairly compatible with the experimental ENT measured, corroborating unilamellarity. Taking the mean molecular area for DODAB as 0.6 nm^2 (23) and assuming that DODAB liposomes are unilamellar, the calculated entrapment efficiency is 12 L/mol, a value slightly below the 16 L/mol obtained experimentally at 3.3 mg DODAB/mL (Table 4). Considering the overestimation in entrapment due to adsorption of radioactive sucrose, which tends to increase with the DODAB amount used in the experiment, one can conclude that the DODAB preparation in water contains unilamellar vesicles. DODAB dispersions in water or in PBS have about the same entrapment efficiency (Table 4). Thus, both preparations do offer a large total surface area to interact with proteins, drugs, DNA or other substances to be delivered in vivo.

TABLE 4. Entrapment efficiency (L/mol) for DODAB liposomes prepared in water or in PBS

Medium	[DODAB]	Entrapment Efficiency
	mg/mL	*L/mol*
Water	3.3	15.5 ± 5.7
Water	5.0	41.0 ± 0.6
PBS	3.3	8.2 ± 4.0

Liposomes were prepared in water or PBS as prescribed for their use as immunoadjuvants by Snippe and coworkers (5). Note the effect of DODAB concentration on the entrapment efficiency measured. Mean value was obtained from 3–5 independent experiments.

DODAB liposomes for induction of delayed type hypersensitivity towards the antigenic 18 kDa heat-shock protein of *Mycobacterium leprae*

The method for dispersing DODAB in PBS followed Snippe's recipe to use DODAB as an effective immunoadjuvant for presentation of virus, proteins and other antigens (2). In this section, DODAB liposomes are used for presentation of the 18-kDa antigenic protein of *Mycobacterium leprae*. P/DODAB-induced delayed-type hypersensitivity (DH) in experimental animals is evaluated using the footpad swelling test. In **Table 5,** the induction of DH by the P/DODAB complex is shown in comparison to the situation in which the inducer is P alone. Footpad swelling is about 10 times larger for P/DODAB than for P as inducer. Also DODAB by itself is allergenic, as seen in the Table 6 for DODAB alone used to sensitize and to elicit the footpad swelling response. DODAB is useful as an effective immunoadjuvant for presentation of the antigenic 18-kDa protein from *Mycobacterium leprae*.

It remains to be discussed how the results presented in sections 1 and 2 could clarify the immunoadjuvant effect of DODAB for P presentation. One should recall that DH was obtained for the P/DODAB complex in PBS (Table 6). In this condition, P is basically "lying over" or "partially inserted" in the DODAB bilayer. (P

TABLE 5. Effect of DODAB liposomes on percentile of footpad swelling induced by the antigenic P protein

Sensitization	Elicitation			
	P 3 μg	P 30 μg	DODAB 100 μg	P 30 μg + DODAB 100 μg
	footpad swelling ± MSD			
P, 30 μg	3.5 ± 0.4	24.7 ± 2.8	—	—
DODAB, 100 μg	0.4 ± 0.7	16.9 ± 4.9	71.5 ± 7.0	—
P, 30 μg + DODAB, 100 μg	55.6 ± 5.2	158.7 ± 18.4	—	129.8 ± 13.0

The P dose effect on the induction of delayed-type hypersensitivity is depicted by comparison between % footpad swelling at two different P doses, 3 μg and 30 μg; MSD, mean square deviation.

partial insertion is an insertion that does not cause leakage of internal contents of the liposome). Therefore, from the P/DODAB complex, there is an efficient exposure of P haptens to the immunological system. These results are of importance for further developments towards a subunit vaccine design against leprae.

CONCLUSIONS

The 18-kDa heat shock protein, P, from *Mycobacterium leprae* can be incorporated successfully in dioctadecyldimethylammonium bromide liposomes both in water or in PBS. In water, P incorporation is driven both by electrostatic and hydrophobic attraction between P and liposomes. These interactions lead to protein insertion in the bilayer with leakage of internal liposome contents. In PBS, the incorporation is driven by the hydrophobic interaction between liposome and protein, though deep protein insertion in the membrane does not occur as seen from the absence of leakage from the internal liposome contents. The bioassay for delayed-type hypersensitivity carried out for the P/liposome complex in PBS yielded a very large immunological response. Aggregation of the N-acyl derivative of P (AP) in water solution reduces affinity of AP for the liposome bilayer and yields lower amounts of adsorbed AP onto the liposome. A "lying-over" P position is suggested for the protein on the liposome that explains both the absence of leakage upon P incorporation to the liposome and the very effective P presentation to the immunological system caused by the DODAB liposomes in PBS.

