Splenic Trapping of Nanoparticles: Complementary Approaches for *In Situ* Studies

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Purpose. To identify more accurately in the spleen, the areas and the cells where nanoparticulate carriers were taken up from the blood flow, a series of complementary approaches were used.

Methods. First, *in* and *ex vivo* examination of the whole spleen led to a global view of all the trapping areas. Then, histological studies on frozen sections of the same organ allowed for a more precise localization of these areas and image analysis gave an evaluation of tissue distribution of the nanoparticles. Finally, immunological and enzymological characteristics of the capturing cells were determined *in situ*, using monoclonal antibodies (F4/80 and anti-sialoadhesin) and cytochemichal reactions (esterases and acid phosphatase). Furthermore incubation of spleen slices with different nanoparticles was used so as to know if the capture was due to a high capturing capacity of these cells or to a high blood flow in their vicinity.

Results. It was shown that more than 90% of the splenic capture was localized in the marginal zone of the follicles. The capturing cells form a special population of macrophages inserted in a reticular meshwork, showing low esterase and acid phosphatase activities, giving faint or no reaction with F4/80 or anti-sialoadhesin antibodies. The circulating nanoparticles were quickly trapped with rather low specificity by these cells.

Conclusions. Combination of coherent approaches allowed for the tracking of capturing cells from *in vivo* observations to their in situ identification on immunological and enzymological criteria.

KEY WORDS: spleen; nanoparticles; capturing cells; histoenzymology; immunohistochemistry; mouse.

INTRODUCTION

Different particulate carriers (*e.g.* liposomes or nanoparticles) have been used to target drugs, but most of them are strongly taken up by the liver and the spleen which represent 80–90% of the phagocytic cells of the Mononuclear Phagocyte System (MPS). This capture may be useful for some applications, but more often, such a profile of distribution hinders targeting towards other tissues. The hepatic uptake of these particulate carriers has been extensively studied and the role of the Küpffer cells is now well documented. On the contrary, spleen sequestration is less precisely known, perhaps because the spleen microcirculation is complex with many interconnected and fenestrated sinuses. Which and where are the first cells able to take particles out of the blood remains unclear, as is how they interact with circulating nanoparticles. Understanding this capture process is critical to allow modulation of splenic targeting. Of the few studies which aim to elucidate the nanoparticle uptake mechanism by the spleen, most were performed using *in vitro* models. Although they gave useful information concerning phagocytic mechanisms, they did not give a clear appreciation on *in vivo* uptake. In fact, the use of isolated cells may induce various artefacts : cell damage may occur, some important cell populations may remain trapped in the spleen reticular meshwork, different subpopulations of splenic macrophages may become mixed and the anatomical architecture and circulation channels are thus destroyed.

So, the aim of this paper is to identify more accurately the areas of the spleen and cells where nanoparticles are taken up from the blood flow *in vivo*. Therefore, *in vivo* observation as well as *ex vivo* investigation on frozen sections were performed.

MATERIALS AND METHODS

Nanoparticles

Polystyrene fluorescent nanoparticles Estapor FX-010 (98nm and 185nm in diameter) were obtained from Prolabo (France). These particles were washed, separated by ultracentrifugation (23 000 rpm, 1h30, Beckmann L7-55) washed twice with distilled water and resuspended in saline. Polyisohexylcyanoacrylate (PIHCA) nanoparticles (200nm in diameter) loaded with doxorubicin were prepared by anionic polymerisation as previously described (1). The suspension was dispensed in vials of 1.3ml for lyophilization in an Alpha 1-4 system (Christ Loc-1, Bioblock Scientific), then stored at 4°C. Resuspension of nanoparticles before injection was performed by addition of 1ml of 0.1M phosphate buffer (pH 6.8) to each vial containing 13.3 mg of polymer and 1 mg of doxorubicin.

In Vivo Studies

For *in vivo* microscopic examinations of the spleen, we used, with slight modifications, the method previously described by Mac Donald (2) as laid out in Figure 1. CD1 female mice 25–30g (Charles River, France) were anesthetized with 60 mg/kg sodium pentobarbital (I.P.) and placed on an acrylic platform, slightly inclined to their left side, so that the spleen could protrude through a transverse incision. Body temperature was maintained near 37°C using a heat lamp.

The mice received an injection of 0.3ml of a 0.1% nanoparticle suspension (12mg of polystyrene/kg) in the caudal vein.

