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Biodistribution of Free ^{99m}Tc-ovalbumin and ^{99M}Tc-ovalbumin Encapsulated in Liposomes

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ABSTRACT

The oral administration of proteic antigens, like ovalbumin, may result in the induction of oral tolerance or immunization. The aim of this work was to label a protein antigen with ^{99m}Technetium, encapsulate it in liposomes and investigate its absorption and tissue distribution after oral administration in mice. Ovalbumin was labeled with ^{99m}Technetium and encapsulated in small unilamellar vesicles. ^{99m}Tc-OVA encapsulated or not in liposomes was administrated to mice that were sacrificed after different times. The radioactivity was measured in various organs of the animals. Differences concerning the biodistribution of ^{99m}Tc-OVA were noticed. The technique may represent alternatives for the induction of immunization or oral tolerance.

Key words*:* Liposomes, oral absorption, mucosal delivery, proteins, nanotechnology.

INTRODUCTION

Pathogenic microorganisms are responsible for infectious diseases that are the major cause of morbidity and mortality. Many of these microorganisms penetrate the organism through the mucosal surfaces of the body, especially the gastrointestinal route. Oral tolerance is the main phenomenon observed in animals that have been orally treated with a soluble protein antigen, such as ovalbumin (OVA), before being immunized. In this case, animals fail to produce an immune response when they are subsequently challenged with the antigen, even in the presence of adjuvants. However, immune responses have also been observed in animals that were treated via the oral route with antigens. For this reason, the oral route is being considered as an appropriate choice for immunization (Boyaka et al, 1999; Cripps et al, 2001; William and Gibbons, 1972; Majumdar and Ghose, 1982; Killian et al, 1988).

Different and contradictory results have been observed when animals are orally treated with the antigen in distinct manners, such as in its free or soluble form; conjugated with lipid residues or encapsulated in liposomes (Oliveira et al, 1998; Fujii et al, 1993; Clark et al, 2002). These results suggest that the form of the antigen may, in some cases, protect it from the attack of digestive fluids and/or modify its absorption and biodistribution, leading either to the induction or the suppression of an immune response. Therefore, further studies are needed to develop either oral vaccines or new therapeutic protocols for treating allergies and autoimmune diseases. In this paper, the biodistribution of ^{99m}Technetium-ovalbumin that

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has been orally administered encapsulated or not into liposomes is investigated.

MATERIALS AND METHODS

Radiolabelling of Ovalbumin

Ovalbumin (OVA) grade V (Sigma, USA) was incubated for 20 minutes at room temperature with 10 μL of $SnCl_2$ (2 mg/mL in 0.25 M HCl), 10 μL of $NaBH_4$ (10 mg/mL in 0.1 M NaOH) and 3.7 MBq of ^{99m}Tc. The labeling yield, assessed by the method described by Araujo et al. (Eckelman, 1990; Pauwels et al., 1993; Araújo et al., 2002), was 95.0% ± 0.5 (n = 45).

The antigenic integrity of ^{99m}Tc-OVA was tested by affinity chromatography. The labeling process did not modify the antigenic property of ovalbumin since ^{99m}Tc-OVA was able to bind to anti-OVA antibodies fixed in Sepharose-4B columns as well as the native molecules of the antigen.

Liposomes

Small Unilamellar Vesicles (SUV) were prepared according to the method of Hope et al. (1985). SUV-liposomes were composed of soybean phosphatidylcholine (PC), cholesterol (CH) and phosphatidylglycerol (PG) or distearoylphosphatidylcholine (DSPC), CH and PG. All liposomes had a molar ratio of 7/2/1, with a 30 mmole/L lipidic concentration and an initial concentration of OVA solution of 100 mg/mL. The diameter of SUV liposomes was determined by light scattering using a N4MD nanosizer.

Animals

Swiss mice of both sexes were obtained from the breeding unit at the Federal University of Minas Gerais (UFMG – Belo Horizonte, Brazil). Groups of mice were used when 7 to 9 weeks old.

