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Ex vivo and in situ PLGA microspheres uptake by pig ileal Peyer's patch segment

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Abstract

We investigated the ability of pig ileal Peyer's patch segments to transport intestinal poly (D,L-lactide-co-glycolide) microspheres (PLGA MS) from intestinal lumen across the mucosae using in situ and ex vivo segments with confocal laser scanning microscopy (CLSM) and transmission electronic microscopy (TEM). From a global aspect, CLSM suggested that PLGA MS were translocated by M cells labelled with a FITC-conjugated anti-cytokeratin peptide 18, and transported through the follicle-associated epithelium (FAE) in the dome area in both types of experiments. At the ultrastructural level, TEM showed the traffic of PLGA MS throughout M cells, their transport into the basolateral invaginations of the M cells and their subsequent migration into the dome area and the follicular area in contact with macrophages and lymphatic vessels. Although in situ experiments allowed following the migration of PLGA MS until mesenteric lymph nodes, an ex vivo model could be used as a useful tool to study the targeting ability of PLGA MS formulations to the gut-associated lymphoid tissue (GALT). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Pig Peyer's patch; PLGA microspheres; Ex vivo experiment; In situ experiment

1. Introduction

Among the compartments of the mucosae-associated lymphoid tissues (MALT), attention has been focused largely on the Peyer's patches (PP) of the intestine. A consistent feature of the gut-associated lymphoid tissue (GALT) is a distinctively differentiated lymphoepithelium called follicle-associated epithelium (FAE) capable of non degradative transport of soluble or solid materials from

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the lumen of the gut to affiliated lymphoid tissues located intra and subepithelially. These tissues play a critical role in immune surveillance of mucosal tissues and by a close interaction with cells of the immune system, immunological response or tolerance can be initiated. The cells thought to play primarily a predominant role in this phenomenon are M cells that are a distinguishing feature of lymphoepithelium (Gebert et al., 1996; Gebert, 1997).

The uptake of large particulates such as microorganisms (Grützkau et al., 1990; Clark et al., 1994; Regoli et al., 1995a; Neutra et al., 1996) across the FAE has been previously demonstrated in different species. It is of interest for the area of vaccine delivery systems to exploit the M cell transcellular pathway to gain entry in the GALT in a similar manner to microorganisms (Eldridge et al. 1990, 1991).

Nano- and microparticles based on polystyrene (Sass et al., 1990; Jepson et al., 1993a,b; Hodges et al., 1995) and, to a lesser extent, on other polymers (Ermark et al., 1995; Mathiowitz et al., 1997) have been successfully used to define and characterize the uptake and translocation in the intestine. Although polystyrene particles are not suitable to deliver vaccines, they have been used to study the effects of various parameters on the uptake efficiency: size, hydrophobicity and influence of surface residues allowing adsorption or covalent binding of specific substrates. However, it is not clear whether data derived from such studies can be suitably extrapolated to poly(D,L-lactide-co-glycolide) microspheres (PLGA MS) (Jepson et al., 1993b).

The efficiency of oral vaccination with PLGA MS has been assessed in laboratory animals, mainly on mice. The induction of systemic IgG and secretory IgA antibody responses following oral immunisation with staphylococcal enterotoxoid B encapsulated in PLGA MS was first reported by Eldridge et al. (1990). Notable results were obtained by O'Hagan and co-workers (Challacombe et al., 1992; Maloy et al., 1994; Challacombe et al., 1997), Uchida et al. (1994) and Tabata et al. (1996), who showed efficient salivary and serum antibody responses after oral immunization with ovalbumin encapsulated in PLGA MS.

In the case of farm animals, oral administration of antigen-encapsulated microparticles via food is, by many aspects, of interest to induce a mucosal immunisation directly at the site of infection.

To our knowledge, few studies are available in the specific area of particles targeting to porcine mucosal lymphoid tissues, despite the paramount economic interest of an oral vaccine in such farm animals.

The other aspect of vaccine delivery is the production of a potent microsphere formulation. However, considering the number of pharmacotechnical parameters involved in MS fabrication, a great number of MS formulations have to be studied. Thus, ex vivo models should be interesting for screening formulations before oral administration to the animal. In previous studies, we have described an ex vivo model based on MS phagocytosis by alveolar macrophages to screen MS according to their uptake characteristics (Torché et al., 1999; Torché et al., in press). Such a simple model using macrophages allowed a rapid screening of MS characteristics. However, a more selective screening has to be performed using the cells of interest, i.e. M cells.

Among the published experiments studying particles intestinal uptake, direct oral administration and in situ ligated loops have both been employed. The goal of the present study was to investigate an alternative model allowing study of the uptake ability of PLGA MS by pig ileal PP using ex vivo intestinal segments and their subsequent transport using confocal laser scanning microscopy (CLSM) and transmission electronic microscopy (TEM). Since intestinal integrity should be checked to assess this model, the experiment was performed in situ to compare with the ex vivo situation.

2. Materials and methods

Intestinal segments washings were achieved with Phosphate Buffered Saline pH 7.4 (PBS). The ex vivo tissue incubation was performed in Krebs–Ringer Bicarbonate buffer (KBR) (Sigma, Saint Quentin Fallavier, France). For immunohistochemistry, the following antibodies were used: monoclonal anti-cytokeratin peptide 18 (clone

KS-B17.2; Sigma, Saint Quentin Fallavier, France), rabbit anti-mouse immunoglobulins (Z0412; Dako, France) monoclonal mouse PAP (P0850; Dako, France) and FITC-conjugated rabbit anti-mouse immunoglobulins (F0313; Dako, France). Antibodies were diluted in ChemMate™ antibody diluent (S2022; Dako, France).

