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Differential biodistribution of encapsulated and surface-linked liposomal antigens

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Abstract

The biodistribution of liposomal antigens either encapsulated in or surface-linked to liposomes of similar composition was studied over time following intravenous injection and the results analyzed in relation to adjuvanticity. The two formulations were shown to behave very differently in vivo. While encapsulated antigen was rapidly focused to liver and spleen as expected, surface-linked antigen exhibited a more disseminated distribution which parallels that of the free protein. In dual-labelling experiments, it was also shown that encapsulated antigen remains associated with its liposomal vehicle in contrast to surface-linked antigen which is rapidly dissociated. This dissociation was apparently neither due to an exchange with plasma lipoproteins nor to a direct action of blood constituents. Besides, it was found that surface-linked antigen was rapidly accumulated in the carcass. We propose that the retention of the surface-linked antigen in the carcass results from a pre-processing of the protein involving more probably mononuclear phagocytes. This pre-processing might in turn favor the dissociation of the protein from the liposomes in a form that allows its dissemination in the whole organism and its interaction with more efficient antigen presenting cells such as for example Langerhans or dendritic cells.

Keywords: Liposomal antigen; Antigen; Biodistribution; Encapsulation; Surface linkage

1. Introduction

Liposomes are artificial membranous vesicles mainly composed of synthetic or naturally occurring phospholipids, which may be associated with other hydrophilic or hydrophobic substances. Their intrinsic properties such as biodegradability, low toxicity or poor immunogenicity have raised hopes of their eventual use as drug carrier systems in body fluids [1]. However, their rapid removal from circulation by mononuclear phagocytes of the reticulo-endothelial system (RES) and their inability to cross the endothelial barrier [2–4] have largely discredited their therapeutic usefulness until the recently described strategies to prolong their survival time in vivo [5]. Although the privileged interactions of standard liposomes with the RES have hampered most of their hypothesized applications, this specific property is nevertheless considered as a potential advantage in immunomodulation studies since RES tissues are the physiological sites in which immune cells are mainly located and in which immune responses are elaborated. Liposomes were, in fact, soon recognized as potent stimulators of the immune response provided they are physically associated with an antigen whether by encapsulation in the aqueous phase or exposition at the liposomal surface [6–8].

The immunoadjuvant character of liposomes first reported by Allison and Gregoriadis in 1974 [9] is generally attributed to the rapid and efficient targeting of antigen to macrophages of the RES capable of initiating the immune response by presenting processed antigen to helper T cells [10–12]. However, the investigations which have attempted to demonstrate the details of this postulated mechanism are somewhat fragmentary and have yielded conflicting results [13–16]. The question is particularly puzzling since macrophages are apparently not the best induc-

Abbreviations: RES, reticuloendothelial system; DMPC, dimyristoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; SPDP, 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester.

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tive antigen presenting cells for virgin T cells [17,18]. Other mechanisms to adjuvanticity that have been proposed for alun or Freund's adjuvant such as depot formation at the site of injection or activation of co-stimulatory signals have also been hypothesized for liposomal antigens but have never been systematically tested [19,20].

While a physical linkage of the antigen to the liposome appears to be the exclusive condition for immunopotentiation, important differences between encapsulated and surface-linked liposomal antigens were reported for all aspects of the immune response analyzed namely kinetics, intensity and quality of the humoral response, in vitro proliferation of presensitized cells or cytokine production [21–24]. These observations suggest that both liposomal formulations are differently handled by the immune system leading consequently to the activation of distinct immune pathways and to the induction of specific effector functions. This behavioral difference could in turn be used to advantage in the design of vaccines capable of stimulating the type of protection relevant to the cure of specific pathogenic diseases. The source of these differences remains to be identified but it might be speculated to reside in the steps that follow the introduction of an antigen in the organism.

