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The Intrasplenic Circulation of Three Formulations of the Same Protein Antigen

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Abstract

This paper presents a kinetic study of the intrasplenic circulation of three formulations of the protein antigen conalbumin including the soluble form and two liposomal formulations, one encapsulated in the internal aqueous milieu and one surface-linked to the liposomal vehicle. These formulations differ not only in their physical status but also in their immunostimulating properties and were chosen in an attempt to correlate the movements of antigen in lymphoid tissues with the immune response elicited. The presence of conalbumin was followed over a period of 21 days using, as a detection system, an antibody that we developed and which allows for the visualization of antigenic peptides such as those presented at the surface of antigen-presenting cells (APC). The results demonstrate that the amount of antigen accessing to the spleen, its time of residency and the pathway it follows are all profoundly influenced by the form under which it penetrates the immune system. The results also indicate that the marked initial preferences of an antigen for either B cells, marginal zone macrophages (MZM) or metallophilic macrophages (MM) are of fundamental importance in determining the fate of this antigen in the spleen. It is concluded that the exact formulation of an antigen is as crucial to the regulation of the immune response as is the nature of this antigen. It is further concluded that liposomes can be used efficiently to modify the formulation of an antigen and can contribute as such to the induction of specific immune functions by driving the antigen towards some privileged immune cell populations.

Introduction

Immune activation occurs mainly in secondary lymphoid tissues disseminated throughout the body (1). These tissues are highly organized and dynamic structures through which body fluids constantly percolate under the immune surveillance of migrating lymphocytes. There are found all the cell types involved in the induction, the maintenance

Abreviations: APC = Antigen-presenting cells; DC = Dendritic cells; DMPC = Dimyristoyl phosphatidylcholine; DPPE = Dipalmitoyl phosphatidylethanolamine; FDC = Follicular dendritic cells; fCa = Fragmented conalbumin; MZ = Marginal zone; MZM = Marginal zone macrophages; MM = Metallophilic macrophages; SPDP = 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester.

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and the control of the immune response (2–4). However, despite the importance of collaboration in all these processes, each cell population is preferentially found in some specific anatomical niche. In the spleen for example, metallophilic macrophages (MM), marginal zone macrophages (MZM) and dendritic cells (DC) are mainly found in the marginal zone (MZ) that delineates the white and the red pulps, T cells segregate in the periarteriolar lymphoid sheath (PALS) that asymmetrically surrounds the branches of the splenic artery while B cells occupy a zone overhanging the T cell area just beneath the marginal zone (3-6). Most of these cells are not permanent residents of the white pulp since they constantly emigrate from the blood, penetrating in the white pulp through the marginal zone from where they reach their specific compartment (7). The antigen also penetrates through the marginal zone, either percolating through the red pulp sinuses or being actively transported by dendritic cells or macrophages (8). By way of its organization, the lymphoid tissue orchestrates the circulation of both, the antigen and the immune cells. So doing, it allows a sequential and ordered cross-talk between the individual cells that need to collaborate for initiating the immune response, optimizing cell-cell interactions as well as antigen localization and cytokine influences (2).

The antigen is the essential driving force leading to the immobilization of sensitized cells in lymphoid tissues and to the formation of activation foci which both contribute to the tissue dynamics (5, 9, 10). It has been proposed that the exact form under which the antigen penetrates the tissue, whether it is soluble, particulate or transported by antigen-presenting cells (APC) determines the outcome of the response by affecting such important parameters as the dose of antigen available in the tissue, the time of residency, the interactions with specific cell types and consequently the kinetics of influences (2, 6). If this hypothesis holds true, any alteration in the lymphoid circulation of an antigen would be expected to influence the quality and the intensity of the induced response. As a corollary, the exact formulation of a given antigen should influence its intra-lymphoid circulation. This question has been addressed in a few studies but controversial results were obtained (11–14) may be as a consequence of the intrinsic properties of the tested antigens or the peculiarities of the method used to evaluate their circulation.

In this study, we tested this hypothesis by comparing the intrasplenic circulation of three different formulations of the protein antigen conalbumin, including the soluble form and two liposomal formulations, one encapsulated in the aqueous internal liposomal milieu and one surface-linked to the liposomal vehicle. All of these three formulations have been extensively characterized and were shown to differ significantly in their immunostimulating properties. While both liposomal formulations considerably potentiate the intensity of the immune response as compared to the soluble form, the response to encapsulated antigen is rapidly turned out and mainly characterized by an increased production of IgG_1 as well as by the secretion of IL-2 and IL-4. In contrast, that to surface-linked antigen is long lasting and associated with an increased production of IgG_{2a} and IgG_{2b} as well as with the predominant secretion of IFN-γ (15–19), characteristics related to cell-mediated immunity and Th1 activation. The mode of antigen association not only influences the characteristics of the immune response but also the antigen biodistribution profile and pharmacokinetics (20). In this paper, we analyzed how this can be related to the pathway by which the antigen penetrates the immune network.