Poly(D,L-lactide-co-glycolide) with a molar ratio of 75:25 (Resomer® RG755; Mw = 15 kDa) was obtained from Boehringer-Ingelheim (Labso Chimie Fine, Paris, France). Fluorescent dye, Rhodamine B base, was supplied by Sigma (Saint Quentin Fallavier, France). The other reagents were poly(vinyl alcohol) (Rhodoviol 4/125; Prolabo, Nogent sur Marne, France), and dichloromethane RPE-ACS (Carlo Erba Reactifs, Val de Reuil, France). All chemical reagents were obtained from commercial suppliers and used without further purification.

2.1. Preparation and characterization of fluorescent microspheres

Fluorescent microspheres (MS) were prepared according an O/W emulsion and solvent evaporation/extraction method previously described (Le Corre et al., 1994). The aqueous phase (pH 8) composed of 0.01 M Tris and 0.1% of poly(vinyl alcohol) was cooled to 5°C using a Ministat cooler (Huber, Germany). The organic phase, composed of rhodamine B base (1%, w/v), PLGA (RG755; 5%, w/v) and dichloromethane, was progressively poured into the external phase and emulsified for 15 min at 3000 rev./min (Polytron, Kinematica, Switzerland). Solvent extraction was performed by dilution of the emulsion with distilled water and subsequent magnetic stirring for 60 min at 15°C under vacuum. The microspheres were collected by filtration through a 1-μm pore size membrane, rinsed with distilled water, frozen, lyophilised and stored at + 4°C.

Microspheres were sized by laser diffractometry using a Mastersizer S (Malvern Instruments, Orsay, France). The particle size was expressed as the number diameter (μm) to allow an estimation of the number of microspheres per g. The surface of microspheres was examined by scanning electron microscopy (Jeol 6400, Tokyo, Japan).

Measurements of the zeta potential were carried out using a Malvern Zetasizer 3000 (Malvern Instruments, Orsay, France). The microspheres were suspended in a low ionic strength solution (1 mM KCl). The experiment was performed in triplicate. Data were expressed as the mean value ± S.D.

2.2. Ex vivo experiment

An 8-month SPF pig was used in this study. The pig had free access to food and water prior to the experiment. The pig was intravenously anaesthetized with 20 mg/kg of thiopental sodium (Nesdonal, Merial, France) and the abdomen was opened aseptically. A distal part of the continuous Peyer's patch ileum was excised, then the animal was killed by exsanguination. The ileal piece was immediately put in a beaker containing cold PBS (+ 4°C) and cut into 14-cm segments. The lumen of the segments was gently cleansed with PBS at room temperature. Then, the segments were ligaturated (length = 10 cm; $n = 2$), filled with a suspension of 10^9 MS in 8 ml of KBR and immediately placed for 1 h in a bath containing KBR gassed with O_2/CO_2 (95:5%). The experiment was carried out at 37°C. After 1 h, the ileal segments were recovered and the MS suspension was removed. The segments were longitudinally cut and washed three times with 20 ml of PBS to remove residual adhering MS. The tissues were maintained in KBR during the preparation of the different microscopic samples. A control ligaturated segment was performed incubated under the same conditions without MS.

2.3. In situ experiment

An 8-month SPF pig was used in this study. The pig had free access to food and water prior to the experiment. The pig was anaesthetized as previously indicated and the abdomen was aseptically opened. A distal part of the continuous Peyer's patch ileum was moved out of the abdominal cavity and the intestinal content was mechanically evacuated to the distal part. The proximal end of a segment drained by a mesenteric node was ligaturated and care was taken to isolate a gut

segment without cutting the connection with blood and lymphatic supplies. A suspension of 10^9 MS in 8 ml of PBS was instilled into the lumen and the distal part of the segment was ligaturated. The loop was then returned to the abdominal cavity for 1 h. Then, the ileal segment and the draining mesenteric lymph node were excised and immediately placed in PBS before washing as described above. After resection of the segment, the animal was killed by exsanguination. The segments were cut longitudinally and washed with PBS. The tissues were maintained in PBS during the preparation of the different microscopic samples. A control ligaturated segment processed as above and instilled with PBS was performed on the same pig.

2.4. Histological studies

2.4.1. Immunohistochemistry

Tissue samples from the ex vivo experiment were fixed in 10% formol. Then, the tissue was sliced into 3- to 4-mm-thick sections, dehydrated in ethanol gradient solutions and embedded in paraffin. Histological sections (5 μm) were cut, deparaffinized and rehydrated in ethanol gradient solutions. The slices, pre-treated with protease, were incubated with anti-cytokeratin peptide 18 antibody diluted at 1/50 for 1 h at 37°C. After washing in PBS, the slices were incubated with diluted rabbit anti-mouse immunoglobulins antibody (1/50) for 30 min at room temperature. After washing, the slices were stained by the peroxidase-anti-peroxidase procedure for 30 min. The chromogenic substrate was developed by incubation in diaminobenzidine (DAB) for 10 min. Finally, the slices were stained with haematoxylin. The slices were examined using a binocular microscope (Leitz Diaplan) equipped with a photographic system.