Biodistribution

Aliquots of ^{99m}Tc-ovalbumin and ^{99m}Tc-ovalbumin encapsulated in liposomes were administrated by gavage to mice. The animals were sacrificed at different times after treatment (30, 60, 90, 120, 180 and 360 minutes). The studies were performed in accordance with the Brazilian Society for Neuroscience and Behavior Guidelines for Animal Experimentation. Stomach, gut, Peyer's patches, mesenteric lymph nodes, spleen and liver were excised from mice that were previously bled. The organs were washed with saline, dried and weighed. The radioactivity of the samples was measured by an automatic scintillation apparatus (ANSR-Abbot, USA). Data were expressed as percentage of the administered dose per gram of each organ's tissue.

Statistical analysis

Statistical significance was evaluated using Student's *t* test. Significance was accepted for $p < 0.05$. All data represent the result of at least three experiments and are expressed as mean ± S.D. for a sample size of seven.

RESULTS AND DISCUSSION

The absorption and the tissue distribution of OVA depend on the size of liposomes and the way that the antigen is administered to the animals. The measured diameter for the SUV-PC/CH/PG liposome was 103 nm (SD±4) and for the SUV-DSPC/CH/PG liposome was 75 nm (SD±4). However, during the first hour after antigen administration, no differences were observed in the amounts of ^{99m}Tc-OVA found in the gut mucosa, independantly of the manner in which the antigen had been administered (Fig. 1). After this period, the amount of antigen delivered by SUV-DSPC/CH/PG liposomes is almost double the quantity delivered in the other two forms (free or encapsulated in SUV-PC/CH/PG liposomes).

The presence of the antigen in the Peyer's patches decreased, homogeneously, during the first 120 minutes. Interestingly, after three hours, a significant increment of the antigen was observed when SUV-DSPC/CH/PG liposomes were used (Fig. 2).

It was also noticed that, although being absorbed by the gut mucosa, including the Peyer's patches, the majority of the antigen was not absorbed and remained inside the gut, independently of the manner in which ^{99m}Tc-OVA was administered. During the first 120 minutes, it was encountered mainly in the content of the small intestines. After this time, it moved to the large intestine (data not shown).

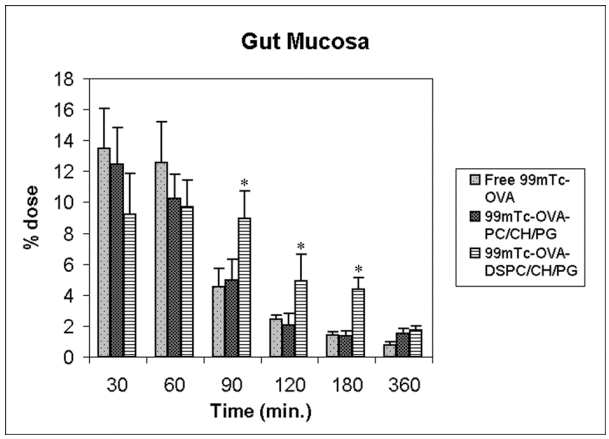

Figure 1 - Percentage of ^{99m}Tc-Ova in the gut mucosa after oral administration. Key: Phosphatidylcholine (PC), cholesterol (CH), phosphatidylglycerol (PG), distearoylphosphatidylcholine (DSPC). ^{99m}Technetium labeled Ovalbumin (^{99m}Tc-OVA) Small Unilamellar Vesicles (SUV). Data are expressed as mean ± S.D. (n = 7) * ($p<0,05$).

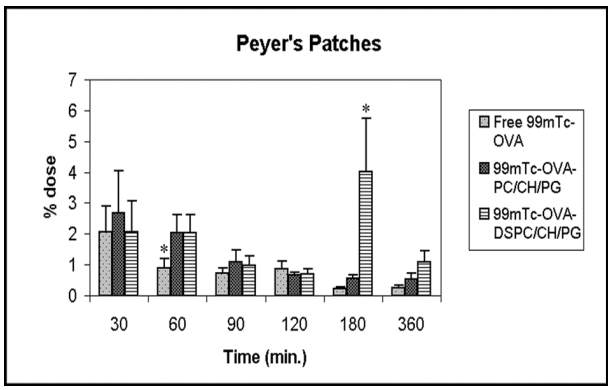

Figure 2 - Percentage of ^{99m}Tc-Ova in the Peyer's Patches after oral administration. Key: Phosphatidylcholine (PC), cholesterol (CH), phosphatidylglycerol (PG), distearoylphosphatidylcholine (DSPC). ^{99m}Technetium labeled Ovalbumin (^{99m}Tc-OVA) Small Unilamellar Vesicles (SUV). Data are expressed as mean ± S.D. (n = 7) * ($p < 0.05$)

