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Poly(anhydride) nanoparticles as adjuvants for mucosal vaccination

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1. ABSTRACT

In the last years, many efforts have been directed toward the enhancement of vaccine delivery by using polymeric nanoparticles as adjuvants for mucosal immunization. However, conventional nanoparticles usually display a low capability to target specific sites within the gut and, thus, the elicited immune responses are not as high as necessary to offer the adequate protection to the host. To overcome these drawbacks, one possible strategy can be the association of nanoparticles with compounds involved in the colonization process of microorganisms. In this biomimetic context, two different examples are shown. In both cases, poly(anhydride) nanoparticles were coated with either flagellin from *Salmonella* Enteritidis or mannosamine. When administered by the oral route both types of ligand-coated nanoparticles induced stronger and more balanced serum titers of IgG2a and IgG1 than control nanoparticles which induced a typical Th2 response. This Th1 response enhancement may be related to the high tropism of both flagellin- and mannosylated-nanoparticles to the ileum and uptake by Peyer's patches rich in antigen presenting cells.

2. INTRODUCTION

Looking for successful vaccines has become one of the driving forces in global health. The history of vaccination is rich in many trials to treat numerous infectious diseases. They are responsible for approximately 25% of global mortality, especially in children younger than five years (1, 2). The safety and effectiveness of a vaccine depends on how it is made and what it contains. It is possible to classify the different vaccines in different classes: (i) live attenuated, (ii) killed whole, (iii), toxoid and (iv) component (subunit) vaccines (3).

Live attenuated vaccines usually are created from the naturally occurring pathogen itself. Live virus vaccines are prepared from strains that are almost or completely devoid of pathogenicity but are capable of inducing a protective immune response (4). They multiply in the human host and provide continuous antigenic stimulation over a period of time. On the contrary, they may cause severe infections in immunocompromised individuals (5). Another important aspect is that the distribution of these vaccines requires a cold chain that may not be readily accessible in developing countries (6).

Inactivated (killed) vaccines contain killed bacteria (bacterins) or inactivated viruses. They cannot cause an infection, but they still can stimulate a protective immune response, and require multiple doses initially to induce immunity and booster doses later to maintain immunity (7). On the other hand, toxoid vaccines stimulate the production of antibodies against an infectious agent secreted by certain bacteria (i.e. tetanus, diphtheria) (8).

In the past decade, several new approaches to vaccine development have emerged that may have significant advantages over traditional ones. These new vaccines are based on the use of well defined parts of the viruses or bacteria, including proteins, peptides or plasmid DNA. Although these "subunit vaccines" offer advantages such as reduced toxicity, they are poorly immunogenic when administered alone. For these reasons, accessory technologies are required to make these defined antigens immunogenic, including new strategies for their optimum physical presentation to the antigen presenting cells (APCs) (9, 10). One of these technologies, and the most popular, is the use of adjuvants to improve the immunogenicity of the active molecule and confer to the host the required protection against infection (11).

2.1. Adjuvants

The term "adjuvant" (from the Latin word *adjuvare*, which means to help or to enhance), originally described by Ramon (12, 13) is used to identify any substance, combination of substances or strategies that augment specific immunity to an antigen as compared to that induced by the antigen or vaccine alone (14).

Chemically, the adjuvants are a highly heterogeneous group of compounds with just only one thing in common: their ability to enhance the immune response (14). An effective adjuvant formulation provides the antigen with both an optimal physical presentation and a boost to create immune recognition and reaction (15-17). They are highly variable in terms of how they affect the immune system and what type of immunomodulation process they induce. In any case, specific antigen/adjuvant combinations preferentially induce type 1 (Th1) or type 2 (Th2) cytokine responses (18, 19). The Th1 subset is characterized by the secretion of cytokines such as interleukin 2 (IL-2) and interferon-γ (IFN-γ), to assist in cell-mediated immune response. On the other hand, the Th2 subset assists preferentially in antibody immune responses after secreting cytokines including interleukin 4 (IL-4).

