Stealth PLA-PEG Nanoparticles as Protein Carriers for Nasal Administration

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Purpose. The aim of the study was to encapsulate a model protein antigen, tetanus toxoid (TT), within hydrophobic (PLA) and surface hydrophilic (PLA-PEG) nanoparticles and to evaluate the potential of these colloidal carriers for the transport of proteins through the nasal mucosa.

Methods. TT-loaded nanoparticles, prepared by a modified water-in-oil-in-water solvent evaporation technique, were characterized in their size, zeta potential and hydrophobicity. Nanoparticles were also assayed *in vitro* for their ability to deliver active antigen for extended periods of time. Finally, ^{125}I-TT-loaded nanoparticles were administered intranasally to rats and the amount of radioactivity recovered in the blood compartment, lymph nodes and other relevant tissues was monitored for up to 48 h.

Results. PLA and PLA-PEG nanoparticles had a similar particle size (137–156 nm) and negative surface charge, but differed in their surface hydrophobicity: PLA were more hydrophobic than PLA-PEG nanoparticles. PLA-PEG nanoparticles, especially those containing gelatine as an stabilizer, provided extended delivery of the active protein. The transport of the radiolabeled protein through the rat nasal mucosa was highly affected by the surface properties of the nanoparticles: PLA-PEG nanoparticles led to a much greater penetration of TT into the blood circulation and the lymph nodes than PLA nanoparticles. Furthermore, after administration of ^{125}I-TT-loaded PLA-PEG nanoparticles, it was found that a high amount of radioactivity persisted in the blood compartment for at least 48 h.

Conclusions. A novel nanoparticulate system has been developed with excellent characteristics for the transport of proteins through the nasal mucosa.

KEY WORDS: nasal administration; stealth nanoparticles; poly(lactic acid)-polyethylene glycol; protein delivery; vaccine delivery; protein mucosal transport.

INTRODUCTION

The exploitation of the new generation therapeutic and antigenic molecules, genes and proteins, has been limited by the lack of appropriate delivery systems. Most DNA and protein molecules show poor transport characteristics through mucosal barriers and, therefore, require parenteral administration. This invasive route limits the practical use of these molecules, thereby stimulating investigators to search for new strategies to overcome mucosal barriers. Among the various approaches investigated, extensive research has been conducted on the ability of colloidal systems (liposomes, nanoparticles) to cross the intestinal mucosa (1,2). Despite contro- versial information on the extent and mechanisms of transport of these colloidal carriers, there is now no dispute over the fact that particulate uptake does take place especially via the M-cells of the Payer's patches and the isolated follicles of the gut-associated lymphoid tissue (GALT), but also via the normal enterocytes (1). This better understanding of the interaction of particles and the GALT has led to one of the most prolific growth areas in research, the development of oral vaccines (3). Nevertheless, the application of this approach for the oral administration of macromolecules is uncertain. In this latter sense, the question that remains to be answered is: whether or not sufficient carrier particles are taken up to produce therapeutically worthwhile levels of drug. To address this point, some considerations related to the oral route of administration should be taken into account. First, particles are very diluted in the gastro-intestinal fluids and, as a consequence, the number of particles which have the chance to interact with the absorptive cells or M cells is low. Second, the particles which are taken up by the M cells are directly drained to the lymphatics, however, those which might cross the epithelium, by a paracellular or trascellular pathway, would reach the submucosal capillary vessels and, then, be drained to the liver (first-pass effect).

Another alternative for the administration of proteins, antigens and genes is the nasal route. This route offers a number of advantages as compared to oral administration: it is easily accessible, the amount of fluids in the nasal cavity is very low, the nasal epithelium is leaky and the absorbed material has direct access to the systemic circulation, thus avoiding passage through the liver (4). Because of these features, the nasal route has been proven successful for the administration of low molecular weight peptides i.e. calcitonin (5). Several investigators have also proposed the nasal route as a very efficient way of immunization (6). The antigens may be delivered locally to the highly concentrated immune active tissues known as nasal associated lymphoid tissue (NALT) and, eventually, reach the bronchus-associated lymphoid tissue (BALT). Both, protection of the upper respiratory tract and potentiation of systemic immunity have been documented for a number of antigens (7). In addition, there is sufficient evidence that, as in the case of the oral route, the association of antigens to an appropriate adjuvant or carrier helps induce effective and long-lasting immunity. For example, results published until now on the utility of liposome-associated antigens for nasal immunization are quite impressive (8,9), whereas, only one reference has been found describing the response to tetanus toxoid adsorbed onto poly(lactic acid) submicron particles following nasal administration (10). The latter could be due, at least in part, to difficulties for the efficient incorporation and delivery of antigens from biodegradable nanoparticles. On the other hand, even though the surface hydrophobicity of the particles seems to play a key role in their recognition by the M-cells (11), there is not a clear understanding of the parameters that affect the interaction and transport of colloidal particles through the nasal mucosa.