2.4.2. Confocal laser scanning microscopy

Tissue samples from ex vivo and in situ experiments were embedded in Tissue Tek OTC compound (Miles, USA), frozen and stored at −20°C. Frozen tissues were cut into 10-μm sections using a cryostat and air-dried. The slices were fixed in ethanol/acetic acid (95:5). After washing with distilled water, the slices were incubated with anti-cytokeratin peptide 18 antibody diluted in PBS for 1 h at 37°C. After washing in PBS, the slices were incubated with diluted FITC-conjugated anti-mouse immunoglobulins antibody for 30 min in the dark. The slices were imaged with oil immersion lenses. The confocal microscope system used in this study was a Leica TCS NT (Leica, France) equipped with Kr/Ar laser and mounted on a Leica DMB microscope. Rhodamine and FITC fluorescence were detected with TRITC and FITC filter sets, respectively.

Tissue samples from ex vivo experiment were fixed in 10% formol. Then, the tissue was sliced into 3- to 4-mm thick sections, dehydrated in ethanol gradient solutions and embedded in paraffin. Histological sections (5 μm) were cut, deparaffinized and rehydrated in ethanol gradient solutions. Then, the slices were stained with haematoxylin and eosin and examined as previously described. A control was achieved with MS treated like histological tissue samples and observed as previously described.

2.4.3. Transmission electron microscopy

Tissue samples for transmission electron microscopy were fixed by immersion in a 2.5% glutaraldehyde solution at 4°C and then stored and rinsed in 0.2 M cacodylate buffer (pH 7.4). Tissues were post fixed in 2% osmium tetroxide, dehydrated in a series of acetone aqueous gradient solutions and then embedded in Epon/araldite. Semi-thin sections stained with toluidine blue were mounted in glass slides for light microscopy. Ultra thin sections cut using glass knives were stained with 5% uranyl acetate and examined with transmission electron microscopy (Jeol-100CX II; Jeol, Japan).

A control was achieved by melting whole blood with MS in a solution containing 2.5% glutaraldehyde at 4°C for 1 h and rinsing in 0.2 M cacodylate buffer (pH 7.4). The suspension was embedded in agar and was post-fixed in 2% osmium tetroxide, dehydrated in a series of acetone aqueous gradient solutions and then embedded in Epon/araldite. Ultra thin sections were prepared and observed as previously described.

3. Results and discussion

3.1. Characterization of fluorescent microspheres

The results of size distribution analysis obtained by laser diffractometry indicated that 50% of the MS were ≤ 2.6 μm and 90% were ≤ 5.8 μm. By SEM examination, the PLGA MS were spherical in shape with few pores on the external surfaces (Fig. 1).

No rhodamine leaching was spectrofluorometrically observed until 3 days and the microspheres were still fluorescent after 7 days, (unpublished results).

Surface properties of MS, i.e. surface charge and hydrophobic character, are known to influence their uptake in the intestine (Florence et al., 1995). The MS used in the current study had a negative surface charge (-20.3 ± 0.4 mV).

3.2. Histology of the FAE

The labelling pattern of M cells by specific antibody directed against cytokeratin 18, the major component of the intermediate filaments, was previously described by Gebert et al. (1994) for Gôttingen minipigs. In fact, all the cells of the intestinal simple epithelium basically express cytokeratin 18, but by comparison with the other epithelial cells, the pig M cells contain a larger amount of cytokeratin 18. In the conventional pig, we showed that M cells were densely labelled by the anti-cytokeratin 18 antibody with a noticeable dense staining of the apical part. According to the labelled cells observed in the FAE representative cell population (Fig. 2), the number of M cells could be estimated as almost 30–40%. These M cells were mainly localized on the flanks of the dome epithelium, rather than on the top of the dome suggesting that MS distribution into the pits should maximize the uptake.

Contrary to what was generally described (Gebert, 1997), we found goblet cells in the FAE (Fig. 2). The M cell labelling allowed seeing clusters of intraepithelial cells interspersed between M cells (Fig. 2) that are mainly known to be lymphocytes (Chu et al., 1979; Pabst, 1987; Regoli et al., 1995b).

On the other hand, the histological observation has shown an intestinal tissue integrity supporting the apparent viability of the tissue during the 1-h incubation in the ex vivo experiment.

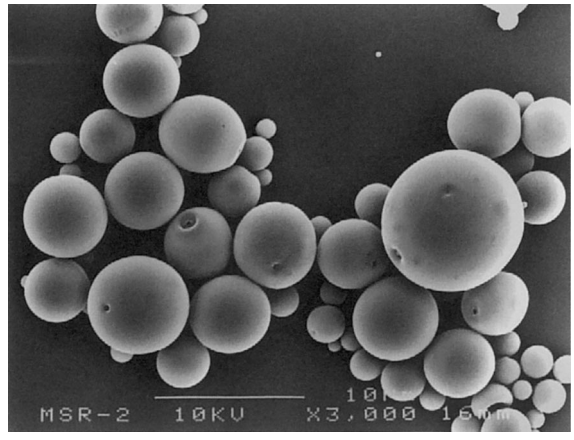


Fig. 1. Scanning electron micrographs of fluorescent microspheres composed of poly(DL-lactide-co-glycolide) and rhodamine B base. Magnification $\times 3000$.