In spite of large list of compounds and strategies described as adjuvants, the only FDA approved adjuvant is alum, a general name for the aluminum-based mineral salt (20). It yields a reasonable antibody response (Th2), but it does not induce a Th1 profile. Th1 immunity is essential for protection against many infective organisms (e.g. intracellular parasites, including virus and some prokaryotic and eukaryotic microorganisms) and even to limit allergenic processes (21). Moreover, aluminium adjuvants have shown limitations in their applicability in vaccines based on small-size peptides or antigen-expressing DNA (22, 23). Another limitation lies in the fact that aluminium-adsorbed vaccines are frost sensitive and thus not lyophilizable.

Nowadays, it is well established that adjuvants can enhance the specific immune response of the co-administered antigens by one or the combination of several of the following effects: (i) depot effect, (ii) effect on antigen presenting cells (APCs) or/and (iii) non-specific immunostimulating effect.

2.1.1. Depot effect

It is well known that antigens in solutions are mostly quickly removed by neutrophils and macrophages, but subsequently, they are unable to induce an immune response. Different types of adjuvants, such as mineral (i.e Aluminium salts) and emulsion-like adjuvants (i.e. FIA, Montanide or Quil A) may retain antigen at the injection site (forming a depot of antigen), from it would be released in minute quantities over a prolonged period of time (24, 25).

2.1.2. Effect on APCs

The adjuvant-induced enhancement of an immune response may be ascribed to the improved delivery of antigens into the draining lymph nodes. This may be achieved by facilitating the antigen uptake by APCs, or by increasing the influx of APCs into the injection site. Whichever is the case, the result is the same: an effective priming of specific T cells derived from an increase in the provision of antigen-loaded APCs, promoting the activation state of APCs by up regulating co-stimulary signals or the major histocompatibility complex (MHC) expression. This results in the corresponding cytokine release, enhancing the speed, magnitude and duration of the specific immune response (26).

Particulate delivery systems would belong to this category of adjuvants (27, 28). APCs have evolved to engulf microorganisms, and thus, it is not surprising that particulate antigen with sizes in the range of pathogens act as adjuvant by direct targeting of antigen to these cells. In fact, the term of particulate delivery systems comprise any strategy addressed to endow an antigen with dimensions of a microorganism (29). These adjuvants can be classified in two major groups (9, 30), according to their lipidic or polymeric composition. Within the group of lipid-based particles it is possible to distinguish liposomes, immunostimulatory complexes (ISCOMs) and virosomes. Among non-lipidic particles, the following systems can be ascribed: virus-like particles, microparticles and nanoparticles.

2.1.3. Non-specific immunostimulating effect

Some agents can stimulate the non-specific component of the immune system and directly activate innate immune cells. Numerous microorganisms contain "alert signals", the so-called "microbial or pathogen associated molecular patterns (PAMPs), not present in mammalian cells. These structures activate immune cells through interaction with specific receptors (i.e. toll like receptors or TLRs). To this category belongs

Table 1. Physicochemical characteristics of poly(anhydride) nanoparticles. Data expressed as the mean ± SD (n = 6-8).

	Size (nm)	Zeta potencial (mV)	Ligand content (μg/mg)	OVA content (μg/mg)
NP	210 ± 1	-50 ± 2	-	-
F-NP	277 ± 1	-43 ± 2	18.7 ± 1.3	-
M-NP	313 ± 2	-45 ± 2	36.4 ± 3.7	-
OVA-NP	277 ± 13	-48 ± 4	-	11.9 ± 1.5
OVA-F-NP	391 ± 5	-34 ± 2	15.2 ± 0.9	7.3 ± 2.4
OVA-M-NP	350 ± 3	-37 ± 3	34.1 ± 3.6	9.0 ± 2.1

lipopolysaccharide, murein, flagellin, muramil dipeptide or CpG sequences among others (31-34).

2.2. Polymer nanoparticles as mucosal adjuvants

Among the different types of particulated delivery systems, polymer nanoparticles is a group of carriers with interesting abilities as adjuvants for both conventional and mucosal vaccination (35, 36).