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The aim of the present work was to assess the merits of biodegradable nanoparticles and the role of their hydrophobicity for the transport of proteins through the nasal mucosa. For this purpose, tetanus toxoid (TT), chosen as a model antigen, was encapsulated within nanoparticles made of PLA and of the block copolymer PLA-PEG and the particles were characterized in terms of size, surface charge, hydrophobicity and in vitro release of the active antigen. The biological fate of the TT-loaded nanoparticles following nasal administration was determined over 48 h.

MATERIALS AND METHODS

Chemicals and Animals

The polymer PLA (molecular weight 50,000 Da) and the diblock copolymer, PLA-polyethyleneglycol (PLA molecular weight 45,000 and PEG molecular weight 5,000) were synthesized by the ring-opening polymerization at 114°C of lactide and, in the second case, in the presence of monomethoxy PEG, by the use of stannous octoate as a catalyst. Details are given elsewhere (12–14). Purified tetanus toxoid ((TT) molecular weight 150 KDa, 85–90% monomeric) dissolved in phosphate buffer saline, pH 7.4, was kindly donated by the Massachusetts Public Health Biological Laboratories (Boston, MA, USA). Antitetanus monoclonal antibody and purified guinea-pig anti-tetanus immuno-globulin G (IgG) were obtained from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom), rabbit anti-guinea pig IgG peroxidase conjugate and the enzyme substrate, 2,2-azino-bis-3-ethylbenzthiazoline sul-phonate (ABTS), were purchased from Sigma Chemical (Madrid, Spain). The gelatine (type A, 60 bloom) and cholic acid (sodium salt) were purchased from Sigma Chemical (Madrid, Spain). The surfactants polyoxyethylensorbitan monooleat (Tween® 80) and polyoxyethylensorbitan monolaurate (Tween® 20) were obtained from Fluka (Madrid, Spain), and Triton-X 100 was from Ana-lema (Madrid, Spain). The solvent ethyl acetate was obtained from Panreac (Madrid, Spain).

Female Stragues-Dawley rats weighing 300 g were used in the *in vivo* study. Animals were allowed free access to food and water during the experiments.

Preparation of PLA and PLA-PEG Nanoparticles

Nanoparticles were prepared by the double emulsion technique, conveniently modified to reduce the size to the submicron range (15). Briefly, 50 µl of the above indicated TT solution, containing 500 µg of the toxoid, were emulsified in a 1 ml solution of PLA or PLA-PEG in ethyl acetate (50 mg/ml) by sonication (Branson 250, Sonifier®) for 15 s (15 W). Then, 2 ml of an aqueous sodium cholate solution (1% w/v) were added to this emulsion and the resulting (w/o)/w emulsion was sonicated for 15 s (15 W). The double emulsion was diluted in 100 ml sodium cholate solution (0.3% w/v) and the solvent was rapidly eliminated by evaporation under vacuum. Finally, the nanoparticles were isolated by centrifugation at 22,000 × g for 30 min (Avanti™ 30, Beckman, Spain) and washed three times with water.

When gelatine was used as an stabilizer for TT, it was incorporated into the inner aqueous phase at a concentration of 2% (w/w respect to polymer).

Physicochemical Characterization of Nanoparticles

The morphological examination of nanoparticles was performed using a transmission electron microscope (TEM, CM12 Philips, Netherlands) following negative staining with sodium phosphotungstate solution (0.2% w/v).

The particle size and zeta potential of nanoparticles were determined, respectively, by photon correlation spectroscopy (PCS) and laser Doppler anemometry (LDA) using a Zetasizer® III (Malvern Instruments, UK).