Materials and Methods

Mice and reagents

Male and female Balb/c mice, 8 to 12 weeks old, were purchased from Charles River, St.Constant, Qc. Conalbumin, dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylethanolamine (DPPE), cholesterol, 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP) and 1,3,4,6-tetrachloro-3a, 6a-diphenylglycouril (IODOGEN) were all purchased from Sigma Chemical Co. (St-Louis, MO, USA). ^{125}I, protein iodination grade was obtained from New England Nuclear Canada Ltd (Lachine, Qc, Canada). CNBr-activated Sepharose was from Pharmacia (Upsala, Sweden). Biotinyltaed secondary antibodies were from Cedarlane Lab. Ltd. (Hornby, Ont, Canada) while avidin-biotinylated alkaline phosphatase and horseradish peroxidase complexes (Vectastain Kit), avidin-biotin blocking kit, enzyme substrates, Texas red-Avidin and FITC-Avidin were purchased from Vector Lab (Burlingame, CA, USA).

Monoclonal antibodies

Anti-Ia^d as well as monoclonal antibodies to mouse CD3ε, CD45R/B220, CD11c and Mac-3 recognizing T cells, B cells, dendritic cells and marginal zone macrophages respectively were obtained from Pharmingen (Mississauga, ON, Canada). MOMA-1, used to identify metallophilic macrophages, was purchased from Cedarlane.

Polyclonal antibodies against conalbumin peptides

A polyclonal antibody against conalbumin fragments was developed, using as immunogen, the peptides obtained by the CNBr proteolysis technique (21). Three New-Zealand 15 weeks-old male rabbits were immunized thrice at 1 month interval with 100 μg of the protein fragments emulsified first in Freund complete adjuvant and then in incomplete adjuvant. The antigen was injected subcutaneously at ten different sites with 100 μL of the emulsion containing 10 μg of the peptide mixture. Blood was collected eight days following the last immunization and specific antibodies were purified by affinity chromatography on CNBr-activated Sepharose using conalbumin fragments as ligand. The antiserum was tested by ELISA and Western blotting against native and fragmented conalbumin (fCa) as well as against unrelated proteins such as ovalbumin, bovine serum albumin and lysozyme. The capacity of the antiserum to detect MHC-II-peptide complexes was demonstrated by double-labeling confocal immunofluorescence microscopy and by Western blotting of anti-Ia^d immunoprecipitates of whole cell lysates according to the method of DAVIDSON et al. (22).

Liposomal antigens

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, Canada) using polycarbonate filters with a pore size of 0,2 μm. Conalbumin was either encapsulated in the course of liposome formation or surface-linked to preformed liposomes containing DPPE previously modified with the heterobifunctional reagent SPDP according to the method of LESERMAN et al. (23). Liposome-associated conalbumin was separated from free conalbumin by two ultracentrifugations (30 min., 145 000×g) carried out at 10°C. All liposomal antigens were prepared at a constant ratio of 25 μg protein antigen/μmole phospholipid. Liposome size and protein:lipid ratios were determined as previously described (16).

Experimental protocol

Balb/c mice were sensitized intraperitoneally with 25 μg of soluble, encapsulated or surface-linked conalbumin in 10mM Hepes, 0,9% NaCl pH 7.4. Animals (2 per group) were sacrificed by cervical dislocation 1h, 5h, 24h, 5d or 21d post-immunization. Their spleen was then rapidly removed, sus-

pended in isopentane and deeply frozen in liquid nitrogen. The overall protocol was repeated three independent times.

Immunohistochemistry

Cryostat sections (12 μm) were fixed for 20 min at room temperature in phosphate-buffered saline (PBS) containing 2% paraformaldehyde, rinsed twice in PBS and then blocked for 20 min with PBS containing1% gelatin, 1% non-immune decomplemented mouse serum, 1,5% goat serum. Endogenous activities were also blocked using the avidin-biotin blocking kit and, when necessary, with 0,3% H_2O_2. After three washes in PBS-0,1% gelatin, the sections were incubated for 45 min with one of the primary antibodies, washed again and incubated for 30 min with the appropriate biotinylated secondary antibody. The ABC-enzyme was then added for 30 min followed by the colored substrate according to the company's recommendations. In multiple labeling experiments, antigens were detected sequentially. Color negatives were digitized with a Photosmart S20 and treated in Adobe Photoshop®4.0. Due to the low intensity of the anti-conalbumin labeling, specially at the longest times studied, sections were not counterstained but the exact position of the antigen labeling was rather analyzed by localizing T cells, B cells and macrophages on serial sections. The specificity of the labeling reaction was assessed using the following controls: animals immunized with buffer alone or omission of the primary antibody.