In principle, these polymer carriers offer a number of advantages including protection of the loaded antigen against its gut degradation or inactivation and controlled release properties (37, 38). Furthermore, nanoparticles can enhance the delivery of the loaded antigen to the gut lymphoid cells due to their ability to be captured and internalized by cells of the gut-associated lymphoid tissue (GALT) (39, 40). However, conventional polymer nanoparticles usually display a low capability to target specific sites within the gastrointestinal tract (i.e. Peyer's patches), and they can be eliminated to some extent by the mucus shed off and intestinal peristaltism (41, 42). As a consequence, the elicited immune response with these antigen delivery systems is usually not as high as necessary for vaccination purposes (43, 44).

In order to overcome these drawbacks and render nanoparticles more efficient as adjuvants for vaccination, one possible strategy can be their association with compounds or molecules involved in the colonization process of microorganisms. So, the idea would be to closely mimic the bacteria and virus abilities to colonize and invade a given cell.

2.3. Biomimetic nanoparticles

The phenomenon of microorganism adhesion to the surface of a cell is the first step and prerequisite for the colonization and invasion of the host (45-47). Microorganisms can invade and colonize the host tissue by using a number of different specific adherence factors including lipoteichoic acids, outer membrane proteins (48), flagella (49, 50), fimbriae and pili (50, 51), lectins (52) and glycoproteins (53). Most of these adhesive factors are also considered as immunomodulators and they are included in the generic denomination of PAMPs.

In order to develop these microorganism-like or biomimetic nanoparticles, the copolymers between methyl vinyl ether and maleic anhydride (Gantrez AN) can be adequate materials to prepare these mucosal adjuvants. Gantrez AN or poly(anhydride) nanoparticles have demonstrated a high ability to develop bioadhesive interactions within the gastrointestinal tract (54). More important, their surface can be easily modified by simple incubation with different excipients or ligands in order to modify their distribution within the gut or/and their bioadhesive potential (55-58). In this context, some ligands such as flagellin or mannosamine can be interesting to improve the targeting properties of these biomimetic nanoparticles.

Flagellin from Salmonella *Enteritidis* is considered as the key element forming the typical flagella of this microorganism. This protein is about 53 kDa and is encoded by the fliCgene (59). Studies have described the importance of the flagella in the salmonella invasion and colonization (49, 60). On the other hand, mannosamine has been used due to the data available about the implication of mannose residues expressed on the surface of some microorganisms, such as *Candida albicans,* in their adhesion and colonization of the mucosal cells (61). This adhesive mechanism is mediated by the high affinity binding of mannose to the so-called mannose-binding proteins which specifically recognizes carbohydrate moieties, terminated with mannose, on the surface of pathogens (62).

3. POLY(ANHYDRIDE) NANOPARTICLES

3.1. Preparation and characterization of poly(anhydride) nanoparticles

To study the adjuvant ability of nanoparticles, ovalbumin (OVA) was used as antigen model. In all cases, ovalbumin-loaded nanoparticles were prepared by a solvent displacement method (54, 56, 57). For this purpose, the poly(anhydride) was dissolved in acetone and incubated with the antigen (ovalbumin) and the ligand (either the flagellin-enriched extract or mannosamine). Then, nanoparticles were obtained by addition of a hydro alcoholic phase under magnetic stirring. The organic solvents were eliminated under reduced pressure and the resulting aqueous suspensions of nanoparticles were cross-linked by addition of 1,3-diaminopropane for 1 min, purified by centrifugation and lyophilized using sucrose as cryoprotector. In the case of mannosylated nanoparticles, mannosamine was also incubated with the ovalbumin-loaded nanoparticles in order to complete the coating of the nanoparticles with this ligand. Figure 1 summarizes the preparative process of nanoparticles.