Surface hydrophobicity of PLA and PLA-PEG TT-loaded nanoparticles was investigated by hydrophobic interaction chromatography (HIC) according to a procedure previously described with some modifications (16). HIC was performed used phenyl-agarose and butyl-agarose as stationary phase (HiTrap® HIC Test Kit, Pharmacia Biotech, Spain). Volumes of 0.5 ml of the nanoparticles suspension (0.5% w/v in PBS, pH 7.4) were loaded into columns and eluted with PBS, pH 7.4. The OD (450 nm) of each eluate was measured (UV-1603, Shimadku, Spain) and compared to that of the original suspension. Particles that interact with the gel were removed by washing with PBS containing Triton® X-100 (0.1% w/v). The hydrophobicity of the samples was quantified by the percentage of particles retained in the column.

Determination of the Encapsulation Efficiency

Tetanus toxoid was radiolabeled with ^{125}I (Na^+ salt) (Amersham Iberica, Spain) using N-chloro-benzene sulfonamide (sodium salt) (Iodo-beads®, Pierce, IL, USA) as iodination reagent. Briefly, 12 µl of 5 mg/ml TT solution was mixed with 500 µl of phosphate buffer (PB, pH 6.5) and 5 µl (500 µCi) ^{125}I. Then, one Iodo-bead® was incorporated to allow the iodination process to occur. Radiolabeled protein was separated from free ^{125}I on a D-Salt™ Dextran Plastic Desalting Column (Pierce, IL, USA) and the activity assessed in a Cobra II-Autogamma (Packard Instruments, CT, USA).

Nanoparticles containing TT together with a trace of ^{125}I-iodinated TT were prepared according to the procedure indicated above, centrifuged and the supernatants were assessed for gamma emission. The amount of TT encapsulated into the nanoparticles was calculated by the difference between the total amount used to prepare the nanoparticles and the amount of TT present in the aqueous phase.

In Vitro Release Studies

Samples of 12 mg of nanoparticles were suspended in 3 ml of phosphate buffer saline (PBS) pH 7.4 containing 0.02% (w/v) Tween® 80 and incubated at 37°C. At predetermined time intervals, the samples were centrifuged at 22,000 × g for 30 min. Two ml of supernatant were removed and replaced by 2 ml of fresh release medium. Antigenically active TT released was determined by the ELISA assay. Results are shown as the percentage of antigenically active material released with respect to the amount of protein encapsulated.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was used to determine the antigenicity of TT released from the nanoparticles. To flat-bottom micro-titration

plates (Corning, NY, USA) 100 µl of anti-tetanus monoclonal antibody at 1 µg/ml in carbonate buffer pH 9.6 was added and allowed to incubate overnight at 4°C in a humid container. The plates were washed 3 times between all steps with PBS containing 0.5% (w/v) Tween® 20 (PBST buffer). To minimize non-specific interactions, 100 µl of PBST containing 2.5% (w/v) of dried skimmed milk powder (PBSTM buffer) were added to all the wells, and plates were incubated for 1 h at 37°C in a humid container. After washing the plates 3 times with PBST, a reference TT preparation and test samples were diluted serially in two-fold steps in PBSTM. The plates were incubated at 37°C for 2 h in a humid container and washed. Then, 100 µl of guinea pig IgG at 25 µg/ml in PBSTM were added to the wells and allowed to react for 2 h at 37°C, followed by 100 µl of rabbit anti-guinea pig peroxidase conjugate diluted 1:2000 in PBSTM for another 1 h at 37°C. The plates were washed and the substrate (ABTS), 0.5 mg/ml in 0.05 M citric acid, pH 4.0 were added. Following color development (30 min) plates were read at 405 nm on an microplate reader (3550-UV, Biorad, Spain).

Absorption and Biodistribution of TT-loaded Nanoparticles

Three groups of rats (n = 8) were used in these experiments, group 1 being treated with TT-loaded PLA nanoparticles, group 2 with TT-loaded PLA-PEG nanoparticles and group 3 with free toxoid. For administration, nanoparticles were suspended in a certain volume of PBS (pH 7.4) in order to obtain the required concentration of TT. Simultaneously, a solution of free TT in the same vehicle and of the same concentration was prepared. Each animal was dosed nasally (i.n.), under light ether anaesthesia, by instillating 80 µl into each nostril (20 µl four times in 3 minutes time-intervals) of the appropriate preparation, containing 40 µg of TT with a trace of ^{125}I (1 µCi).