Results

Characterization of anti-fragmented conalbumin antibodies

Antibodies against fragmented conalbumin were raised in an attempt to detect *in situ* some of the immunologically relevant forms of the antigen such as those generated through the endocytic and cytosolic antigen processing and presentation pathways. The antibodies were characterized for their specificity towards fragmented conalbumin and for their capacity to recognize the antigen even when associated with MHC products. By ELISA (Fig. 1), the antibodies were shown to react strongly with fragmented conalbumin and to a much lesser extent with the native protein. Only minimal reactivity was observed towards the unrelated proteins ovalbumin and bovine serum albumin. As expected, under the denaturing conditions of SDS-gel electrophoresis (Fig. 2), the native form of conalbumin became as strongly detected as the fragmented forms. However,

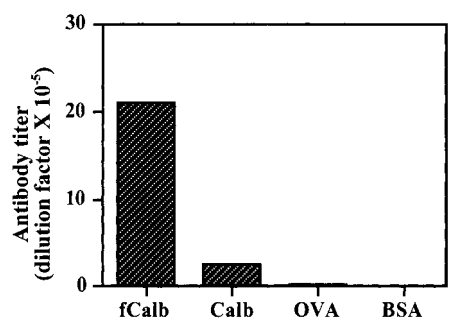

Figure 1. Antibody titers of anti-fragmented conalbumin against various proteins as assessed by ELISA. fCa: fragmented conalbumin; Calb: native conalbumin; OVA: ovalbumin; BSA: bovine serum albumin.

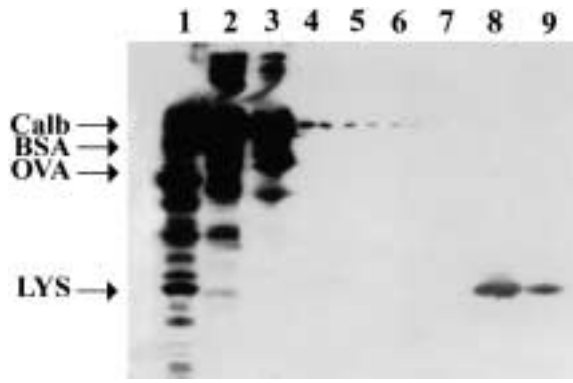

Figure 2. Western blot of different proteins probed with anti-fCa antibodies. 1: fragmented conalbumin (5 μg); 2 and 3: native conalbumin (5 and 1 μg); 4 and 5: bovine serum albumin (5 and 1 μg); 6 and 7: Ovalbumin (5 and 1 μg); 8 and 9: Lysozyme (5 and 1 μg). The arrows indicate the relative position of native conalbumin (Calb), bovine serum albumin (BSA), ovalbumin (OVA) and lysozyme (LYS).

even at the high load of protein put on the gel, bovine serum albumin and ovalbumin were completely ignored while lysozyme was only faintly reactive. Evidences that our antibodies can recognize some MHC-conalbumin peptide complexes were obtained using APCs derived either from bone marrow or from peritoneal exudates (24, 25). The APCs, mainly dendritic cells and macrophages, were first incubated with liposomal conalbumin for periods of time sufficient to allow for antigen processing (between 3 and 24 hours). The cells were then subjected to anti-Ia^d immunoprecipitation or double-labeling immunofluorescence microscopy. Some of the expressed MHC-II molecules are known to be stabilized by their bound peptide (22). These complexes readily dissociate when boiled in SDS sample buffer but remain associated during electrophoresis if solubilzed at room temperature. As shown in Figure 3a, in unboiled samples of anti-Ia^d immunoprecipitates of detergent extracted antigen-loaded APCs, the anti-fragmented conalbumin antibodies identify a band of similar electrophoretic mobility as that detected by anti-Ia^d. Since this band is not detected in boiled samples, we conclude that our anti-fragmented conalbumin antibodies do in fact recognize some of the conalbumin peptides complexed to MHC-II molecules. The same conclusion was reached when analyzing the colocalization of anti-Ia^d and anti-fragmented conalbumin by confocal microscopy in conalbumin-loaded APCs (Fig. 3b). This conclusion was further supported by an immunoelectron microscopic study of liposomal trafficking in macrophage (24), by an immunocytochemical study of DC/liposomal antigen interaction in permeabilized and non-permeabilized cells (unpublished observations) as well as by the histochemical study presented in this paper.