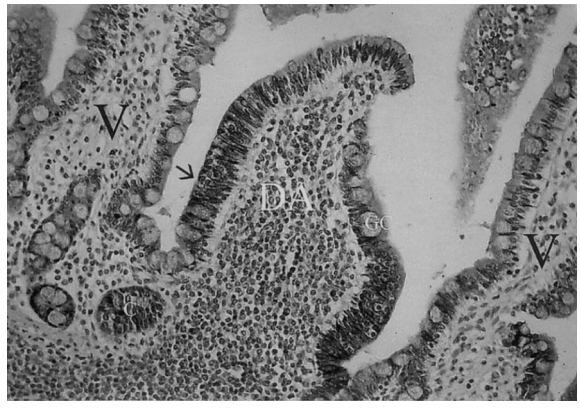


Fig. 2. Immunostaining of histological section of pig ileal Peyer's patch for cytokeratin 18 M cells marker. Ex vivo intestinal segments were incubated with PLGA MS for 1 h. M cells were detected in the follicle-associated epithelium (FAE) by the dark staining in the cytoplasm. M cells were mainly located at the flanks of the dome area (DA). Intraepithelial spaces infiltrated by clusters of intraepithelial lymphocytes were detected (arrow). DA was densely filled with lymphocytes. No cytokeratin reactivity was identified in the adjacent villi (V) but cytokeratin staining was detected in the crypt (C). Goblet cells (GC) were identified in the FAE. Magnification $\times 140$.

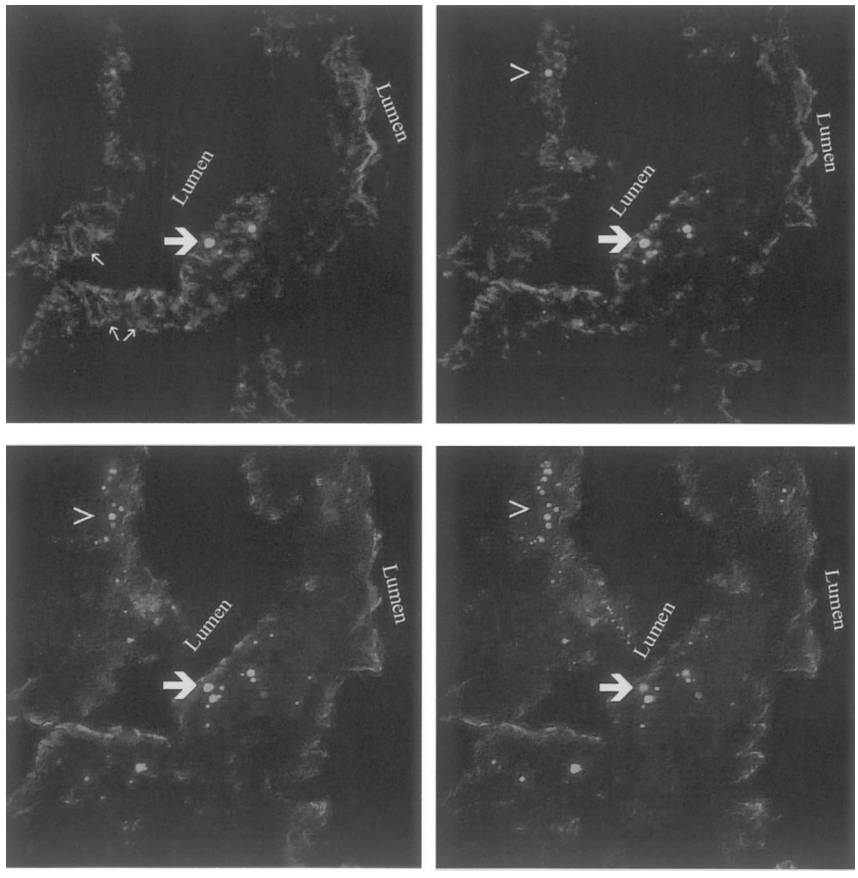


Fig. 3. Montage of four confocal microscope images obtained by indirect immunofluorescence of the same longitudinal cryosection of pig ileal Peyer's patch segment. In situ intestinal segments were incubated with PLGA MS for 1 h. M cells were detected in the follicle-associated epithelium by the green fluorescent labelling using an anti-cytokeratin 18 antibody. Fluorescent PLGA MS appear in red. The images are separated by intervals of approximately 4 μm. Small PLGA MS are indicated by an arrowhead and an example of a large MS is indicated by an arrow. Noticeable cytokeratin rings of M cells are indicated by small arrows. Magnification $\times 112$.

3.3. Observation of microspheres uptake

Many physiological and physicochemical factors are known to affect intestinal uptake of particles, e.g. particle size, particle surface characteristics, animal species, age of the animal, administered dose (O'Hagan, 1996; Seifert et al., 1996).

The determination of the maximal size of MS above which uptake apparently does not occur has been explored in a number of studies with contradictory results, even in the same animal species. Such discrepancies may result from different evaluation techniques: microscopy (Sass et al., 1990; Jani et al., 1992a,b; Damgé et al., 1996), flow cytometry (Jenkins et al., 1994; Smith et al. 1995), tissue dissociation (Sass et al., 1990; Ebel, 1990; Le Ray et al., 1994; Hodges et al., 1995), that should explain differences reported in the literature.

According to Eldridge et al. (1990), microspheres in a size range of 5–10 μm stay rather in the lamina propria while the smaller (1–5 μm) particles leave the follicle. Several observations in rodents (Eldridge et al., 1990; Jenkins et al., 1994; Carr et al., 1996) showed that transcytosis occurred for PLGA and polystyrene particles in the same size range as the MS we used, i.e. between 1

and 10 μm. Hence, it was of interest to investigate the uptake of polymeric particles in the micron range in pig PP that had not yet been studied.