Numerous biodistribution and pharmacokinetics studies have analyzed the in vivo destiny of injected liposomes as a function of dose, charge, fluidity, size, surface hydrophilicity or route of injection in order to identify the liposomal formulations capable of avoiding the RES and hence survive for longer periods in body fluids, with a view for their exploitation for drug transport. Large doses, neutral charges or shielded negative charges, small size, bilayer rigidity, surface hydrophilicity and subcutaneous or intramuscular route of penetration were all shown to correlate with prolonged survival in body fluids through decreased uptake by the RES [2,20,25–27]. The relationship between RES avoidance and adjuvanticity has however been less frequently investigated [20,28] in spite of the numerous studies of those liposomal properties necessary for adjuvanticity [11,29].Since surface-linked antigens induce a more potent stimulation of immune responses than their encapsulated counterparts, both in intensity and duration, and since the immunopotency of liposomal formulations is generally associated with their uptake by the RES, it seems reasonable to suppose that surface-linked antigens are focused and retained more efficiently by the RES than encapsulated antigens. We approached this question by comparing the biodistribution of both liposomal antigenic formulations following intravenous injection. This route of penetration was chosen since it was previously shown to induce the strongest response [24,30]. The results we obtained invalidate our initial hypothesis since surface-linked antigen was neither focused to nor retained more efficiently by RES tissues. However, they also suggest some alternative explanation to the increased adjuvanticity of surface-linked antigen.

2. Materials and methods

2.1. Materials

Conalbumin, dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylethanolamine (DPPE), cholesterol, 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP), 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (IODOGEN) were purchased from Sigma (St. Louis, MO). ^{125}I, protein iodination grade was obtained from New England Nuclear Canada (Lachine, Quebec) and [^{3}H]cholesterol (46 Ci/mmol) from Amersham Canada (Oakville, Ontario). ScintiVerse II, a micro-emulsified scintillation cocktail was obtained from Fisher Scientific.

2.2. Animals

Male and female BALB/c mice, the offsprings of breeding pairs obtained from Charles River Canada (St. Constant, Quebec), were used between 8 and 12 weeks of age.

2.3. Liposomes

Liposomes made of DMPC, cholesterol and DPPE in a molar ratio 63:31:6 were prepared by an extrusion technique (Lipex Biomembranes Inc., Vancouver, BC) using polycarbonate filters with a pore size of 0.2 μm as previously described [31]. Conalbumin, our model antigen, was either encapsulated in the course of liposome formation or surface-linked to preformed liposomes containing DPPE modified with the heterobifunctional reagent SPDP by the method of Leserman, Machy and Barbet [32].

2.4. Biodistribution studies

Liposomes labelled with either [^{3}H]cholesterol (1 μCi/μmol) or ^{125}I-conalbumin (1100 cpm/μg) prepared by the IODOGEN method of Salacinski et al. [33] were injected into mice via the tail vein. Each animal received a total of 30 μg conalbumin either free or associated to liposomes at a mean protein/lipid ratio of 20 μg/μmol phospholipid.

At varying times after injection, mice were bled by cardiac puncture under ether anaesthesia, killed by cervical dislocation and dissected. All tissue samples were weighed and the level of radioactivity determined either by gamma- or scintillation counting depending on the isotope analyzed.

For tritiated cholesterol measurements, tissues were first extracted with isopropanol/heptane/1 N H_2SO_4 (40:10:1, v/v) according to the method of Kedar et al. [34].

Tissue samples were obtained from liver, spleen, lung, heart, kidney, brain, inguinal lymph nodes and blood. In some cases, the remaining carcass was also considered.

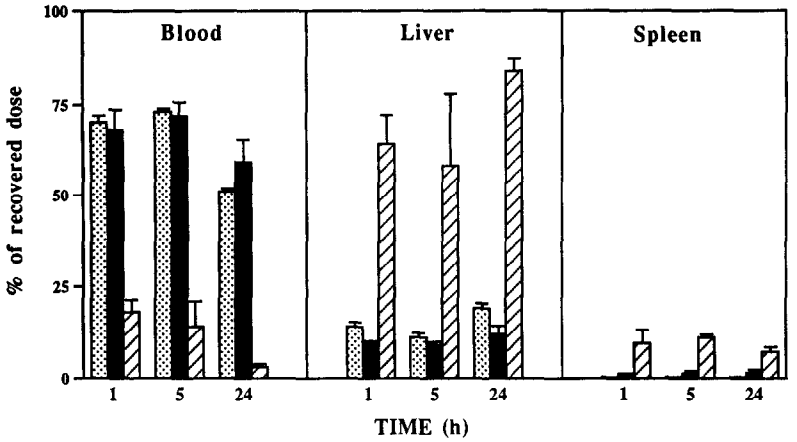

Fig. 1. Three to five mice were injected intravenously with either free (stippled bars), encapsulated (hatched bars) or surface-linked (black bars) iodinated conalbumin as described in Section 2. Radioactivity was measured in blood, liver, spleen, kidneys, brain, heart, lungs and inguinal lymph nodes. The percentage of recovered radioactivity was calculated for each tissue. The results obtained for blood, liver and spleen are expressed as mean ± S.E.