Intrasplenic circulation of conalbumin

Differences in the anti-fCa staining of spleen sections obtained from animals sensitized with either soluble or liposomal antigens were already evident 1 hour post-immunization. At this time point, the soluble antigen was diffusely recovered over the B-cell areas

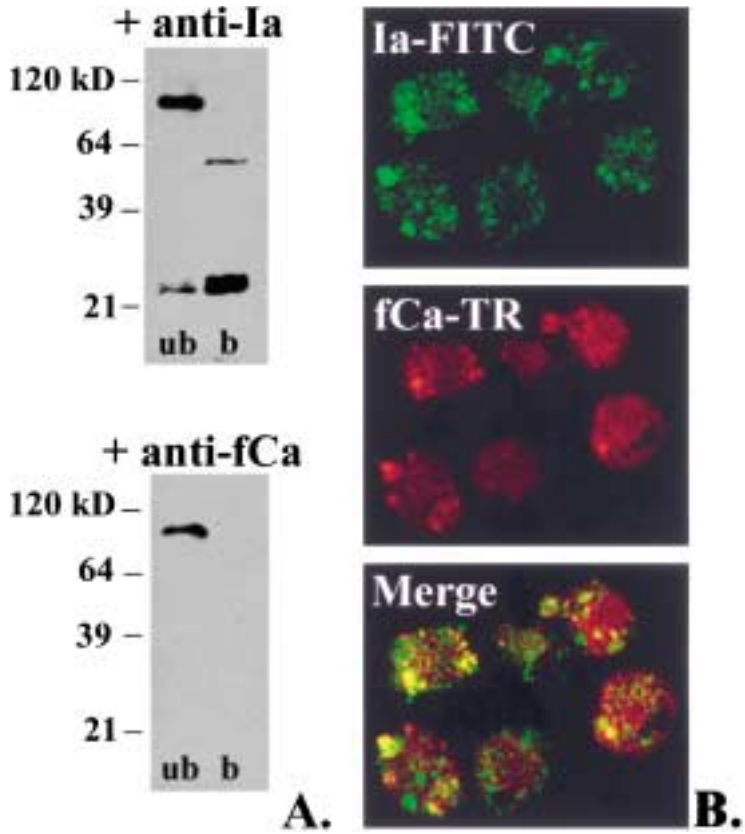

Figure 3. Detection of MHC-II/peptide complexes with anti-fCa. a. Boiled (b) and unboiled (ub) samples of anti-Iad immunoprecipitates of detergent extracted antigen-loaded APCs. In the top figure, samples were probed with anti Iad while in the bottom figure, the same samples were probed with anti-fragmented conalbumin. The position of the molecular weight markers is shown on the left. b. Antigen-loaded APCs were fixed and processed for double immunofluorescence microscopy using biotinylated anti-Iad + FITC-Avidin and anti-fragmented conalbumin (fCa) + anti-rabbit Texas Red as detection tools. The cells were finally analyzed by confocal microscopy. The top and middle images are those obtained for each of the primary antibodies while the bottom image is the merged image of the two individual figures.

(Fig. 4b) indicating that B cells were the main cells responsible for the splenic retention of this antigenic formulation, a process that more probably involves interaction with surface immunoglobulins. After 5 hours, the labeling became concentrated at the surface of some cells of the follicles and by 24 hours, it has almost totally disappeared, being essentially characterized by the very occasional presence of dendritic profiles in very few outer B cell regions.

In contrast, one hour after immunization, both liposomal formulations were mainly detected in the marginal zone of the white pulp and to a lesser extent in the red pulp. However, the two formulations did not localize similarly in the marginal zone. While encapsulated antigen was distributed uniformly over the zone (Fig. 4c), surface-linked