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REFERENCES

1. Carmona-Ribeiro, A. M. 1992. Synthetic amphiphile vesicles. *Chem. Soc. Rev.* **21:** 209–214.
2. Katz, D., C. A Kraaijeveld, and H. Snippe. 1994. Synthetic lipoid compounds as antigen-specific immunostimulators for improving the efficacy of killed-virus vaccines. *In* The Theory and Practical Application of Adjuvants. D. E. S. Stewart-Tull, editor. John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapore. 37–50.
3. Gall, D. 1966. The adjuvant activity aliphatic nitrogenous bases. *Immunology*. **11:** 369–386.
4. Prager, M. D., M. C. Kanar, J. L. Farmer, and J. Vanderzee. 1985. Effect of dimethyl-dioctadecylammonium-bromide-induced macrophages on malignant cell proliferation. *Cancer Lett.* **27:** 225–235.
5. Snippe, H., J. M. N. Willers, J. K. Inman, and B. Merchant. 1980. The specificity of antibody formation in mice following immunization with hapten-carrier complexes mixed with the surfactant, dimethyl-dioctadecyl-ammonium-bromide. *Immunology*. **39:** 362–365.
6. Coon, J., and R. Hunter. 1973. Selective induction of delayed hypersensitivity by a lipid conjugated protein antigen which is localized in thymus dependent lymphoid tissue. *J. Immunol.* **110:** 183–190.
7. Dailey, M., and R. L. Hunter. 1974. The role of lipid in the induction of hapten-specific delayed hypersensitivity and contact sensitivity. *J. Immunol.* **112:** 1526–1534.
8. Doherty, T. M., R. J. Booth, S. G. Love, J. J. Gibson, D. R. K. Harding, and J. D. Watson. 1989. Characterization of an antibody-binding epitope from the 18-kDa protein on *Mycobacterium leprae*. *J. Immunol.* **142:** 1691–1695.
9. Harris, D. P., H-M. Vordermeier, S. J. Brett, G. Pasvol, C. Moreno, and J. Ivanyi. 1994. Epitope specificity and isoforms of the mycobacterial 19-kilodalton antigen. *Infect. Immun.* **62:** 2963–2972.
10. Mustafa, A. S., K. E. Lundin, and F. Oftung. 1993. Human T cells recognize mycobacterial heat shock proteins in the context of multiple HLA-DR molecules: studies with healthy subjects vaccinated with *Mycobacterium bovis* BCG and *Mycobacterium leprae*. *Infect. Immun.* **61:** 5294–5301.
11. Pinho, J. R. R., P. J. Barr, E. J. Vicente, and A. C. Schenberg. 1994. Expression of the 18 kDa protein of *Mycobacterium leprae* in *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **16:** 1241–1246.
12. Costa, M. H. C., C. M. P. M. Ueda, R. A. S. Sato, C. Liberman, and I. Raw. 1995. Procedures for scaling up the re-

combinant 18kDa-hsp lepra protein production. *Biotechnol. Tech.* **9:** 527–532.
13. Schales, O., and S. S. Schales. 1941. A simple and accurate method for the determination of chloride in biological fluids. *J. Biol. Chem.* **140:** 379–884.
14. New, R. R. C. 1994. Preparation of liposomes. *In* Liposomes: a Practical Approach. R. R. C. New, editor. IRL Press, Oxford. 84–85.
15. Booth, R. J., D. P. Harris, J. M. Love, and J. D. Watson. 1988. Antigenic proteins of *Mycobacterium leprae*. Complete sequence of the 18-kDa protein. *J. Immunol.* **140:** 597–601.
16. Jackson, S., M. D. Reboiras, I. G. Lyle, and M. Jones. 1986. Adsorption of phospholipid vesicles on solid surfaces. *Faraday Discuss. Chem. Soc.* **85:** 291–301.
17. Tsuruta, L. R., M. M. Lessa, and A. M. Carmona-Ribeiro. 1995. Interactions between dioctadecyldimethylammonium chloride or bromide vesicles in water. *Langmuir.* **11:** 2938–2943.
18. Snippe, H., L. Johannesen, J. K. Inman, and B. Merchant. 1978. Specificity of murine delayed-type hypersensitivity to conjugates of large or small haptens on protein carriers bearing lipid groups. *Immunology.* **34:** 947–954.
19. Smith, R. H., and B. Ziola. 1986. Cyclophosphamide and dimethyl dioctadecyl ammonium bromide immunopotentiate the delayed-type hypersensitivity response to inactivated enveloped viruses. *Immunology.* **58:** 245–250.
20. Sicchierolli, S. M., and A. M. Carmona-Ribeiro. 1995. Incorporation of the cholera toxin receptor in phospholipid-covered polystyrene microspheres. *Colloids Surf. B-Biointerfaces.* **5:** 57–61.
21. Sicchierolli, S. M., and A. M. Carmona-Ribeiro. 1996. Biomolecular recognition on phospholipid-covered polystyrene microspheres. *J. Phys. Chem.* **100:** 16771–16775.
22. Carmona-Ribeiro, A. M., and H. Chaimovich. 1983. Preparation and characterization of large dioctadecyldimethylammonium chloride liposomes and comparison with small sonicated vesicles. *Biochim. Biophys. Acta.* **733:** 172–179.
23. Claesson, P. M., A. M. Carmona-Ribeiro, and K. Kurihara. 1989. Dihexadecylphosphate monolayers: intralayer and interlayer interactions. *J. Phys. Chem.* **93:** 917–922.