Although MS size is an important parameter in particle uptake, other characteristics, such as physicochemical properties of the MS surface (Florence et al., 1995; Foster et al., 1998), may influence and affect the extent of particle uptake since they govern their interactions with the biological environment.

Direct oral administration and in situ models give overall information about uptake and further distribution of translocated particles. The former should provide a more realistic assessment of MS uptake since, under physiological conditions, the mucus turn over, the fed state of the animal and the sweeper wave may affect the MS transport. Although, it could be suggested that the in situ model alters the homeostatic environment and the high local concentration of MS increases the uptake, the effectiveness of this approach is the interest to assess accessibility of the MS to M cells in a concentrated follicle area such ileal PP, which could not be clearly confirmed without using explants ex vivo or a ligated intestinal loop.

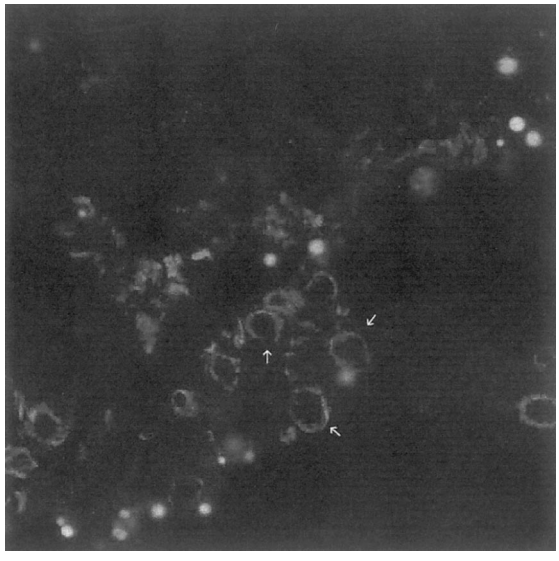


Fig. 4. Confocal microscope images obtained by indirect immunofluorescence of a cryosection cut in a plane parallel to the mucosal layer of pig ileal Peyer's patch segment. Ex vivo intestinal segments were incubated with PLGA MS for 1 h. M cells were detected in the follicle-associated epithelium by the green fluorescent staining using an anti-cytokeratin 18 antibody, some of the cytokeratin rings of M cells were indicated by small arrows. PLGA MS appeared in red. The image came from the first third of the image gallery (observation from the luminal side to the follicle depth), PLGA MS being clearly localized deeper in the mucosae. Magnification × 350.

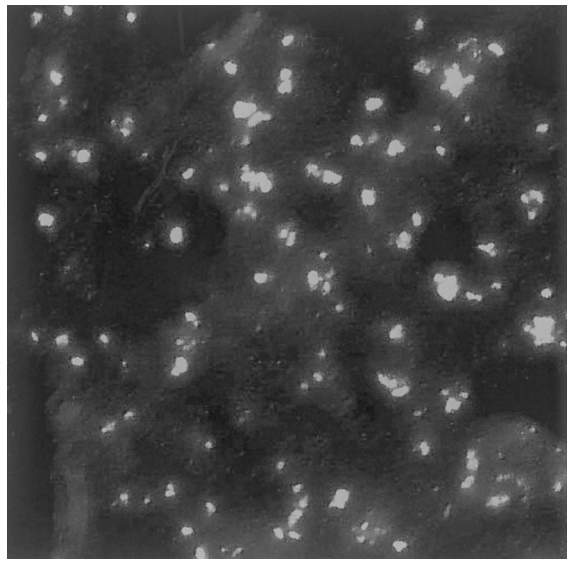
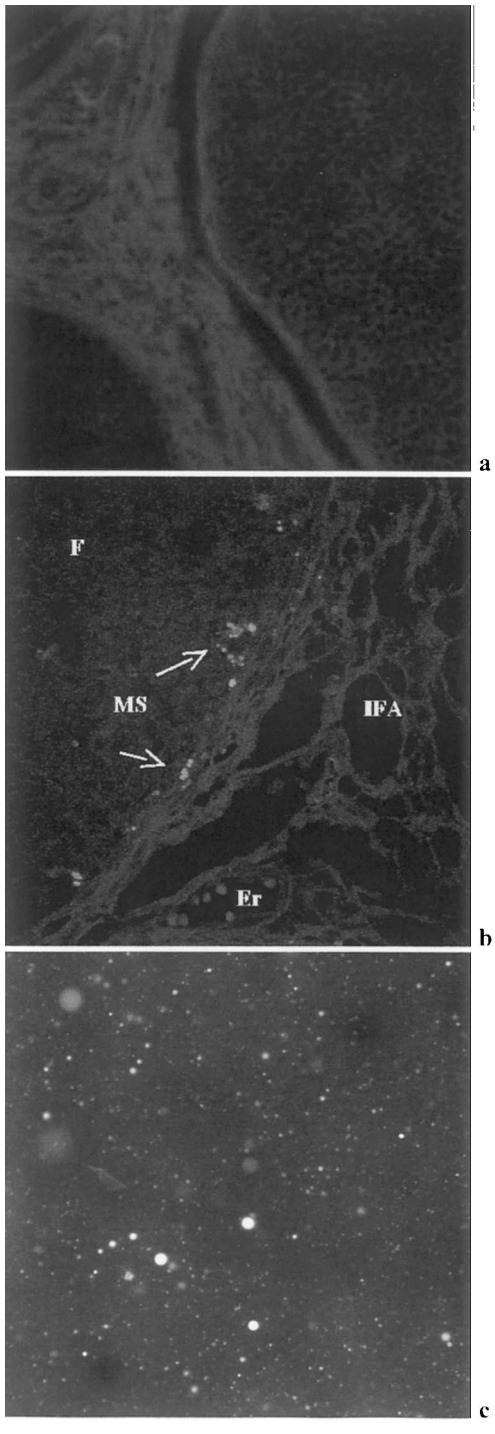