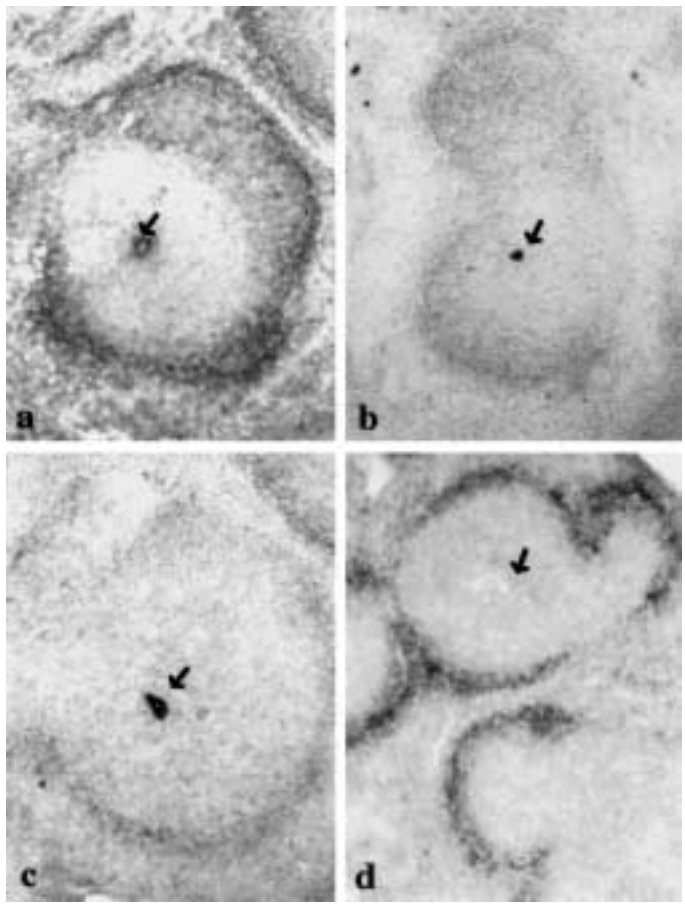

Figure 4. Localization of fCa in the spleen 1h post-immunization with one of the three formulations of conalbumin. Mice were immunized intraperitoneally with 25 μg of soluble, encapsulated or surface-linked conalbumin in 10mM Hepes, 0.9% NaCl, pH 7,4. The spleens were recovered one hour post-immunization and processed for immunoenzymatic detection by bright-field microscopy as described in Materials and methods. Colour negatives were transformed in the grayscale mode with Adobe® Photoshop 4.0. All sections probed with anti-fCa were labeled using the Vector®Blue substrate for alkaline phosphatase. a. Control section in which metallophilic macrophages (MM) and B cells were labeled using anti-MOMA-1 and anti-CD45/B220 respectively. Following transformation of the colored pictures in the grayscale mode, MM, initially labeled in blue with the Vector®Blue substrate for alkaline phosphatase, appear now in dark black while B cells, initially labeled in brown with the DAB substrate for horseradish peroxidase, now appear in light gray. b. Anti-fCa labeling observed after immunization with soluble conalbumin. c. Anti-fCa labeling observed after immunization with encapsulated conalbumin. d. Anti-fCa labeling observed after immunization with surface-linked conalbumin. a. and c. are serial sections. The arrows indicate the position of the central artery. Original magnification: 100X.

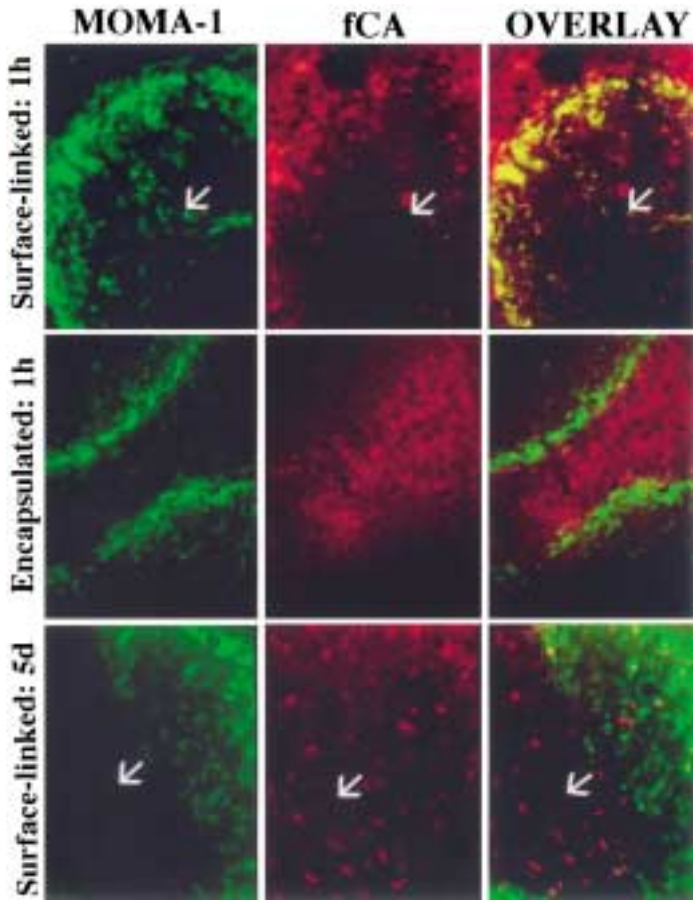

Figure 5. Simultaneous detection of fCa and MM by immunofluorescence microscopy. Double immunofluorescence microscopy carried out on spleen sections obtained from mice immunized with either encapsulated or surface-linked conalbumin. The spleens were recovered either one hour or five days post-immunization as indicated and the sections were sequentially probed with anti-MOMA-1+ FITC-Avidin and anti-fCa + biotinylated anti-rabbit antibodies + Texas Red-Avidin. The arrows indicate the position of the central artery when visible in the field. Original magnification: 200X.

antigen was recovered as a thin intensely stained rim overhanging an inner region of more diffuse staining. It was also more strongly detected than its encapsulated counterpart suggesting that it was more efficiently or more rapidly processed. The differences observed in the distribution pattern of the two liposomal antigens further suggested that different cell populations of the marginal were more specifically involved in their trapping by the spleen. We demonstrated that this was in fact the case using double-labeling immunofluorescence microscopy. As shown in Figure 5, a strong interaction could be detected between surface-linked antigen and MM while encapsulated antigen was almost totally excluded from this zone, being more exclusively localized in the region occupied by MZM.