The spleen rested on a glass window mounted in the platform and was covered with Saran film to minimize dehydration and motion. The tissue was irrigated with saline periodically to keep it moist and to remove blood cells which could interfere with the view.

The viewing platform was placed on the stage of an inverted microscope (Olympus, Japan) equiped with ×10, ×33 and ×66 objectives and epi-fluorescence illumination (excitation wavelength 490nm, barrier filter 495nm) and the spleen was observed for 1hr.

Images were obtained with a KCD-1 digital integrating camera with a CCD colour head (Olympus), digitized and ana-

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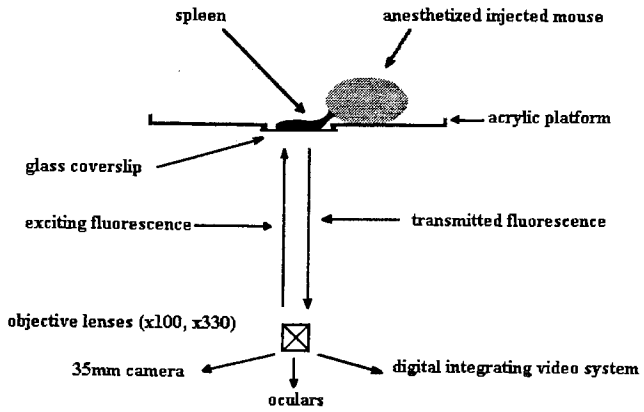

Fig. 1. Schematic representation of the experimental setup for *in vivo* observation. The mouse was lying on an acrylic platform with its partially exteriorized spleen resting on the glass coverslip window. The viewing platform was placed on the stage of an inverted epi-fluorescence microscope.

lyzed using a Visiolab 1000 system (Biocom, France). They were stored as RGB bitmaps.

Some of these images were also recorded on video tape (Sony, Hi8).

Ex Vivo Studies

To improve the identification of the spleen areas and the cells where the nanoparticles were trapped, three complementary approaches were used on the spleens of mice injected intravenously with polystyrene nanoparticles (0.3ml, 12mg/kg of polymer) or with PIHCA nanoparticles containing doxorubicin (0.4ml, 11mg/kg of doxorubicin, 146mg/kg of polymer) and killed at different time intervals after injection (30mins, 3hrs, 24hrs and 96hrs). Firstly, spleens were taken out and tightly pressed against the observation window as described for *in vivo* studies (Fig.1), thus allowing improved images to be obtained from the top and bottom sides of the spleen.

Secondly, histological studies were carried out. Spleens were frozen in OCT Compound (Miles, USA) and frozen sections were cut (5μm) on a Cryomin cryostat (DIS, France), fixed with paraformaldehyde 10% in isotonic saline. Some of these sections were coloured with Harris Haematoxylin for a better identification of the cells. They were observed under an Olympus microscope combining epi-fluorescence and transmitted light examination. Non-coloured sections were observed in the same conditions.

Representative photographs were also taken with a PM10-AD microphoto system (Olympus, Japan) and a Kodachrome film (EPY-135-36, 64T). Confocal studies were realized in the laboratory of "Imagerie Cellulaire" (Dr Laurent, URA CNRS 1116, University of Paris-Sud).

Thirdly, spleens were sliced and tissue dissociated by digestion 1hr at 37°C, pH 7.3, with 1mg/ml of collagenase (CLS_3, 103U/mg, Whorthington). Slides were prepared by cytocentrifugation and coloured with Harris Haematoxylin or May-Grunwald-Giemsa.

Semi-Quantitative Determination of the Spleen Capture of Nanoparticles

The spleen capture was semi-quantitatively analyzed using a Visiolab 1000 image analyzer on groups of five mice. Spleens were taken out 3hrs after polystyrene nanoparticle injection, quickly frozen and cryocut along the longitudinal axis. Five histological sections representing different levels of the spleen were studied under the epi-fluorescence microscope (×100). For each preparation, the green fluorescence contained in granulations was evaluated for each of three spleen zones (marginal zone, white pulp, red pulp) and in four different areas of the preparation (four images were digitized from each section, giving twenty images per spleen). With this method, it was possible to compare the spleen nanoparticle capture in the three above mentioned zones and to evaluate the variations of the distribution profile depending on the nature of the nanoparticles used. Moreover, observation of the cells with a higher magnification (×660 or ×1000) made cytological identification also possible.