The radioactivity of each sample was corrected for blood content according to Allen [35].

The effect of blood on the antigen association to liposomes was analyzed using heparinized whole blood. Briefly, 1 ml of freshly drawn blood was incubated at 37°C for various time periods in the presence of identical amounts of either free, encapsulated or surface-linked antigen (0.15 ml). At the end of incubation, samples were centrifuged at 4°C for 30 min at 145 000 × g, after which the radioactivity of the pellet and supernatant was measured.

3. Results

The distribution in blood, liver and spleen of iodinated conalbumin, in the free state, surface-linked to, or encapsulated within liposomes is shown in Fig. 1. As can be seen, the antigen, when encapsulated, leaves the circulation rapidly and is concentrated mainly in liver and spleen, in agreement with its expected behavior. Surprisingly however, surface-linked antigen which our previous work has shown to be the most immunopotent formulation, behaves as the free protein, remaining predominantly in the circulation and being cleared at a comparable rate.

The specific radioactivity of different tissue samples, an index of the accumulation of antigen that should be tightly related to the biological activity of the formulation, is presented in Table 1. From these data, it can be seen that encapsulated antigen is not only focused rapidly and equally to liver and spleen but also retained more efficiently in these organs; whereas the specific activity of most tissues decreases roughly by an order of magnitude during a 24 h period, that of spleen and liver only decreases by a factor of 2. The surface-linked antigen, on the other hand, like the free protein, is more widely disseminated throughout the organism and does not exhibit any specific tissue accumulation. Although 1 h following injection the protein

Table 1
Specific radioactivity (cpm/g tissue) of various organs of mice injected with iodinated conalbumin, free, encapsulated or surface-linked to liposomes

Organ	Radioactivity recovered (cpm per g tissue)								
	after 1 h			after 5 h			after 24 h		
	free ag	encaps.	linked	free ag	encaps.	linked	free ag	encaps.	linked
Blood	13028	2088	10399	5445	1326	4538	292	120	658
Brain	229	27	165	83	30	58	9	36	50
Heart	5633	1156	5533	2193	1255	1119	152	173	377
Lung	3246	2239	4339	1453	2121	1062	183	559	323
Spleen	383	13081	1337	229	14853	368	27	5817	66
Liver	3018	11196	1718	796	8935	485	121	6729	134
Kidney	3861	1534	3042	1491	3173	972	250	255	196
L.N. [a]	1996	1647	1697	1262	162	529	364	0	160
RE/blood	0.26	12	0.29	0.19	18	0.19	0.51	104	0.30
Liver/spleen	7.9	0.86	1.29	3.47	0.6	1.32	4.42	1.16	2.03

All tissue counts were normalized to 63 000 cpm injected per animal.
[a] L.N. = inguinal lymph nodes.

is detected in the circulation as well as in heart, lungs, kidney or inguinal lymph nodes, the specific activity of these tissues rapidly declines with time without showing any significant tissular preference. The only difference that can be demonstrated between surface-linked and free antigen is a slight bias of the latter in favor of the liver over the spleen. The biodistribution was followed over 21 days but neither free, encapsulated nor surface-linked antigen could be recovered in significant amount even 5 days after injection.

The possibility that the unexpected behavior of surface-linked antigen could be related to a massive dissociation of the protein from its liposomal vehicle was analyzed by examining the biodistribution of liposomal cholesterol, one of the major constituent of the liposomal membrane. Fig. 2 compares the percentage of labelled cholesterol and labelled conalbumin recoveries in the three major tissues (blood, liver and spleen) and for both liposomal formulations. The results confirm that liposomes with encapsulated conalbumin are mainly focused to liver and that the protein remains essentially associated with the liposomal vehicle over the period examined (A). However, in the case of surface-linked antigen, a large discrepancy appears between the distributions of labelled conalbumin and labelled cholesterol (B); the liposomes exit from the circulation to gain access to liver and spleen whereas the protein remains in the circulation suggesting thereby that it is dissociated from the liposomes. The difference observed between both distributions is already highly significant 1 h post-injection with a P value < 0.005. It is also observed that the disappearance of liposomes from the circulation occurs at a slower rate than that of encapsulated homologues suggesting that the surface association of the protein prolongs the half-life of liposomes within the circulation ($P < 0.005$ 1 h post-injection).