The profiles observed for the mesenteric lymph nodes (Fig. 3) and spleen (Fig. 4) are very similar. After three hours, a significant increment of the antigen was observed in these organs when SUV-DSPC/CH/PG liposomes were used. This fact characterized a second peak of the antigen in the organs. Differences were also found earlier: ^{99m}Tc-OVA peak was observed later when freely administered (120 minutes). As a matter of fact, ^{99m}Tc-OVA encapsulated in SUV-DSPC/CH/PG liposomes peaks earlier (first peak at 60 minutes) than OVA encapsulated in SUV-PC/CH/PG liposomes (90 minutes).

When ^{99m}Tc-OVA was freely administered, a significant increment of the antigen was encountered in the liver after 120 minutes (Fig. 5). The presence of ^{99m}Tc-OVA delivered by SUV-PC/CH/PG liposomes increased in a more constant manner, reaching maximum values at the same time. Differently from the other organs, the amount of ^{99m}Tc-OVA delivered by SUV-DSPC/CH/PG liposomes reached maximum values after 60 minutes, although a second wave can be observed later (between 120 and 180 minutes).

^{99m}Tc-OVA was observed to be quicker and in higher concentration in the blood when administered encapsulated in SUV liposomes (PC/CH/PG or DSPC/CH/PG) (Fig. 6). As observed in other tissues, when DSPC/CH/PG liposomes were used, two peaks were observed: the first one at 60 minutes and a second one at three hours.

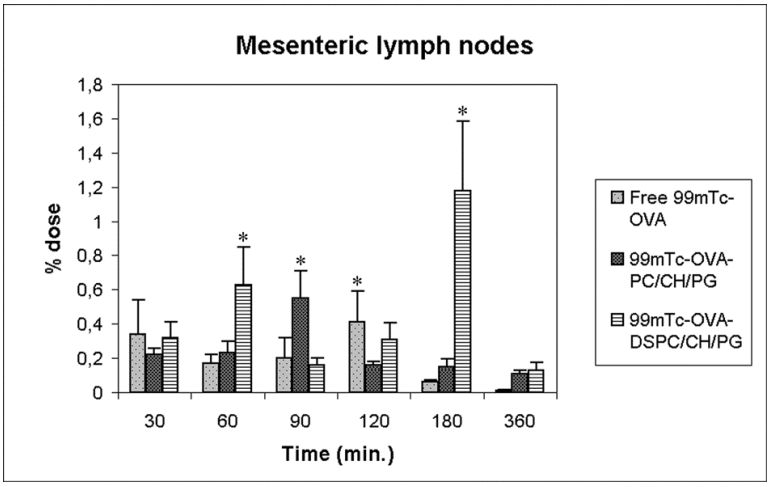

Figure 3 - Percentage of ^{99m}Tc-Ova in the mesenteric lymph nodes after oral administration. Key: Phosphatidylcholine (PC), cholesterol (CH), phosphatidylglycerol (PG), distearoylphosphatidylcholine (DSPC). ^{99m}Technetium labeled Ovalbumin (^{99m}Tc-OVA) Small Unilamellar Vesicles (SUV). Data are expressed as mean ± S.D. (n = 7) * ($p < 0.05$)