Results have been corrected according to the respective surface areas of the marginal zone, white pulp and red pulp.

Immunohistological Studies

To characterize *in situ* cells involved in the nanoparticle capture, antibodies known to react with mice spleen macrophages were used. Spleen cryostat sections (5μm) were air-dried overnight in the dark and fixed in 95° ethanol for 5mins. After drying, sections were washed in 0.01M phosphate buffered saline (PBS), pH 7.2, for 10 minutes. Non-specific reactions were inhibited with a skimmed milk solution for 10mins. After washing in PBS for 10mins, slides were incubated for 30mins, at room temperature with 1/400 dilution of rat anti-mouse macrophage antisialoadhesin or anti F4/80 (Diaclone Research, France) in PBS. The endogen peroxidases were inactivated with a 0.3% H_2O_2 solution. After washing in PBS for 10mins, slides were covered with 1/100 dilution of rabbit anti-rat peroxidase-labelled IgG (Diaclone Research, France). After another wash in PBS, the slides were stained for peroxidase activity with 3-3′ Diaminobenzidin-tetrahydrochloride (Fast DAB, Sigma-Aldrich, France) for 10mins. Control slides were incubated in the same way, omitting the first antibody.

Histoenzymological Studies

To analyse enzymological characteristics of spleen macrophages, two enzyme activities present in most of macrophage populations were studied : esterase and acid phosphatase.

Spleen cryostat sections (5μm) were prepared as previously mentioned then, non-specific esterase was tested according to Flandrin and Daniel (3) with alpha-naphtol acetate as substrate and fast blue BB as coupling agent.

Acid phosphatase was tested according to Li *et al.* (4) with naphtol-As-Bi-phosphate as substrate and pararosaniline as a coupling reagent.

In Vitro Binding Assay

Spleens were cut in slices (250μm thick) with a vibratome (VT 1000 E, Leica). Tissue sections were incubated with polystyrene nanoparticles (50mg/l of polymer) or with 1% Indian ink (Lefranc Bourgeois, Le Mans, France) in IMDM (Gibco, France) supplemented with 10% FCS (Foetal Calf Serum, Gibco, France) at pH 7.4, 37°C, 5% CO_2 for 3hrs under rotation. Tissue sections were observed either with epi-fluorescence (polystyrene) or transmitted light (Indian ink) microscope (Olympus, Japan).

RESULTS

In Vivo Trapping of Nanoparticles

As early as 5mins after IV administration of fluorescently labeled polystyrene nanoparticles (98 and 185nm in diameter), fluorescent zones, the intensity of which increased during the first half hour following injection, began to appear. Although these sequential images were not of perfect quality, due to the respiratory movements of the live animal, they were very useful to identify in which splenic sites nanoparticles were trapped and at which rate. Moreover, they allow further *ex vivo* examinations of these precise tissue areas.

Ex Vivo Examination of Whole Spleen

When the spleens were removed at different time intervals after I.V. administration of nanoparticles and pressed against the observation window, images were much better, confirming *in vivo* observations—the first indentifiable fluorescent areas appeared 5mins after injection and reached a near maximal intensity at 3hrs. These areas appeared as being the same round zones as observed *in vivo* and according to their size and repartition, they were assumed to correspond to white pulp follicles (Fig. 2). But from this observation of the whole spleen, it was not possible to know if the fluorescence was located inside or around the follicle. The same results were observed with the two types of nanoparticles—polystyrene and doxorubicin-loaded PIHCA nanoparticles. However, it was noteworthy that if the spleen was taken off more than 24hrs after injection, a small part of the fluorescence was observed in the red pulp too. During the *ex vivo* examination of the whole spleen, no cell population was lost by manipulations and a global view of all the trapping areas was obtained, giving a good starting document for further investigations. Nevertheless, trapping areas were roughly identified by an outside view using a low magnification and histological studies were necessary for a more precise localization.