Table 1 summarizes the main physicochemical of empty and OVA-loaded nanoparticles. In all cases, the encapsulation of ovalbumin in poly(anhydride) nanoparticles increased the size of the resulting particulate delivery systems (from 200-300 to 300-400 nm). On the other hand, both OVA-ligand nanoparticles (OVA-F-NP and OVA-M-NP) displayed homogeneous sizes, which were significantly higher than for OVA-loaded conventional nanoparticles (OVA-NP). Furthermore, the

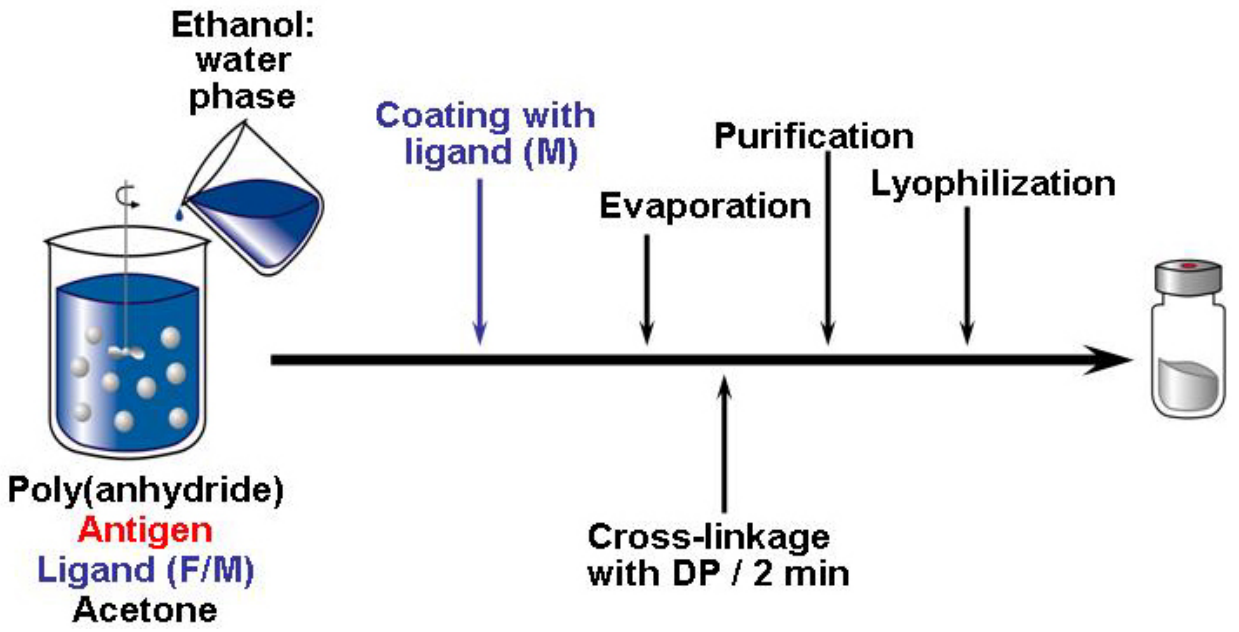

Figure 1. Illustration of the preparative process of poly(anhydride) nanoparticles. M: mannosamine; F: Flagellin; DP: 1,3-diaminopropane.

presence of flagellin or mannosamine on the surface of the nanoparticles decreased the surface negative charge compared to control nanoparticles. By scanning electron microscopy (SEM), it was visualized that at least in part, the ligands were located at the surface of nanoparticles. In fact, conventional nanoparticles displayed a smooth, homogeneous and regular surface whereas ligand-coated nanoparticles displayed irregular and rough surfaces with some appendages or structures apparently bound to the surface of nanoparticles (Figure 2).

The amount of OVA loaded in nanoparticles was calculated after dissolution of nanoparticles with a mixture of DMF and acetone followed by separation in a SDS-PAGE. Then, the band corresponding to OVA was measured using Micro Image® software (Version 4.0; Olympus Optical Co., USA) and an OVA standard calibration curve in the range between 2.5-0.25 µg/well. The amount of mannosamine associated to mannosylated nanoparticles was estimated by quantification of mannosamine content in the supernatants collected from the nanoparticle purification step using the O-phthalaldehyde (OPA) fluorimetric assay of primary amines (63). Finally, the amount of flagellin associated with nanoparticles was quantified using SDS-PAGE as described previously (56).