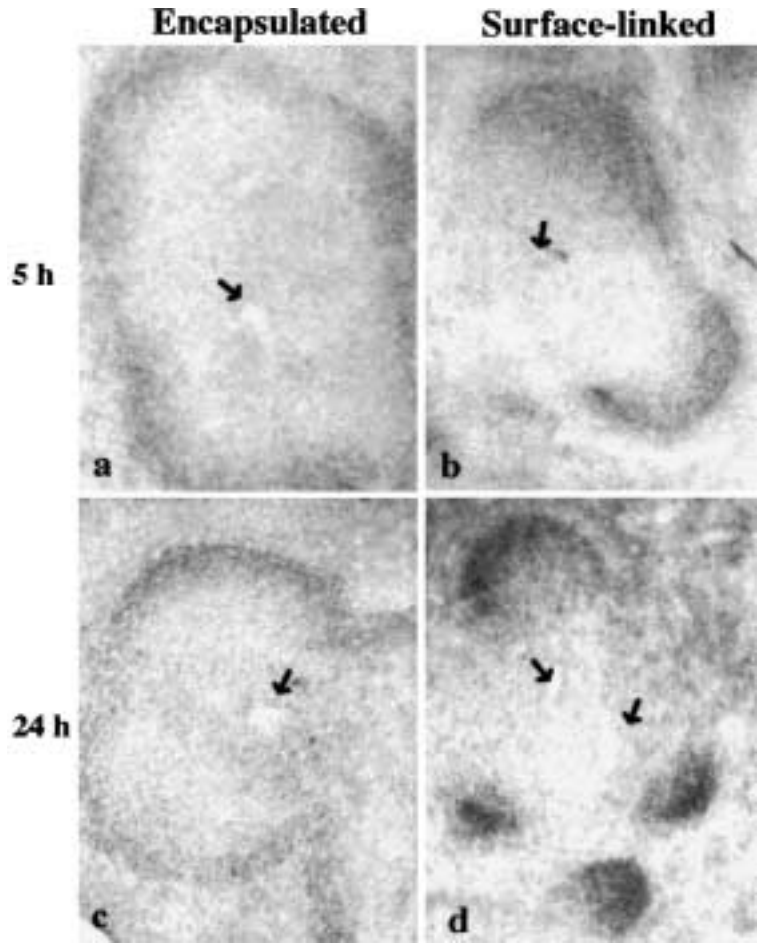

Figure 6. Localization of fCa in the spleen 5h and 24h post-immunization with either encapsulated or surface-linked liposomal conalbumin. The spleen of mice immunized with either encapsulated (a and c) or surface-linked (b and d) liposomal conalbumin was recovered 5h (a and b) or 24h (c and d) post-immunization and probed with anti-fCa. Note in a) and c) the relatively low penetration of the encapsulated antigen in the B-cell area when compared to its surface-linked counterpart. Note also at 24h, the presence of labeling around the central artery (arrow) in response to the encapsulated antigen and the dense masses formed in the B-cell areas in response to surface-linked antigen. Original magnification: 100X.

Four hours later, the red pulp staining has almost totally disappeared. By that time, surface-linked antigen has left the interfollicular rims of the marginal zone to colonize more specifically the follicles into which it penetrates deeply (Fig. 6b) while encapsulated antigen remained mostly located in the marginal zone, penetrating only minimally into the outer B cell area (Fig. 6a). After 24 hours, encapsulated antigen was still mainly found in the marginal zone and in the outer B-cell area although some penetration of the periarteriolar lymphoid sheath via the zone of interruption of the marginal zone