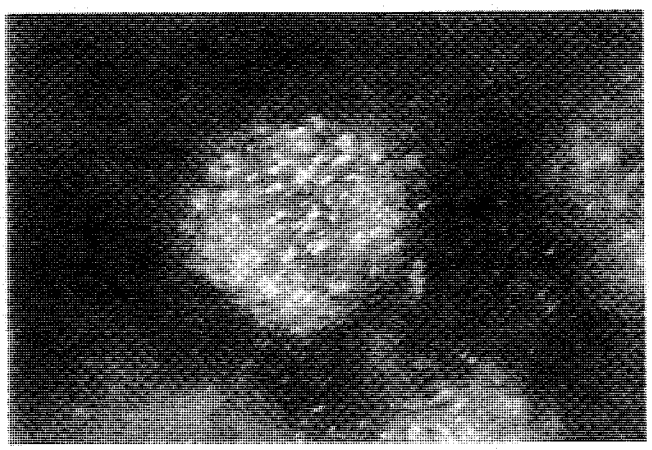

Fig. 2. *Ex vivo* examination of the whole spleen held against the observation window, 3hrs after I.V. injection of fluorescent polystyrene nanoparticles (epi-fluorescence, excitation wavelength 490nm, barrier filter 495nm). Capture was located in round areas, which according to their size and repartition were assumed to correspond to white pulp follicles. (magnification × 100)

Histological Studies

The observation of histological slices revealed that most of the fluorescence was located in a zone circling the white pulp follicles, with a very low fluorescence in the red pulp and almost no fluorescence in the white pulp (Fig. 3). This marginal zone has been described as an important site of spleen capture (5).

Combining fluorescence and Harris Haematoxylin coloration, it was observed that the cells containing most of the nanoparticles had a cytoplasm with long and multiple projections between neighboring lymphocytes. These cells seemed to be identical to the macrophage subpopulation with round or oval nucleus previously described by Humphrey and Grennan (6).

Moreover, confocal examination of some of these preparations showed that, 3hrs after injection, the nanoparticles were inside these cells and not adsorbed on their surface.

Cytological Studies

As marginal macrophages are tightly bound to the reticular meshwork and have long processes present at different levels of the histological slices, for this reason, it was not possible to obtain pictures showing clearly the cytological aspects of those cells using a high magnification. When we used mechanical dissociation of splenic tissue, most of the fluorescent trapping cells were lost. So, we have chosen to dissociate the tissue after collagenase treatment and to spread cells on glass slides by cytocentrifugation. Examination of the same area of these slides with epi-fluorescence (Fig. 4a) and transmitted light (Fig. 4b) showed that the capturing cells were large angular macrophages corresponding to the cells observed in the marginal zone on histological slices. Furthermore with transmitted light (Fig. 4b), these cells were characterized by a round nucleus and thin processes adhering to surrounding lymphocytes as previously described by Dijkstra *et al.* (5).

Semi-Quantitative Determinations

Results of image analysis (Fig. 5) showed that 3hrs after administration, fluorescently labeled polystyrene nanoparticles

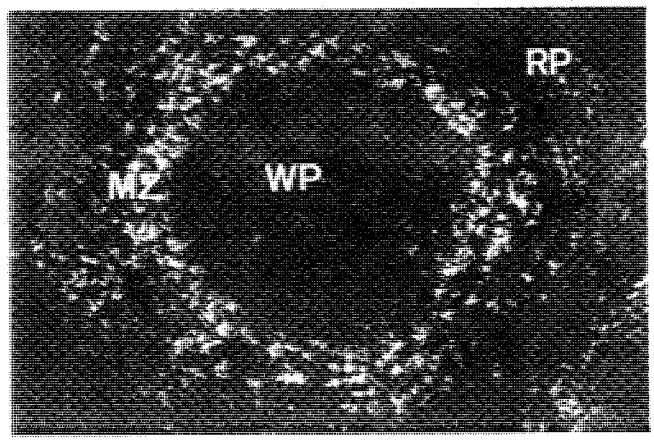

Fig. 3. Cryostat sections of mouse spleen (3hrs after I.V. administration of fluorescent polystyrene nanoparticles (epi-fluorescence) showing a rim of trapping cells in the periphery of white pulp follicles, corresponding to the areas observed *in vivo*. (magnification ×100)

were mainly located in the marginal zone (96%) with far fewer within the white pulp and the red pulp (about 2% each).

Image analysis, as experimented here, gave a much more precise evaluation of the distribution of nanoparticles among the splenic areas than a simple microscopic examination.