Samples of blood were collected from the animals at times 1, 2, 6, 24 (n = 8) and 48 h (n = 4) post-administration by cardiac puncture. At 24 h post-instillation each group of animals was divided in 2 sub-groups (n = 4); animals from one subgroup were sacrificed immediately and the rest at 48 h post-instillation. The lymph nodes, lung, liver, spleen and small bowel were removed from the sacrificed animals. The aliquots of blood and tissues were weighed and the ^{125}I radioactivity was measured. Results of the amount of radioactivity recovered in the organs were presented as the percentage of the total amount of radioactivity administered.

RESULTS AND DISCUSSION

Nanoparticles Characterization

To assess to feasibility of encapsulating a protein within submicron particles, TT, a high molecular weight (150,000 daltons) protein, was chosen as a model compound. The encapsulation was achieved by forming a double emulsion, a technique which has been typically used for the encapsulation of proteins within relatively big particles (more than 0.5 µm) (17); however, in the present work, the technique was adapted to produce sub-200 nm nanoparticles. Results in Table I indicate that the size of the nanoparticles was similar (130–150 nm) irrespective of the polymer composition and of the co-encapsulation of the stabilizer gelatine. In addition, both PLA and PLA-PEG nanoparticles were spherical and formed a monodisperse population (polydispersion index lower than 0.2) as corroborated by TEM (Fig. 1). Accordingly, the encapsulation efficiency values achieved for TT were not influenced by the formulation conditions. The values of the zeta potential were, however, affected by the presence of PEG in the PLA chain. The high negative values obtained for PLA nanoparticles (–50 mV) probably reflect the presence of carboxyl-end groups at the particle surface. A marked decrease in the surface charge for PLA-PEG nanoparticles is evident (–30 mV). This could be related to a shift of the hydrodynamic phase of shear to greater distances from the nanoparticles surface. These results are, therefore, an indication of the presence of the PEG at the surface of the

Table I. Physicochemical Properties and Encapsulation Efficiency (E.E.) of TT-loaded PLA and PLA-PEG Nanoparticles (#stabilizer co-encapsulated with TT)

Polymer	Stabilizer#	Size* (nm)	ζ Potential* (mV)	E. E.* (%)
PLA	—	153.1 ± 3.1	–47.9 ± 1.5	36.7 ± 0.3
PLA-PEG	—	142.8 ± 1.5	–30.1 ± 4.2	31.1 ± 0.5
PLA-PEG	Gelatin	136.8 ± 1.1	–31.7 ± 0.8	35.3 ± 0.6

*mean (n = 3–4) ± standard deviation

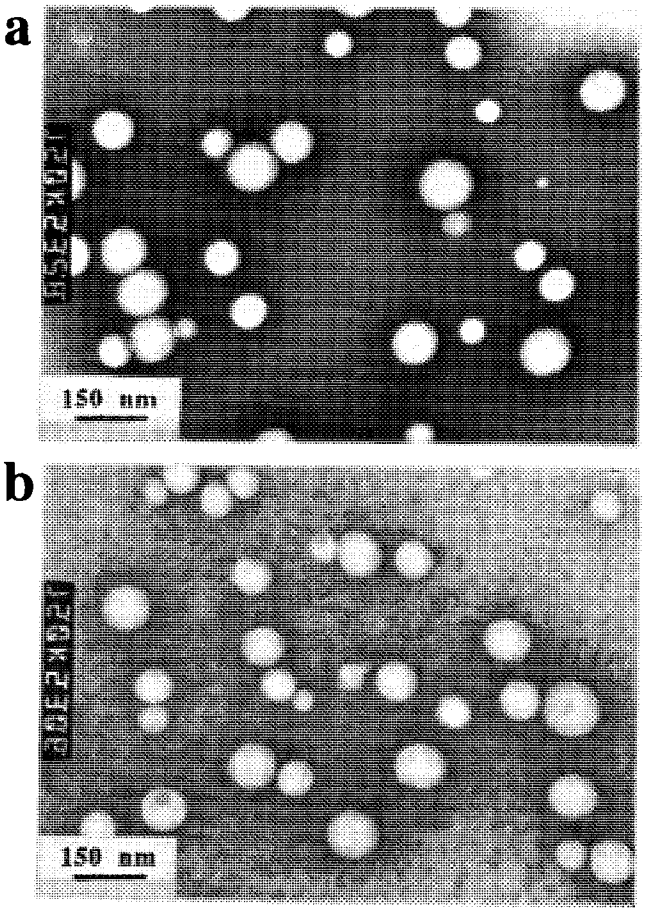

Fig. 1. TEM photographs of PLA (a) and PLA-PEG (b) nanospheres containing TT.