Fig. 5. Confocal microscope image obtained of a cryosection of a pig mesenteric lymph node. In situ intestinal segments were incubated with PLGA MS for 1 h. PLGA MS corresponded to white spots. Magnification × 448.

Interspecies and/or interanimals differences are factors which may prevent the comparison to others models. Actually, physiological differences should be considered since PP, and especially M cells number, varied between species (Torres-Medina, 1981; Jepson et al., 1995), and could have an influence on the extent of particle uptake. For example, within the same species and within the same animal, i.e. pig, the extent of yeast uptake has been shown to be variable along the GALT (Beier and Gebert, 1998).

In order to reduce variability and to target the intestinal lymphoid follicles, the technique of in situ closed intestinal loop has been used (Sass et al., 1990; Porta et al., 1992; Damgé et al., 1996). However, in situ models are time- and cost-consuming to screen a large number of particle formulations and it is hardly conceivable to make several ligated loops in one animal.



Thus, ex vivo models should be interesting for screening formulations before oral administration to the animal. If intestinal particle uptake has been extensively characterized in situ in laboratory animals, few systematic studies have been performed ex vivo using a pig intestinal loop with a MS suspension (Scherer et al. 1993). In the current study, the PLGA MS uptake ability by ileal PP was studied both in situ and ex vivo. The ex vivo experiment was performed under incubation conditions commonly used to study intestinal permeability.

The pig presents a long continuous ileal PP ($\#$ 2.5 m) that regresses from maturity (Pabst et al., 1988). For oral vaccination, it is of interest to exploit and target this lymphoid tissue before its involution. Such a long intestinal loop could be divided into several segments allowing the investigation, with the same animal, of experimental variables such as MS characteristics and incubation conditions. From a complementary manner, in situ investigation on an isolated segment drained by a mesenteric lymph node should allow the study of the first step of the subsequent dissemination of the MS in the other compartments of lymphoid tissue.

With epifluorescence microscopy, the major problem is the high background induced by the thickness of the observed slice and results from the excitation of all the fluorochromes that are situated on the light ray course. With confocal microscopy, the object is not illuminated and imaged as a whole at the same time but at one point after an other like an optical sectioning, thus improvements of the image definition were obtained. The low depth of the focal plane allows the reduction or even the elimination of the fluorescent background. The scanning of the object was successively processed with the filters for rhodamine and for FITC and the final image

Fig. 6. Confocal microscope images obtained from histological section, cut in a plane parallel to the mucosal layer of pig ileal Peyer's patch segment. (a) Control tissue incubated without microspheres. (b) Ex vivo intestinal segments were incubated with PLGA MS for 1 h. PLGA MS (arrow) appeared in clear spots in the follicular area (F) and erythrocytes (Er) were localized in a capillary or venule of the interfollicular area (IFA). (c) Control microspheres. Magnification $\times$ 230.

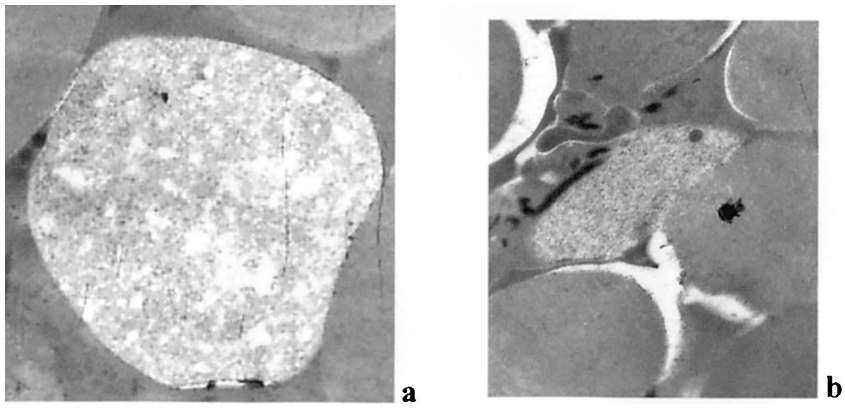


Fig. 7. Transmission electron microscopic observations of PLGA-MS (asterisks) resulting from the mixing of MS with red blood cells and subsequently processed like intestinal tissues. PLGA MS were not fully degraded after acetone treatment and became randomly distorted, the observed variability being increased according to the PLGA MS cutting plane. Magnification × 10 250.

results from the overlay treatment of the software. Confocal microscopic study avoids a bias due to a potential contamination by residual MS during slice preparation that could occur in traditional fluorescence microscopy. However, the main interest is the observation of the distribution and depth of penetration of fluorescent MS in the tissue. In both the ex vivo and in situ experiments, PLGA MS were identified at different levels throughout the PP indicating that PLGA MS were able to transcytose and to migrate through mucosae. Such observations indicated a functional viability under both ex vivo and in situ conditions. Fig. 3 shows four different depths of the same slice obtained in the in situ experiment. Some MS have just left the FAE, while other MS were deeper in the dome area and few MS were still localized in the FAE. As observed previously, the green labelling corresponding to M cells was stronger at the apical part of the cells. These images clearly showed the presence of MS facing the green labelling indicating that uptake occurred via M cells. Furthermore, since the images were separated by intervals of approximately 4 μm, the progressive appearance and disappearance of fluorescent spots allowed distinguishing small and large MS. According to these images, it was obvious that smaller MS were taken up to a greater extent, however porcine M cells were able to transcytose large MS.