We also investigated the possibility that surface-linked antigen is either detached from the liposomal vehicle by hydrolysis of the disulfide linkage or degraded to some extent by plasma proteinases. The results (Table 2) indicate that after 1 h, neither encapsulated nor surface-linked antigen appears to be significantly dissociated from liposomes in the presence of blood since only about 15% of

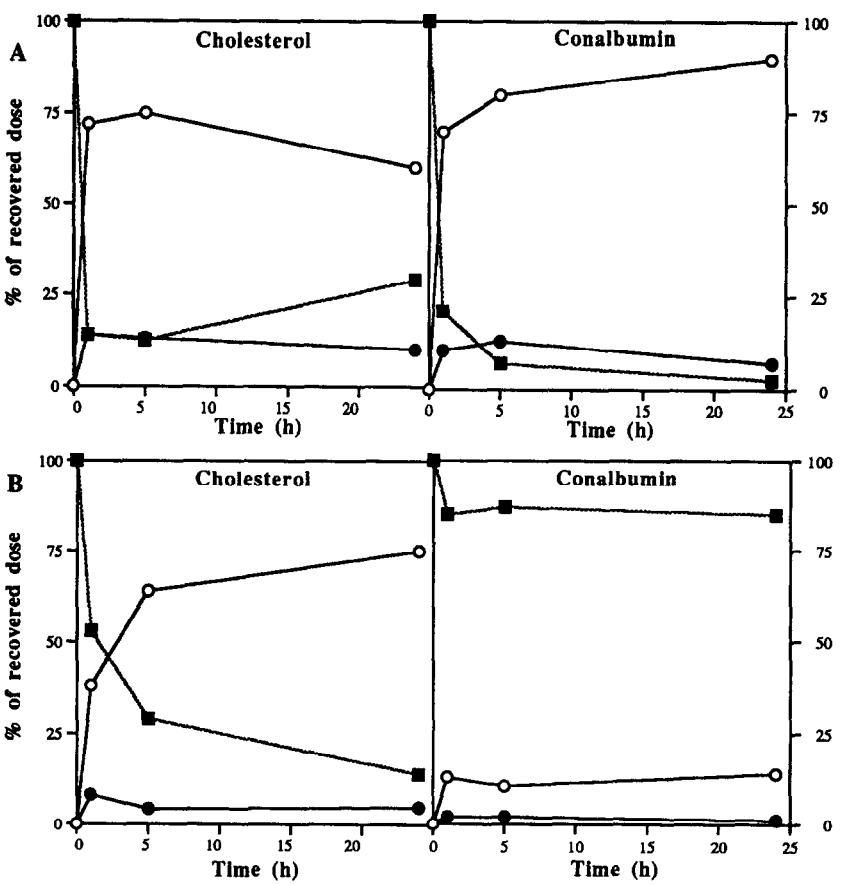

Fig. 2. Encapsulated (A) and surface-linked (B) antigens were made with either tritiated cholesterol or iodinated conalbumin. At different times following injection, the radioactivity was measured in liver, blood and spleen. The results represent the mean of 3–5 mice ± S.E. Error bars were omitted for S.E. < 2%. ○: liver. ■: blood. ●: spleen.

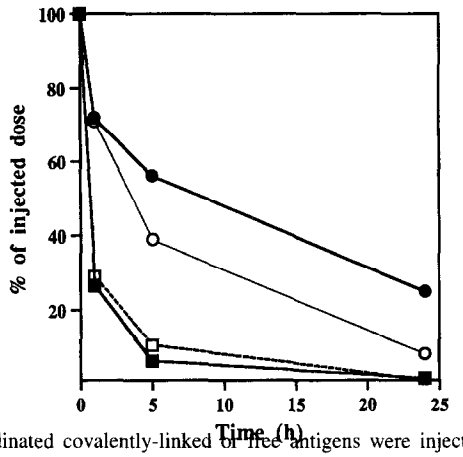

Fig. 3. Iodinated covalently-linked or free antigens were injected intravenously and all animal tissues were analyzed for their radioactivity content at various times post-injection. The total radioactivity recovered is compared to that recovered in blood. The results are expressed as the mean percentage ± S.E. of the recovered radioactivity relative to injected dose. S.E. values inferior to 2% are not indicated. ●: surface-linked antigen, total recovery. ■: surface-linked-antigen, blood recovery. ○: free antigen, total recovery. □: free antigen, blood recovery.

the protein can be recovered in the supernatant after a 30 min ultracentrifugation at 145 000 × g, conditions used to separate excess free antigen from liposomes during their preparation. The amount of protein recovered in the supernatant increases slightly over time, indicating that the exposed antigen is accessible to dissociation processes in the circulation. The possibility that this dissociation involves the transfer of conalbumin to plasma lipoproteins was also considered, but under our experimental conditions spectrophotometric analysis shows that the lipoproteins remain primarily in the supernatant.