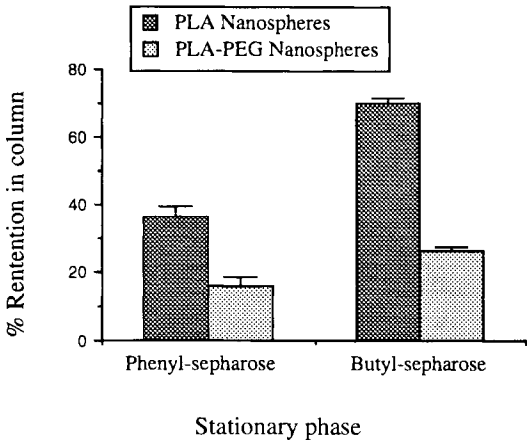

Fig. 2. Hydrophobicity of the nanospheres towards two different stationary phases (mean ± sd, n = 4).

nanoparticles. The same observations were recently reported by Hawley *et al.* for PLGA-PLA:PEG coated nanoparticles (18). Nevertheless, in order to assess more efficiently the surface characteristics of the particles we determined their surface hydrophobicity by using HIC. Results in Fig. 2 indicate that the percentages of particles retained in two different stationary phases: butyl sepharose (low hydrophobicity) and phenyl sepharose (high hydrophobicity) are dependent on their polymer composition. PLA nanoparticles are highly retained, especially in the more hydrophobic phase, whereas PLA-PEG nanoparticles are only slightly retained in both phases. This provides evidence of the more hydrophilic surface of the PLA-PEG nanoparticles, presumably due to the projection of the PEG chains towards the external aqueous medium during the nanoparticle formation process. In fact, previous studies aimed to investigate the surface chemical composition of PLA-PEG nanoparticles, prepared by a single emulsion water-in-oil technique, revealed the presence of the PEG coating on the surface these nanoparticles (12). Thus, the protein-containing nanoparticles have a similar size and loading and also a negative surface charge but they mainly differ in that PLA nanoparticles have a more hydrophobic surface than PLA-PEG nanoparticles.

In Vitro Release of Active Antigen from PLA and PLGA Nanoparticles

Our previous experience in the encapsulation of TT within PLGA microspheres led us to recognize the important problems of stability of this antigenic protein, not only during its encapsulation, but mainly during the microsphere's degradation process (19). Looking for the major causes of inactivation we found that TT interacted irreversibly with the polymer and, especially, with its acidic degradation products and, therefore, we concluded that the use of a blocking agent which would favourably interact with the polymer, i.e. gelatine type A, should help stabilize TT (20). Because of the particular problems encountered for this protein, we thought that it would be a useful model for evaluating the potential of nanoparticles for the delivery of very sensitive molecules. It was also possible that, since the copolymer PLA-PEG has the PLA carboxyl groups blocked by PEG, its interaction with the antigenic protein could be inhibited.

Results of the antigenically active toxoid released at different time-intervals from three formulations are shown in Table II. After a one day incubation, a significant amount of antigen was released (6–18%) from the three formulations. This fast release could be related to the dissolution of the protein molecules close to the nanoparticles surface. After this initial time, the nanoparticles made of PLA-PEG released a greater amount of active antigen than those made of PLA. In experiments conducted to investigate the effect of PEG on the in vitro release of TT from PLA and PLA-PEG microspheres we found that the amount of total protein (not only active antigen) released from PLA-PEG microspheres was larger than from PLA microspheres. This faster release from PLA-PEG microspheres could not be related to the polymer degradation rate, which was similar for PLA and PLA-PEG, but to the different inner structure and reduced protein-polymer interactions (results not shown). In fact, in the PLA-PEG nanoparticles, it could be expected that the PEG chains would not only oriented towards the external aqueous medium but also towards the inner aqueous phase containing the protein. Thus, the protein reservoirs would be theoretically surrounded by a PEG barrier which would reduce the interaction of the protein with the PLA matrix. The stabilizing effect of this steric barrier was further pronounced by encapsulating gelatine together with the toxoid. These new nanoparticles were able to deliver small amounts of active antigen for at least 28 days (table II). Thus, these results illustrate the potential of PLA-PEG nanoparticles for the sustained release of active proteins and the value of co-encapsulating protein stabilizers.