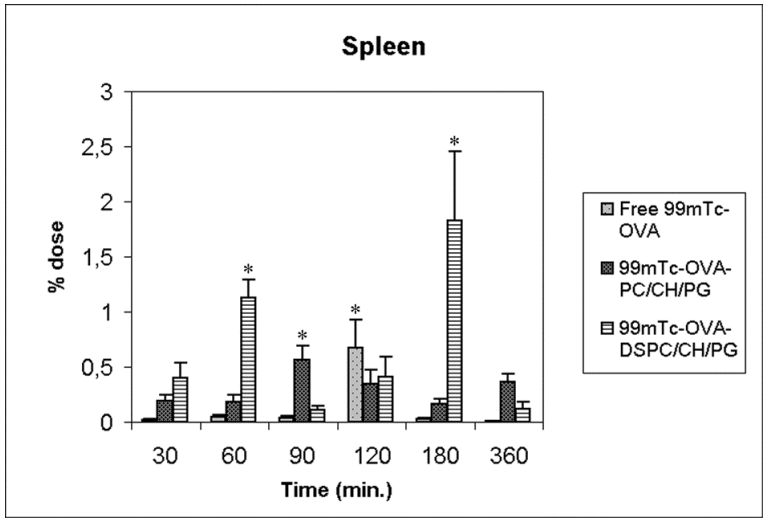

Figure 4 - Percentage of ^{99m}Tc-Ova in the spleen after oral administration. Key: Phosphatidylcholine (PC), cholesterol (CH), phosphatidylglycerol (PG), distearoylphosphatidylcholine (DSPC). ^{99m}Technetium labeled Ovalbumin (^{99m}Tc-OVA) Small Unilamellar Vesicles (SUV). Data are expressed as mean ± S.D. (n = 7) * ($p < 0.05$)

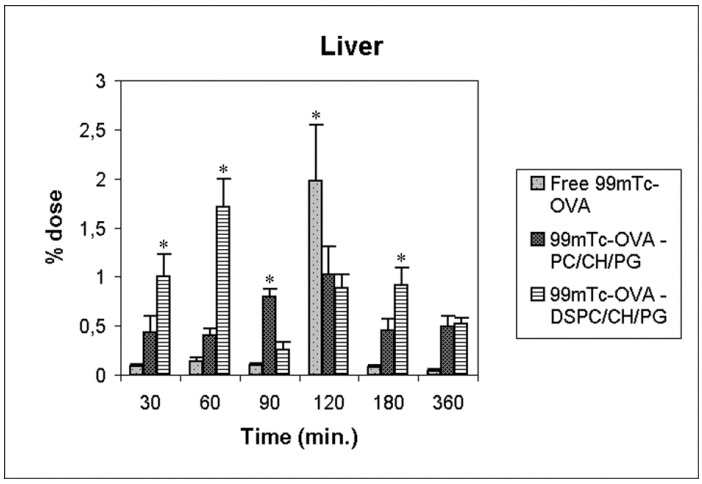

Figure 5 - Percentage of ^{99m}Tc-Ova in the liver after oral administration. Key: Phosphatidylcholine (PC), cholesterol (CH), phosphatidylglycerol (PG), distearoylphosphatidylcholine (DSPC). ^{99m}Technetium labeled Ovalbumin (^{99m}Tc-OVA) Small Unilamellar Vesicles (SUV). Data are expressed as mean ± S.D. (n = 7) * ($p < 0,05$)

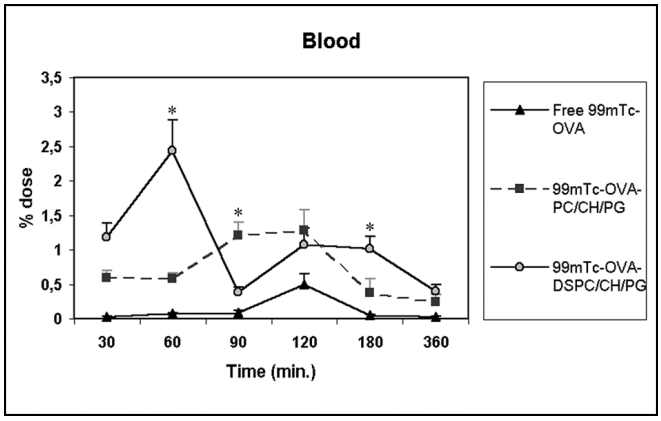

Figure 6 - Percentage of ^{99m}Tc-Ova in the blood after oral administration. Key: Phosphatidylcholine (PC), cholesterol (CH), phosphatidylglycerol (PG), distearoylphosphatidylcholine (DSPC). ^{99m}Technetium labeled Ovalbumin (^{99m}Tc-OVA) Small Unilamellar Vesicles (SUV). Data are expressed as mean ± S.D. (n = 7) * ($p < 0,05$)