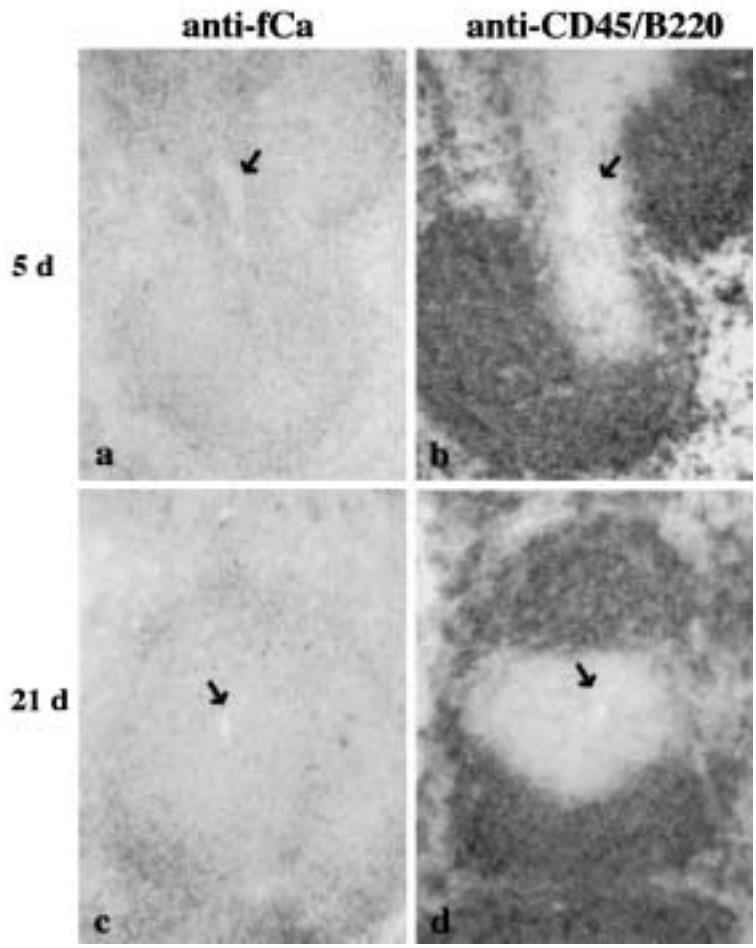

Figure 7. Localization of fCa in the spleen 5d and 21d post-immunization with surface-linked liposomal conalbumin. The spleen of mice immunized with surface-linked antigen was recovered 5d (a and b) or 21d (c and d) post-immunization and probed with either anti-fCa (a and c) or anti-CD45/B220 (b and d) on serial sections. Note that the anti-fCa labeling, mainly observed as dendritic profiles around the central artery (arrow) and in the outer B-cell regions after 5 d, is still observed after 21d and is now found widely dispersed through the entire white pulp nodule. Original magnification: 100X.

could also be seen (Fig. 6c). The staining was, however, faint and diffuse and by 5 days, the antigen has almost completely disappeared from the white pulp with only few cells of dendritic appearance being occasionally observed in some outer B cell regions. In contrast, the presence of surface-linked antigen was still intensely demonstrated 24 hours following immunization (Fig. 6d). The antigen has now penetrated deeply into the follicles where it forms dense, heavily stained masses. Four days later, the antigen has penetrated more deeply into the white pulp, being found both, in the periarteriolar region and in the B-cell area. However, the labeling was no more localized in some specific

zones of these anatomical niches but rather concentrated at the surface of some cells of dendritic morphology dispersed throughout the areas (Fig. 7a and Fig. 5). This widely distributed labeling pattern could still be observed 21 days post-immunization (Fig. 7c) although, with time, one also observed a decrease in the number of labeled nodules.

Discussion

The rapid localization and distribution of a variety of antigens between the different compartments of lymphoid tissues have been recognized since long, as have been the morphological changes and the cell migrations they induced over time in these compartments (6, 26–29). However, although it is believed that these movements are of prime importance in regulating the sequential interactions and activation of immune cells, the exact relation of these movements to immune activation still remains poorly characterized and thus poorly understood.

In this paper, three formulations of the same protein antigen that differ in their physical status, in their interaction potential with immune cells and in their inductive capacity of the immune response were used to sensitize animals in order to analyze this possible relationship.

This detection method significantly differs from those currently used to analyze the circulation of antigen in lymphoid tissues where the presence of antigen is generally assessed using a radioactive or a fluorescent tag associated to antigen prior to immunization (11, 12, 26, 27, 30). While these probes certainly allow for a good localization of intact antigen and in some cases, even of antigenic degradation products, they are not particularly well suited to trace the immunologically significant fraction of the antigenic load. It is believed that our detection method gives the opportunity to look at the intra-lymphoid circulation of antigen from a different perspective. In its principle, the approach is similar to that of REIS e SOUSA and GERMAIN (31) who analyze the lymphoid distribution of soluble hen egg lysozyme using a monoclonal antibody recognizing specifically one of the MHC-peptide complexes. However, the approach also significantly differs in that, during the kinetics, our antibody can recognize different forms of the antigen, among which some of the various peptides generated in the endocytic pathway. Moreover, only a fraction of the relevant MHC-peptide complexes can certainly be identified and any type of rescued fragments, whether it is by MHC molecules or other ligands can eventually be detected.