In Vivo Absorption and Biodistribution of TT-loaded PLA and PLA-PEG Nanoparticles

PLA-PEG nanoparticles were created with the idea of forming particles with a steric PEG barrier which would prevent their rapid uptake by the mononuclear phagocyte system (MPS) (12,21). The surface modification of these nanoparticles

Table II. *In Vitro* Distributive Release of Antigenically Active Tetanus Toxoid from Nanoparticles

		Polymer Stabilizer Antigenically Active TT Released, %#						
		1 d	4 d	8 d	12 d	16 d	21 d	28 d
PLA	—	7.39 ± 1.56	1.50 ± 0.04	0.35 ± 0.04	0.60 ± 0.13	0.32 ± 0.06	n.d.*	n.d.
PLA-PEG	—	6.22 ± 0.84	6.42 ± 0.01	1.69 ± 0.56	0.48 ± 0.01	0.29 ± 0.01	n.d.	n.d.
PLA-PEG	Gelatin	18.21 ± 2.50	1.06 ± 0.03	0.61 ± 0.02	1.33 ± 0.49	0.62 ± 0.16	0.27 ± 0.01	1.61± 0.02

#Mean (n = 3) ± standard deviation
*No detected

was aimed to achieve site-specific drug delivery because of the possibility of influencing particle biodistribution and tissue-targeting potential. As advances continue to be made in improving the circulatory half-life and biodistribution of intravenously administered nanoparticles (18,22) we decided to explore the potential benefit of these colloidal drug carriers for other routes of administration i.e. the nasal route. In this sense, it is surprising that, although it is generally accepted that hydrophobic particles are transported through the intestinal mucosa more favourably than hydrophilic particles (23), little is known on the particle requirements for overcoming the nasal mucosa.

In an attempt to explore this new area, we administered radiolabeled TT-loaded nanoparticles intranasally and determined the amount of radioactivity recovered in the blood circulation. Results in Fig. 3 show that the PLA-PEG resulted in a significant enhancement of the toxoid concentration in the blood stream. In fact, the percentage of radioactivity recovered in the blood 1h after administration of PLA-PEG nanoparticles was 10 fold that observed for the PLA nanoparticles. Furthermore, it can be seen that the blood-associated radioactivity remained almost constant for 24 h and declined slightly after 48 h. Data presented in Fig. 3 refer to the percentage of radioactivity recovered per g of blood, therefore, by taking into account the total weight of the rat blood (15–20 g) we find that 15% of the radioactive toxoid was recovered in the blood at 1-h post-administration.

To elucidate whether the nanoparticles composition would affect not only the nasal absorption of the toxoid but also its biodistribution we determined the concentration of ^{125}TT in some relevant tissues related to the MPS. Results in Fig 4a show that, after 24 h i.n. administration, the percentage of radioactivity detected in lymph nodes, lungs, liver and spleen was between 3–6 fold higher for PLA-PEG nanoparticles than for PLA nanoparticles. These values were also considerably higher for PLA-PEG nanoparticles than for the control (TT aqueous solution). The same pattern was observed at 48 h post-administration (Fig. 4b), although the concentrations in all tissues investigated were lower. Fig. 4 also shows that the radioactive toxoid was particularly concentrated in the lymph nodes at 24 and 48 h post-

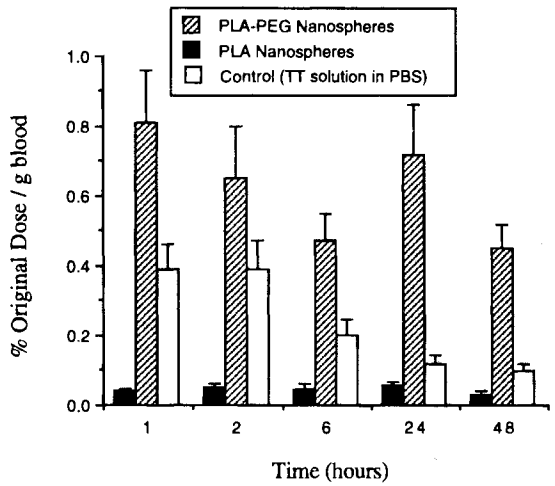

Fig. 3. Blood levels of ^{125}I-TT following nasal administration of ^{125}I-TT-loaded PLA and ^{125}I-TT-loaded PLA-PEG nanospheres (mean ± sd, n = 8 at times 1 to 24 h and n = 4 at 48 h).