Considering all these data, one may assume that Ovalbumin encapsulated in SUV liposomes is much more highly absorbed than when the antigen is freely administered. This group has already shown that SUV liposomes are preferably captured by the Peyer's Patches, while MLV liposomes are not (Ramaldes et al, 2002). Several studies have been conducted to observe the distribution of polymeric particles in the gut mucosa after oral administration (LeFevre et al, 1989; Eldridge et al, 1990; Jani et al, 1992; Hillery and Florence, 1996). It is difficult to compare the results obtained so far, since the methods used in these studies varied from group to group. However, it has been clearly shown that polymeric particles adhere to the intestinal mucosa after being transported to the

mucosal lymphoid tissues. It is important to consider the size, hydrophobic nature and composition of the nanoparticles (Brayden, 2001). A special emphasis has been given to the Peyer's patches that seem to be one of the major sites for the capture of nanoparticles. It is important, as well, to examine the relevance of such organs in terms of the consequences of the oral administration of antigen to the immune system. The "M cells" that are found in the epithelium layer above these structures are known to be involved in the transport of macromolecules to the interior of the lymphoid tissue. In this respect, depending on the nature of the antigen, among other factors, oral tolerance or systemic immunization can be achieved. This group has already shown that oral tolerance induction can be prevented when lipids are coupled to the antigen (Oliveira et al, 2002). In this respect, in another study that is also being conducted by this group, ovalbumin coupled to palmitoyl groups shows a different profile of absorption and tissue distribution after oral administration when compared to the native form of the antigen (paper submitted for publication).

Perhaps because of the low stability of liposomes in digestive fluid, few studies have been conducted regarding their use as a system for oral delivery of antigen (Brayden and Baird, 2001). Only two works were encountered in the literature that have addressed the question of whether oral administration of antigen encapsulated in liposomes triggers oral tolerance or immunization (Clarke and Stokes, 1992; Ouadahi et al, 1998). In both studies, oral tolerance was achieved.

In this paper, the kinetics and tissue distribution of OVA, after being administered orally to mice, have been shown to depend on whether it had been administered encapsulated or not in SUV liposomes. The main difference consisted of a significant increment of the antigen in most of the tissues studied after 180 minutes, when SUV-DSPC liposomes were used. This profile somehow resembles that observed in the study mentioned in the previous paragraph, when palmitoyl-OVA conjugates were orally administered to mice. Studies concerning the consequences to the immune system of mice that are given OVA encapsulated or not in liposomes by the oral route are being planned.

CONCLUSION

The data obtained indicate that it is possible to label ovalbumin efficiently with ^{99m}Tc and that this procedure does not result in the degradation of the antigen. Although most of the antigen that had been orally administered to mice was not absorbed, differences were found concerning the biodistribution of OVA (encapsulated or not in liposomes). It may represent alternatives for the induction of immunization or oral tolerance, a state of immunological unresponsiveness. In particular, SUV-DSPC liposomes represent an interesting formulation since a significant increment in the presence of antigen after 180 minutes in most of the tissues studied was observed. The increased bioavailability of ovalbumina encapsulated in SUV-DSPC liposome may be related to a greater protection of the antigen. On the other hand, the kinetics of tissue distribution of OVA after its oral administration in SUV-PC liposomes is much more similar to that observed when the antigen was freely administered.

RESUMO

A administração oral de antígenos protéicos pode levar a indução ou supressão da resposta imune. A indução da resposta imune é de grande importância para o desenvolvimento de vacinas orais. Já a supressão, denominada de tolerância oral, pode vir a representar uma solução terapêutica a numerosas enfermidades. O objetivo deste trabalho foi de compreender o comportamento *in vivo* de um antígeno modelo, a ovalbumina (OVA), marcado com um radioisótopo, o ^{99m}tecnécio (^{99m}Tc), e administrado por via oral na sua forma livre e encapsulado em lipossomas de pequeno tamanho (SUV). As amostras foram administradas por gavagem à camundongos, sacrificados após 30, 60, 90, 120, 180 e 360 minutos. A radioatividade foi medida no estômago, intestinos, placas de Peyer, linfonodos mesentéricos, baço, fígado e sangue dos animais. Os resultados mostraram que a OVA livre ou encapsulada em lipossomas SUV apresenta biodistribuições distintas. As diferenças encontradas na biodistribuição da OVA livre ou encapsulada podem representar mecanismos diferentes para a indução ou não de tolerância oral.

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