Our results, nevertheless, clearly demonstrate that the amounts of antigen accessing lymphoid tissues as well as the circulation of this antigen in the lymphoid tissues are profoundly influenced by the form under which this antigen penetrates the immune system. When soluble, a protein antigen such as conalbumin appears to be rapidly although inefficiently taken up by the spleen. These observations agree with the empirically recognized poor immunogenicity of protein antigens which induce responses of low intensity and short duration unless adjuvants or high immunizing doses containing some aggregated particles are used for sensitization (31–33). Both of these conditions might be essential to achieve the high local antigen concentration required for the efficient loading of individual cells and for the sensitization of DCs (34–35).

The particularization of our soluble model antigen by association with a liposomal vehicle through encapsulation or surface-linkage, results in radically different localiza-

tion profiles from the beginning to the end of the kinetic study. Under these forms, conalbumin is now rapidly and efficiently trapped in the spleen and is initially recovered in the marginal zone where macrophages as well as dendritic cells (3). The initial targeting of liposomal antigens to the marginal zone is *per se* an unsurprising observation since the rapid accumulation of liposomes in the spleen following in *vivo* injection and their interaction with macrophages, both *in vivo* and *in vitrro* have been repeatedly demonstrated (36–38) as has been reported their interaction with dendritic cells (39, 40). This initial localization at the marginal zone is not a specific attribute of liposomal antigens since it has also been observed with different kinds of particulate materials including bacteria, red blood cells and immune-stimulating complexes (ISCOMs) (11, 12).

Although encapsulated and surface-linked antigens are both initially targeted to the marginal zone, they do not localize similarly in the zone as a consequence of their differential interaction with macrophage sub-populations. While encapsulated antigen mainly interacts with MZM, surface-linked antigen is, in contrast, mainly associated with MM. These initial interactions have profound impact on both, the circulation and the retention of the antigen in the spleen. The observation that encapsulated antigen penetrates only minimally into the white pulp and rapidly disappears from the spleen suggests that MZM are more efficient at scavenging than at processing antigen as already proposed by others (12). In contrast, the initial interaction of surface-linked antigen with MM is followed by a rapid penetration of the antigen into the white pulp, a wide distribution of antigenic peptides and a long retention by the spleen, indicating that MM are more efficient than MZM at propagating and sustaining immune activation. The preferential interaction of surface-linked antigen with MM might explain the specific immune properties of this antigenic formulation since MM have been recognized as a major source of IFN-α/β in the spleen (41), a cytokine associated with the development of cell-mediated immunity (42). Although the exact mechanism by which splenic macrophages discriminate between the two liposomal formulations remains to be elucidated, the specific involvement of some Toll-like receptors could be reasonably envisaged. These receptors, which recognize a wide variety of biological patterns some of which are associated with lipopeptides and phospholipids, have been shown to be differently expressed by macrophage sub-populations and to lead to the induction of different biological responses (43–45).

Both encapsulated and surface-linked antigens, accumulate with time in areas where B cells and FDCs are concentrated. Although B cells have been shown to be sensitized directly although not very efficiently by surface-exposed antigen (46), such direct sensitization has not been demonstrated with the encapsulated formulation and is neither supported by our kinetic study. The delay we observed between the staining of B cell areas and that of the marginal zone rather suggests that the interaction of liposomal antigens with cells of this region is in great part mediated by antigenic peptides that have been released by more professional endocytic cells. Such a release, already reported with macrophages (47), has been observed with both liposomal formulations (24). Once sensitized, B cells, if not anergized by a lack of cognate interaction with primed-T cells (48), can then sustain extensive T cell proliferation and T cell memory induction while FDCs might contribute to the retention of the antigen in lymphoid tissues as suggested by the dendritic profiles observed at the latest times of the kinetics, especially with the surface-linked antigen.

In this study, the involvement of DCs, although addressed, could not be unequivocally demonstrated. We suspect that the paucity of this cell population in the spleen, the limited fraction of processed peptides that can be detected with our antibody as well as the massive involvement of other cell types have obscure the phenomenon since DCs are apparently required to turn on naive T cells and since both, DCs and macrophages, have been shown to internalize liposomal antigens (39).

The understanding of the complex interactions between antigens and immune cell populations is a key to the understanding of their effects on the immune response. Our results demonstrate that initial influences are of fundamental importance in driving the response by tracing the path for the antigen and more probably by establishing the specific cytokine context in which the immune response has to evolve. Those observations lend support to some of the hypothesized mechanisms of liposomal adjuvanticity such as efficient targeting to APCs and they also strongly support the view that the immune response can be modulated by altering the lymphoid circulation of antigens, what can be achieved with properly designed liposomal formulations.

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