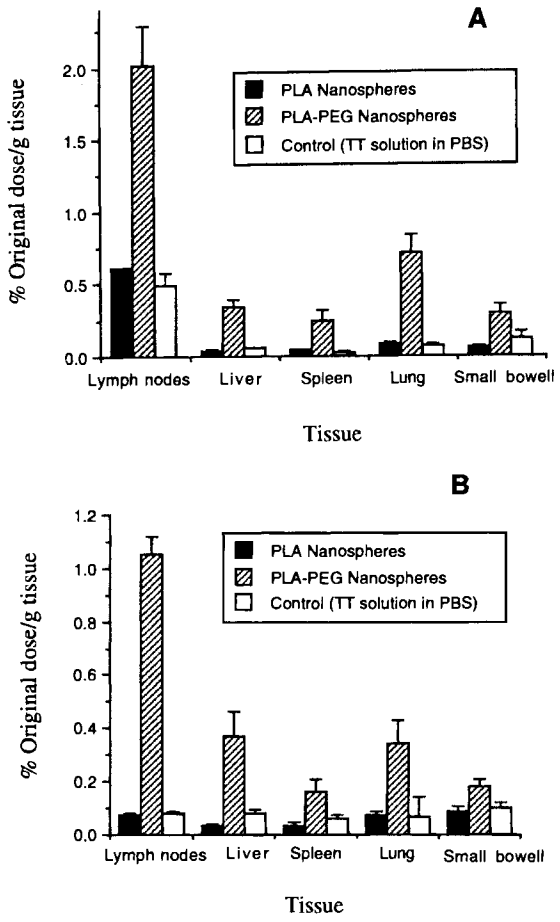

Fig. 4-A and 4-B. Tissue distribution of ^{125}I-TT at 24 h (4a) and 48 h (4b) after nasal administration of ^{125}I-TT-loaded PLA and ^{125}I-TT-loaded PLA-PEG nanospheres (mean ± sd, n = 4).

administration of the PLA-PEG nanoparticles, these values being twice those observed in blood.

These results are in good agreement with those previously reported by Alpar *et al.* (2) for the blood absorption of TT-containing liposomes, although the percentages presented here for the PLA-PEG nanoparticles are considerably higher than those observed for liposomes. These authors suggested that TT-loaded liposomes could be transported through the nasal mucosa and reach the subepithelial layer of the nose which is highly irrigated by lymph and capillary vessels. Then, it was presumed that a quantity of liposomes will pass directly into the systemic circulation and, some liposomes would probably be taken up and delivered to the underlying lymphoid cells of the NALT. The same mechanism of transport was later proposed by Almeida *et al.* (10) to justify the presence of fluorescent latex particles in the blood circulation following i.n. administration. The same authors, being conscious of the possible uptake of the instillated particles by the GALT, analyzed comparatively the absorption of particles administered nasally in normal and tracheotomized rats and concluded that the uptake does take place through the nasal epithelia as it does through the gut epithelia (24).

The results of the present work led us to propose the hypothesis that PLA-PEG nanoparticles could be partially taken up by the M cells of the NALT, but also that they could be trans-

ported, by a transcellular or paracellular pathway, to the submucosa layer and be drained to the lymphatics and blood. In addition, we should accept that some particles could have reached the gastrointestinal tract and the low respiratory tract and been taken up by the lymphoid tissue present in these mucosal surfaces (BALT and GALT). The assumption of the particles uptake by the lymphoid tissue could be also supported by the fact that the free toxoid (control solution) was absorbed in a much lower extent than the PLA-PEG nanoparticles and was rapidly eliminated from the blood. In addition, in a control experiment, we observed that the radiolabeled TT remained mostly (90%) associated with the nanoparticles upon their incubation in plasma for 48 h. Therefore, the blood-associated radioactivity could be taken as an indication of the nanoparticles absorption through the mucosae. If this hypothesis is accepted, the long persistence of the toxoid in the blood stream following the administration of the PLA-PEG nanoparticles could be explained by the slow removal of these particles by the MPS (12).

In conclusion, the results show that PLA-PEG nanoparticles have a great potential for the delivery of proteins, either to the lymphatic system or to the blood circulation, following nasal administration. Experiments are currently underway to investigate the mechanism of interaction and transport of the nanoparticles through the nasal epithelium.

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