Our results thus raise the question of the immunopotency of surface-linked antigen in a situation where no accumulation can be demonstrated in liver or spleen and where its behavior is apparently undistinguishable from that of the free protein. Part of the answer to this question is provided by our investigations of the distribution of

Table 2
Percentage of iodinated conalbumin recovered in a 145000 × g supernatant following incubation in presence of blood

	Conalbumin recovered in supernatant (%)		
Exp. 1	1 h		
Free	87 ± 3.0		
Encapsulated	13 ± 1.7		
Surface-linked	9 ± 0.4		
Exp. 2	1 h	5 h	24 h
Surface-linked	17 ± 5.3	24 ± 5.1	32 ± 2.3

Free or liposomal antigenic formulations were incubated in the presence of heparinized murine blood for the time periods indicated as described in Section 2. Each result represents the mean ± S.E. of three independent determinations.

antigen in the whole animal. Although surface-linked antigen disappears from the circulation at the same rate as free conalbumin and is not preferentially localized in any major organ, the recovery of linked antigen is more important (Fig. 3). As shown in Fig. 4, this difference can be essentially attributed to a significantly increased accumulation of the antigen in the carcass and more specifically in skin and muscles of the limbs.

4. Discussion

This study is the first to compare the biodistribution of two liposomal antigenic formulations of similar lipid composition but differing by their type of physical association with a protein antigen. Our results clearly show that the covalent linkage of a protein to the surface of liposomes differently influences the tissue distribution of the protein when compared to encapsulation.

Most studies of the immunopotentiating properties of liposomes have been carried out with encapsulated antigen

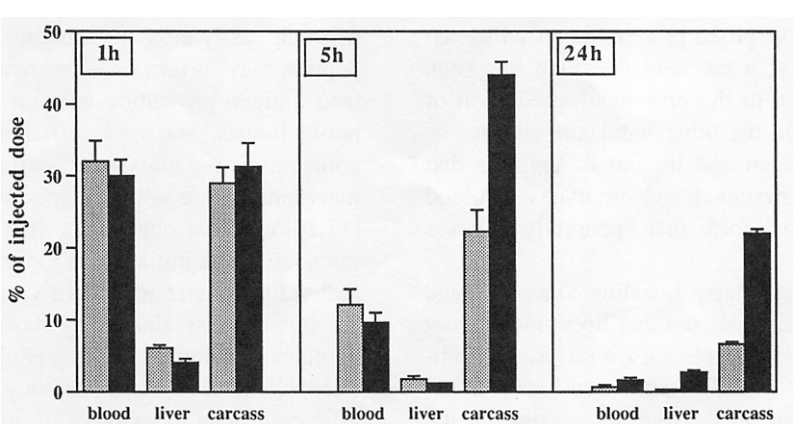

Fig. 4. Iodinated surface-linked (■) or free (□) antigens were injected intravenously and the percentage of injected dose recovered in blood, liver and carcass was calculated. The results are expressed as mean percentage ± S.E.

while biodistribution studies have been carried out with empty liposomes of various composition or with drug-loaded liposomes in the context of drug-targeting strategies. The biodistribution studies have concluded unanimously that liposomes administered parenterally are rapidly focused to macrophages of the major organs of the RES, the liver and spleen, a major disadvantage for most of their eventual pharmacological uses [36]. These studies have also largely contributed to set the dogma that macrophage targeting constitutes the main justification to liposomal adjuvanticity by facilitating the rapid processing and efficient presentation of antigen [20,21,37]. The dogma was comforted by several evidences showing that macrophage depletion strongly suppresses the immune response to liposomal antigens [38,39].

Our own findings with encapsulated antigen are in agreement with this established dogma: the antigen and its vehicle are rapidly cleared from the circulation and are both accumulated predominantly by the liver and spleen. The antigen remains in these organs at a significant level for the first 24 h and then subsequently disappears so that after 5 days, the antigen is no longer detectable within the limits of sensitivity of our assay techniques.