Immunohistological Studies

Cryostat sections of mouse spleen stained with anti-macrophage monoclonal antibodies (anti-sialoadhesin and F4/80) with a two-step immunoperoxidase method showed no reaction on capturing cells (Fig. 6a, 6b). On the contrary, red pulp macrophages (RPM) were positive with F_{4180} (Fig. 6b) whereas marginal metallophilic macrophages (MMM), circling the white pulp just under the ring of marginal zone macrophages (MZM), were strongly positive with anti-sialoadhesin (Fig. 6a).

Histoenzymological Studies

Simultaneous examination by transmitted light and epi-fluorescence of the slides allowed evaluation of enzyme activity in the capturing cells. There was a ring of strongly esterase positive cells around the white pulp follicles; these cells trapped few particles and were positioned like marginal metallophilic macrophages (MMM). Outside this area, a larger zone with moderate esterase and mild acid phosphatase positive cells showed a high trapping activity. These cells corresponded to marginal zone macrophages (MZM) (Fig. 7a, 7b). The low level of acid phosphatase and negative reaction for esterases made these trapping cells stand out as a rather special population, clearly different from the strongly positive ring of marginal metallophilic macrophages (MMM).

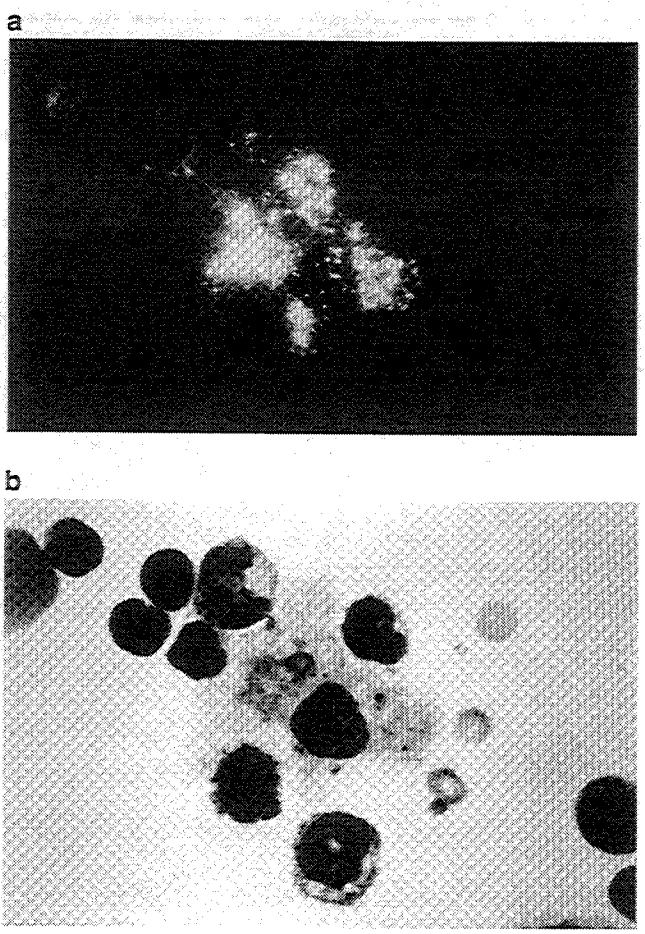

Fig. 4. Observation of capturing cells cytocentrifuged after dissociation of splenic tissue with collagenase. The same field of cytocentrifugated preparations stained with May-Grunwald-Giemsa was observed either with epi-fluorescence (4a) or transmitted light (4b). It showed a large central macrophage with surrounding lymphocytes. (magnification ×1000)

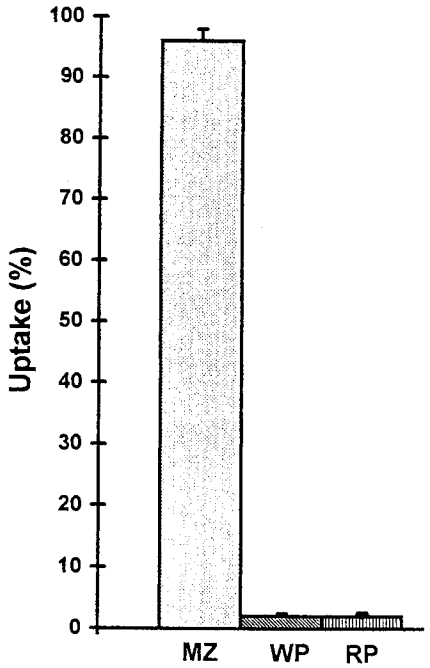

Fig. 5. Capture distribution of fluorescent polystyrene nanoparticles in different spleen areas evaluated by image analysis of cryostat sections (marginal zone MZ, red pulp RP, white pulp WP). 96% of the capture was localized in the marginal zone.