The transverse section, cut in a plane parallel to the mucosal layer, passing through the FAE, showed that cytokeratin 18 formed a dense ring in the cytoplasm of the M cells which is correlated with its function within the cytoskeleton and MS which have been released in the intra-epithelial pockets in both ex vivo and in situ experiments. Fig. 4 illustrated such a phenomenon obtained ex vivo. The suitability of the ex vivo model investigation is supported by the fact that the distribution of MS in the FAE and follicles was similar to that observed with the in situ model. However, ex

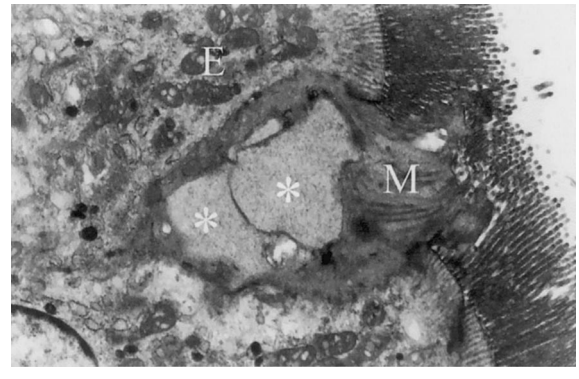


Fig. 8. Transmission electron microscopic image obtained from a section of the pig ileal Peyer's patch. Ex vivo segments were incubated with PLGA MS for 1 h. At the apical part of an M cell (M) the beginning of the transcytosis of PLGA MS (asterisks) is shown. The M cell is surrounded by two enterocytes (E). Magnification × 8400.

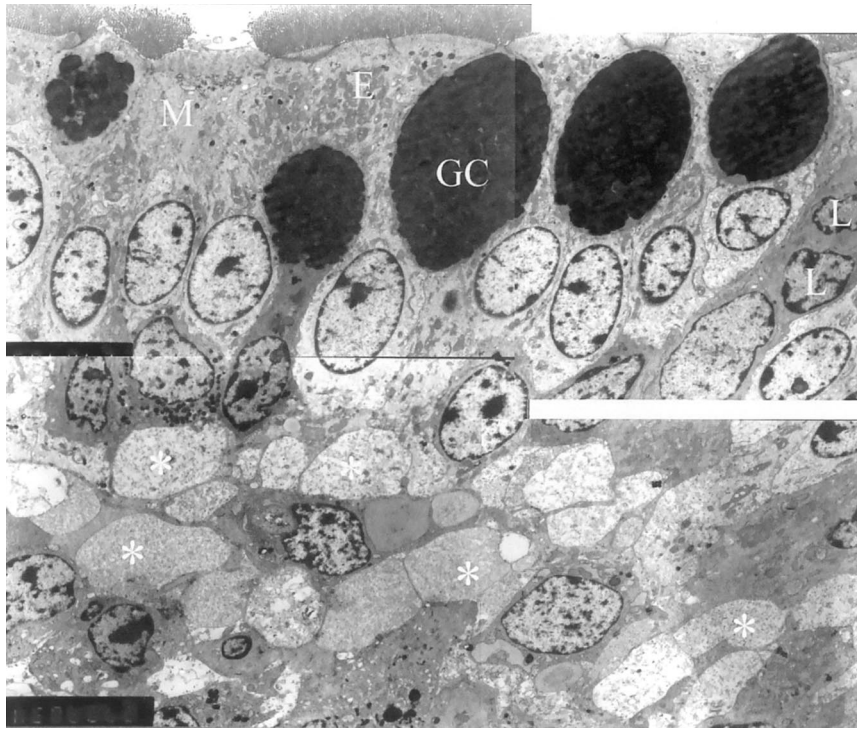


Fig. 9. Montage of four transmission electron microscopic images obtained from a section of pig ileal Peyer's patch segment. Ex vivo segments were incubated with PLGA MS for 1 h. The apical part of M cell (M) is distinguished between adjacent enterocytes (E) and goblet cells (GC). PLGA MS (asterisks) are densely packed beyond and above the basal lamina. Intraepithelial lymphocytes (L) are present in the invagination of the basal part of the M cell inducing intraepithelial pockets of the FAE. Magnification $\times 3200$.

vivo experiments have some limitations, the study of the transport in the subsequent compartments of the mucosal-associated lymphoid tissues, i.e. mesenteric lymph nodes, being precluded. The migration of MS to the draining mesenteric node was observed in situ (Fig. 5). Indeed, in the current study, we used PLGA MS with a size that could induce only a transcytosis process.