The behavior of surface-linked antigen, on the other hand, is in flagrant disagreement with the established dogma. Our initial finding was that the antigen remains mainly in the circulation, being somewhat disseminated in other tissues but apparently avoiding the spleen and liver. Moreover, after 24 h, the recovery of surface-linked antigen was significantly less (2.6%) than that of encapsulated antigen ($15.4 \pm 2\%$) but comparable to the 1.4% observed for the free antigen. We were thus forced to conclude that surface-linked antigen was neither rapidly focused to, nor efficiently retained by RES tissues in spite of the pronounced adjuvant properties of this liposomal formulation.

By following the fate of the lipid component of liposomes carrying surface-linked antigen, we also observed that these liposomes have a longer half-life (by a factor of 3–4) in comparison to liposomes carrying encapsulated antigen. This may be the result of the shielding of the lipid membrane by the surface-exposed protein, thereby increasing surface hydrophilicity, a mechanism which has been invoked as an explanation to the prolonged circulation of stealth liposomes [40]. On the other hand, the absence of colocalization of the antigen and its carrier suggests that the protein is rapidly dissociated and circulates in blood mainly in a non-liposomal form that apparently behaves like free antigen.

The question was particularly puzzling since we and others have previously demonstrated that liposomes do not potentiate the specific humoral response to a protein antigen unless the two are physically associated [6–8]. If the protein was indeed dissociated from its carrier in the circulation, not only would we have expected it to behave similarly to free protein but also to have similar adjuvant properties. Since this was not the case, we were forced to question more deeply the fate of the surface-linked antigen.

Using heparinized blood, we found no evidence of significant proteolytic degradation of the exposed antigen. This result suggests that blood constituents only play a minor role, if any, in the observed distribution of surface-linked antigen. Since our experimental conditions did not however reproduce the exact conditions prevailing in vivo, a role for blood constituents cannot be totally excluded. More importantly, we found that the surface-linked antigen escapes from the circulation and reaches peripheral tissues such as the skin and the limbs in significant amount. Although the free antigen was also recovered in the same areas, only 5% of the injected dose was recovered in these sites 24 h following injection as opposed to the 20% recovered with surface-linked antigen. The results taken together indicate that the surface-linked antigen recovered in tissues, while dissociated from liposomes, nevertheless differs from free antigen since the two behave differently. The possibility that the protein-DPPE complex has been exchanged between liposomes and plasma lipoproteins may be ruled out since under the centrifugation conditions used to analyze the role of blood in the dissociation process, lipoproteins are only pelleted to a limited extent.

The nature of the difference between free and surface-linked antigen remains to be established. While we are not able at this time to totally exclude a role for blood components, the difference in adjuvanticity reported for free and surface-linked antigen together with the reported importance of macrophages in adjuvanticity calls for some alternative explanations. It may be speculated that some transformations occur outside the circulation in areas where liposomes initially accumulate, such as for example the liver or spleen. The linked antigen that gains access to these major lymphoid tissues may be sufficient for the observed adjuvanticity of this type of liposomal formulation. On the other hand, we may also imagine that surface-linked antigen is partially degraded in endocytic compartments of phagocytic cells and regurgitated in the circulation under a modified, possibly lipopeptidic form, that can easily cross the endothelial barrier. The modified peptide may in turn be more readily accessible to specialized antigen-presenting cells of the skin or diffuse lymphoid tissues, and more efficiently presented. This latter conjecture is consistent with the observation that macrophages are not very good activators of virgin T cells [17,18] and that other cells may have to be more directly involved in the initiation of the immune response to liposomal antigens. Dendritic cells could be potential candidates for this process since they have been shown to take up peptides generated by extracellular proteolysis or regurgitated by other cells, since they can retain antigen more efficiently than other types of antigen presenting cells and since their uptake function, unlike that of macrophages, does not appear to be related to antigen clearance or destruction [18]. This hypothesis may also explain the

results of Su and Van Rooijen [39] showing that macrophage depletion is more detrimental to the adjuvant properties of surface-linked antigen than to that of the encapsulated form. If encapsulated antigen can be released in a free form accessible to non-phagocytic antigen-presenting cells even in the absence of macrophages, phagocytosis may be an obligate prerequisite to the processing or pre-processing of surface-linked protein. Our supposition that lipopeptides are involved is based on the finding that lipopeptides enhance both major histocompatibility complex class II-restricted presentation to T cells [41] and cell-mediated immunity [42]. Surface-linked antigen has also been shown to stimulate the production of interferon-γ, the characteristic cytokine of this type of immunity [24].

In conclusion, this comparative biodistribution study of two liposomal antigenic formulations reveals a clear difference in the circulation of antigen depending on its mode of association with the liposomal vehicle that have to be considered in the understanding of their adjuvant potential.

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