In Vitro Binding Assays

After *in vitro* incubation of nanoparticles with thick spleen slices, images showed that the distribution of these nanoparticles between the splenic zones was the same as that observed after intravenous administration: a predominant capture in the marginal zone (Fig. 8).

DISCUSSION

Several *in vivo* and *ex vivo* methods were used in this study to keep track of the cells capturing nanoparticles in the spleen, from the organ level to the cellular level. On the other hand, simultaneous examination by transmitted light and epi-fluorescence of the preparations led to more accurate localization and identification of the nanoparticles trapping cells.

In vivo microscopic examination of the whole spleen allowed the visualization and the description of the early capture of nanoparticles through the various areas of the spleen. In fact with the method used, a significant uptake of nanoparticles was observed, even 5 mins after injection. The distribution suggested follicular localization.

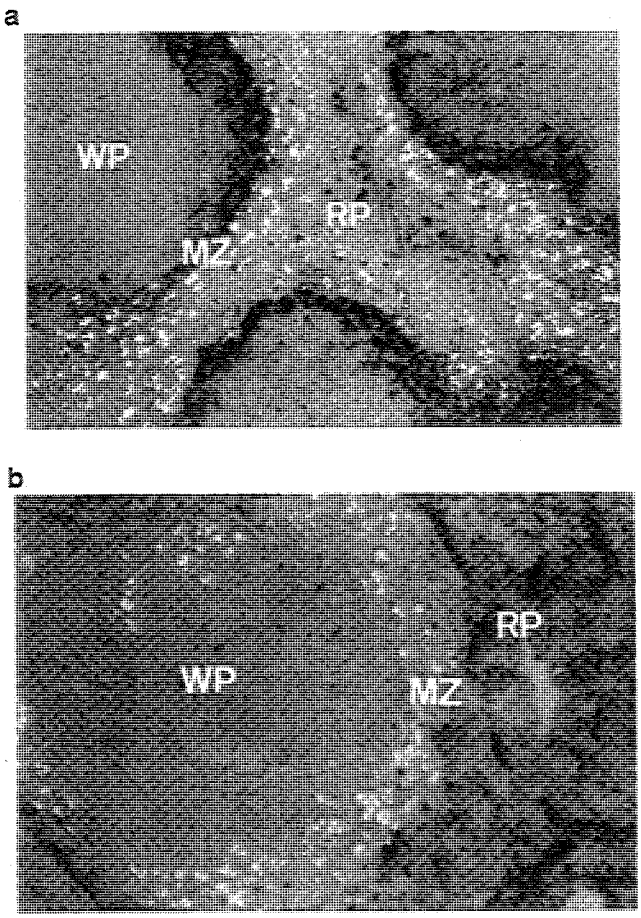

Fig. 6. Cryostat sections of treated mice spleens stained with anti-sialoadhesin (6a) and F4/80 (6b) antibodies with a two-step immunoperoxidase method. The capture observed with epi-fluorescence corresponded to cells showing no reaction with these antibodies. Red pulp cells were positive with F4/80 (6b). Marginal metallophilic macrophages were strongly positive with antisialoadhesin (6a). (magnification ×100)