For OVA-F-NP the amount of flagellin content was calculated to be about 15 µg/mg whereas for OVA-M-NP, the amount of ligand was about 34 µg mannosamine per mg nanoparticles. The encapsulation of the antigen model slightly decreased the amount of ligand incorporated in the nanoparticles. Similarly, the presence of flagellin or mannosamine slightly decreased the amount of OVA in the resulting nanoparticles.

The functional integrity of flagellin or mannosamine on the nanoparticles was confirmed by a simple agglutination test after addition of either Concanavalin A (mannose specific lectin) or Salmonella H antiserum poly a-z (1:4 dilution in PBS), respectively. The agglutination results clearly demonstrated that in both cases, either the lectin or the antibody, were able to recognize the presence of mannosamine residues or native flagellin on the surface of nanoparticles when studies were performed after incubation of nanoparticles under simulated gastric or intestinal fluids (56, 57).

3.2. Bioadhesive properties of poly(anhydride) nanoparticles

The distribution of nanoparticle formulations within the gut and their capabilities to develop bioadhesive interactions with components of the mucosa was studied after labeling of the different nanoparticles with rhodamine B isothiocyanate (RBITC). For this purpose, 10 mg of fluorescently labeled nanoparticles formulations were administered after dispersion in 1 mL water by the oral route to male Wistar rats. At different times, the animals were sacrificed and the gastrointestinal tract was removed and cut in different segments. Each mucosa segment was opened lengthwise, rinsed with PBS and digested with 3 M NaOH, for 24 h. RBITC was extracted from the digested samples by addition of methanol. Finally the samples were diluted with water and assayed for RBITC content by spectrofluorimetry to estimate the fraction of adhered nanoparticles to the mucosa.

Figure 3 describes the distribution of the adhered amounts of nanoparticles in the gut mucosa 1 and 3 h post-administration. In all cases, the different types of nanoparticles displayed a higher adhesion in the small

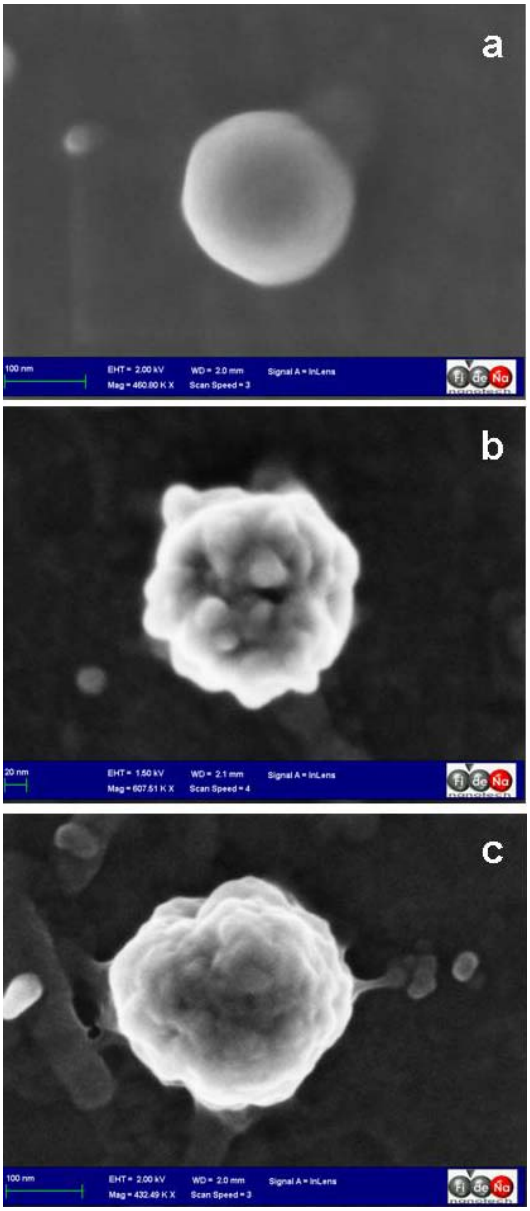

Figure 2. Microphotographs obtained by scanning electron microscopy (SEM) of conventional nanoparticles (NP; a), flagellin-coated nanoparticles (F-NP; b) and mannosamine-coated nanoparticles (M-NP; c).