Since MS were fluorescent, preparations achieved for histological study could be observed using confocal microscopy. The staining with eosin and haematoxylin avoided any confusion due to the high refringence of the unstained erythrocytes and allowed differentiation of these cells (diameter around 8 μm) from MS. The observation of the stained slices gave two distinct kinds of spots, some were small and clear and others were larger and blurred (Fig. 6b). Erythrocytes in capillary or venule were evidenced based on their difference in fluorescence and size (around 7–8 μm) compared to MS and moreover, in both cases, no haematoxylin-stained nuclei were observed under light microscopy. According to the PLGA MS control (Fig. 6c), MS have not been deformed after the histological processing of the tissue allowing the comparison between small and large microspheres.

The size of a red blood cell is 7–8 μm and the size of a lymphocyte is 9 μm, so that the two types of cells could not be confused with small microspheres with respect to size. Fig. 6b showed the presence of MS lining the external part of the follicular area near the interfollicular area where migration occurred; these spots have not been observed on control tissue (Fig. 6a). Given the difference in the size between MS and erythrocytes, the smaller MS left the follicle which confirmed previous observation of the size-dependent dissemination (Eldridge et al., 1990).

Transmission electron microscopy analysis is more sensitive since MS can be localized in the different parts of the Peyer's patch.

In the control structure obtained by melting PLGA MS with blood and treated as a normal tissue, it appeared that PLGA MS were not fully degraded after acetone treatment but lost their spherical shape and became randomly distorted, the observed variability being increased according to the PLGA MS cutting plane (Fig. 7). Consequently, in the observed sections of the Peyer's patches, it was advisable not to attribute the empty vacuoles to MS even if the size corresponded to MS diameters.

Due to their specific invaginated shape, a whole M cell could not be observed. However, M cell apical sides were distinguished by their thick and short microvilli compared to the ordered brush border surface of the enterocytes. The different levels of PLGA MS translocation through the mucosae were identified. The beginning of the transcytosis process was observed in Fig. 8, where closed MS are present in the apical part of the M cell, indicating that the internalized phagosome could contain several MS depending on their size. Then the subsequent transport of MS was observed in Fig. 9, where MS are present in the basolateral pockets at the contact of intraepithelial lymphocytes. Just beyond the basal lamina, at the beginning of the dome area, an accumulation of MS was found showing that MS are able to pass through the basal lamina. Thus, the porosity of the basal lamina, which was demonstrated by Lowden and Heath (1994) in pig, has allowed the transport of the MS into the sub-epithelial dome region. Deeper in the follicular area, the progression of the PLGA MS continued since similar features were found indicating the migration of MS through the follicle area. However, if MS were found externally to cells, some MS were found partially or completely engulfed in macrophages (Fig. 10). Otherwise, MS were observed in the interfollicular area in close apposition to lymphatic vessel (Fig. 11). These observations suggest that MS are able to migrate freely in the mucosae.

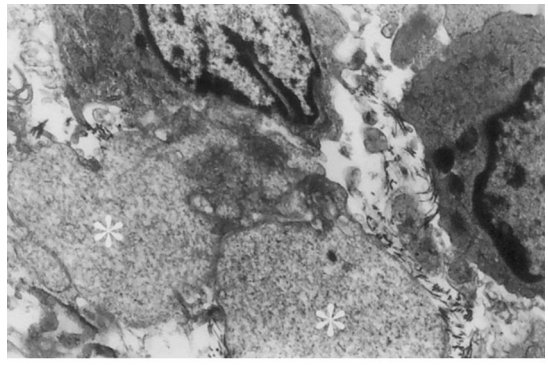


Fig. 10. Transmission electron microscopic image obtained from a section of pig ileal Peyer's patch segment. Ex vivo segments were incubated with PLGA MS for 1 h. Macrophages were found in the follicle with PLGA MS (asterisks) (a) being phagocytosed and (b) in macrophages after phagocytosis. Magnification $\times 8400$.

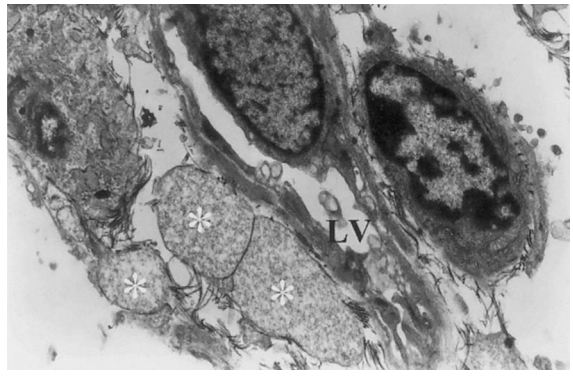


Fig. 11. Transmission electron microscopic image of the interfollicular area obtained from pig ileal Peyer's patch segment. Ex vivo segments were incubated with PLGA MS for 1 h. PLGA MS (asterisks) were detected in close apposition to the external membrane of a lymphatic vessel (LV). Magnification $\times 8400$.

4. Conclusion

These experiments have focused on the ability of porcine GALT to transcytose PLGA MS in both in situ and ex vivo models. The investigations performed with pig ileal PP using CLSM and TEM have indicated that intestinal segments studied ex vivo were able to uptake PLGA MS like isolated in situ segments. Such ex vivo segments should be used as a convenient alternative model to study the accessibility and the transcytosis of MS. Furthermore, since it appeared to be difficult to compare results between species and according to the technical methods such a model may facilitate interspecies comparisons of the transport through the intestinal mucosae.

Further investigations will be necessary to quantify the extent of uptake of PLGA MS and to screen formulations of PLGA MS with different characteristics, e.g. size and modified surface properties.

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