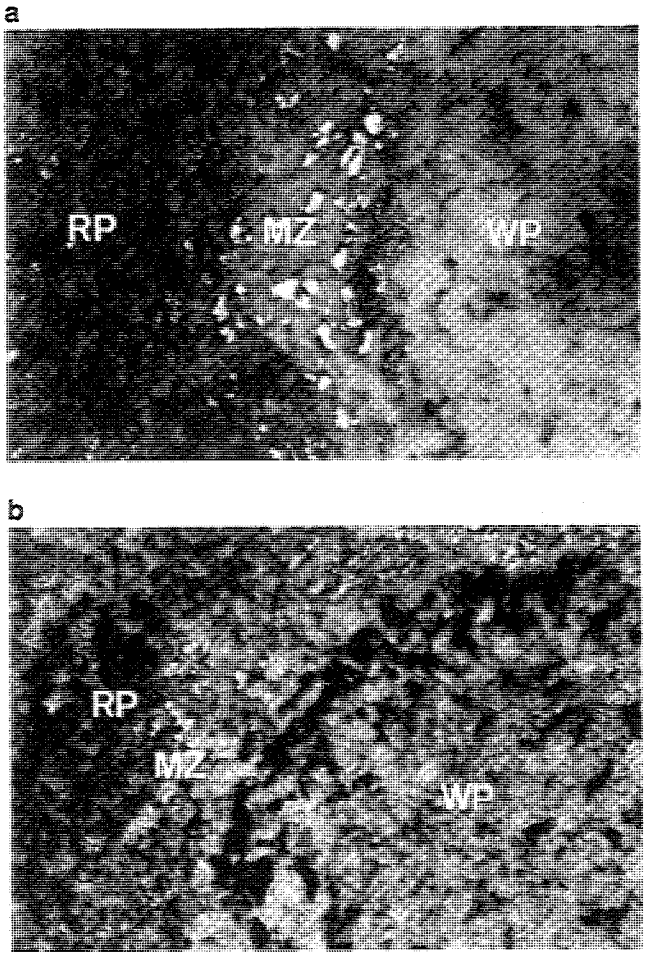

Fig. 7. Cryostat sections of treated mice spleens stained for acid phosphatase and non-specific esterase. The capture observed with epi-fluorescence corresponded to cells with moderate acid phosphatase (7a) and faint esterase (7b) activities. Red pulp cells and the rim of marginal metallophilic macrophages showed high positivity for the two enzymes. (magnification ×250)

The *ex vivo* observation of spleens confirmed these *in vivo* data, showing a high level of nanoparticle capture from 3hrs to 96hrs after administration. Moreover, we noticed that nanoparticle fluorescence was mainly located in angular cells of the perifollicular marginal zone. But it was difficult with this method to appreciate the differences of fluorescence intensity between 3hrs, 24hrs and 96hrs after I.V. injection because of fluorescence intensity saturation. Nevertheless, image analysis of sections obtained 3hrs after injection showed the highly predominant role of the marginal zone (96% of the fluorescence) in capturing the nanoparticles. Thus, *in vivo* and *ex vivo* microscopy fluorescence examination made clear the distribution profile of nanoparticles through the various areas of the spleen, whereas most of the methods reported in the literature did not allow such investigation. Previously published γ-scintigraphy studies in animals, only allowed observation of the capture in the whole organs (7,8,9). In addition, kinetic studies previously described, generally began 10mins after administration for the spleen (10) while earlier kinetics have been realized for hepatic, renal or lung uptake only (7).

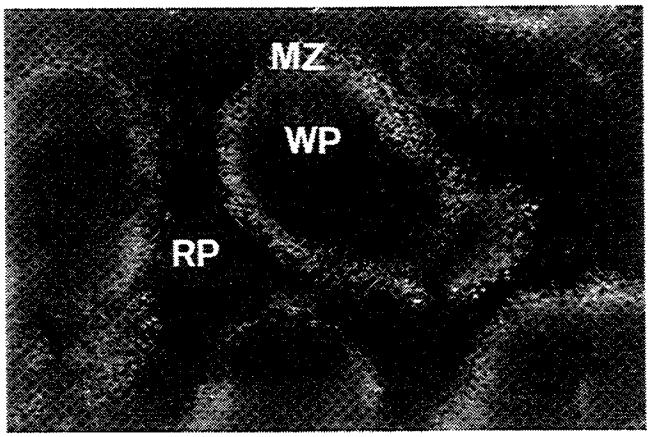

Fig. 8. *In vitro* binding assays: 250μm slice of spleen incubated 3hrs at 37°C with fluorescent polystyrene nanoparticles, viewed with epi-fluorescence, showing a preferential capture by the marginal zone. (magnification ×40).

The present histological and cytological studies also specified the cells involved in nanoparticle splenic capture. These actively capturing cells, tightly bound to a reticular meshwork by their long cytoplasmic processes, are partly lost by classical techniques of cells suspension preparation, so that their role was generally underestimated and their properties only partially known. These cells were called reticular cells and described by Kraal as marginal macrophages (11). Nevertheless, we showed that these particular cells share only part of the macrophages characteristics: they have low esterase and mild acid phosphatase activity, do not react with F4/80 or anti-sialoadhesin antibodies, contrary to the behavior of most of mice macrophages. These immunological and enzymological particularities may be useful for identification of these cells on histological and cytological preparations.

In our studies, the two sizes of polystyrene nanoparticles used (98 and 185 nm) led to similar spleen capture intensity and distribution: they are both trapped in the same area by the same cells and with comparable kinetics. This is in contradiction with previous studies by Porter *et al.* (12), Moghimi *et al.* (13) and Storm *et al.* (14) showing an increased spleen capture by increasing the size of the nanoparticles used (60, 150 and 250nm). This difference could be attributed to the different animal species used—rabbits and rats (sinusoidal spleen) instead of mice (non-sinusoidal spleen) (15).

Concerning the nature of the polymer, non-biodegradable highly fluorescent polystyrene nanoparticles were mainly used in this study, because they were easier to localize, avoiding decrease or diffusion of fluorescence due to polymer biodegradation. It is, however, important to point out that these nanoparticles were only used as a model to understand the first steps of nanoparticle spleen uptake. Using biodegradable PIHCA nanoparticles, it appeared that the type of polymer did not change either the nature of the splenic trapping areas or the cells involved in this capture: whatever the type of nanoparticles and whether biodegradable or not, they were trapped in the marginal zone by the same angular cells.

The massive spleen marginal capture of nanoparticles might be attributed to the high blood flow passing through this area, leading to a sieving effect (16). However, as spleen slices directly incubated *in vitro* with nanoparticles showed that most of them were associated with the marginal zone, other mechanisms are certainly involved. Moreover, as explained before, this capture was observed to have limited specificity since doxorubicin-loaded PIHCA nanoparticles were captured in this area as well as polystyrene. Thus, adhesive proteins present in the marginal zone are likely to be involved in nanoparticle spleen capture. Experiments are under way to explore this possibility and to further identify the underlying mechanisms.

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REFERENCES

1. C. Verdun, F. Brasseur, H. Vranckx, P. Couvreur, and M. Roland. *Cancer Chemother. Pharmacol.* **26**:13–18 (1990).
2. I. C. Mac Donald, D. M. Ragan, E. E. Schmidt, and A. C. Groom. *Microvascular Res.* **42**:60–76 (1991).
3. G. Flandrin and M. Th. Daniel. *Path. Biol.* **19**:9–10, 547–555 (1974).
4. C. Y. Li, L. T. Yam, and K. W. Lam. *J. Histochem. Cytochem.* **18**:901 (1970).
5. C. D. Dijkstra, E. Van Vliet, E. A. Döpp, A. A. Van Der Lelij, and G. Kraal. *Immunology.* **55**:23–30 (1985).
6. J. H. Humphrey and D. Grennan. *Eur. J. Immunol.* **11**:221–228 (1981).
7. L. Grislain, P. Couvreur, V. Lenaerts, M. Roland, D. Deprez-Decampaneere, and P. Speiser. *Int. J. Pharm.* **15**:335–345 (1983).
8. L. Illum and S. S. Davis. *FEBS Lett.* **167**(1):79–82 (1984).
9. L. Illum, S. S. Davis, R. H. Müller, E. Mak, and P. West. *Life Sci.* (1987), **40**:367–374 (1987).
10. J. E. O'Mullane, K. Petrak, L. E. F. Hutchinson, and E. Tomlinson. *Int. J. Pharm.* **63**:177–180 (1990).
11. G. Kraal. *Int. Rev. Cytol.* Academic Press. Inc. 1992, pp. 31–74.
12. C. J. Porter, M. C. Davies., S. S. Davis, and L. Illum. *Polymeric Site Specific Pharmacotherapy.* A. J. Domb, John Wiley & Son Ltd, 1994, pp. 57–203.
13. S. M. Moghimi, C. J. Porter, I. S. Muir, L. Illum, and S. S. Davis. *Biochem. Biophys. Res. Commun.* **177**(2):861–866 (1991).
14. G. Storm G., S. O. Belliot, T. Daemen, and D. D. Lasic. *Adv. Drug Deliv. Rev.* **17**:31–48 (1995).
15. T. Snook. *Am. J. Anat.* **87**:31–61 (1950).
16. A. C. Groom, E. E. Schmidt, and I. C. Mac Donald. *Scanning Microscopy* **5**:159–174 (1991).