intestine than in the stomach or the cecum. In the stomach, the amount of nanoparticles adhered was found to be two-times higher for ligand-coated nanoparticles (F-NP or M-NP) than for conventional nanoparticles (NP). Similarly, the capability of nanoparticles to develop bioadhesive interactions within the small intestine significantly increased when either flagellin or mannosamine were used to coat these carriers. Thus, for F-NP, about 40% of the given dose was found adhered in the intestine 3 hours post-administration. For M-NP, one hour post-administration about 36% of the given dose was found adhered at the intestine mucosa whereas, two hours later, only 23% of the dose remained adhered. On the contrary, mannosamine-coated nanoparticles appeared to display a higher tropism to reach and interact with the cecum mucosa (see Figure 3). In summary, it can be deducted the significant increase of the bioadhesive capacity of surface modified nanoparticles with flagellin or mannosamine compared to control nanoparticles.

In order to confirm the phenomenon of bioadhesion, mucosa portions from different regions of the gut were treated with the tissue-embedding medium OTC and frozen in nitrogen. The tissue samples were cut into 5 μm longitudinal sections in a cryostat, attached to poly-L-lysine precoated slides and visualized by fluorescence microscopy. From these experiments it was clear that both

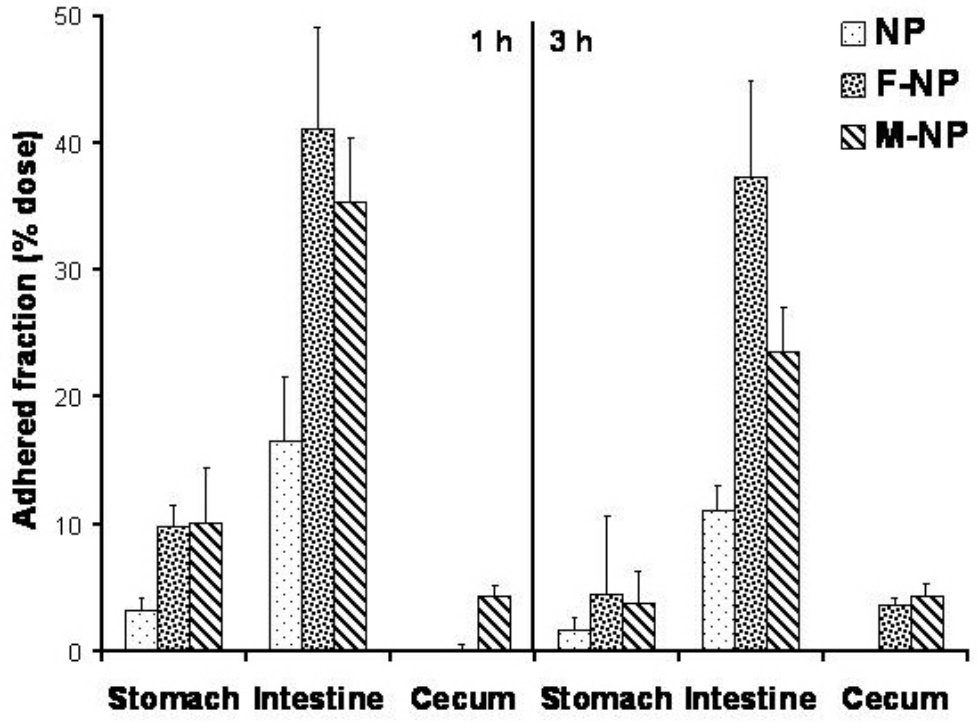

Figure 3. Gut distribution of nanoparticle formulations in the different regions of the gastrointestinal tract at 1 and 3 h post-administration. Each animal received a single oral dose of 10 mg RBITC-loaded nanoparticles. Each value represents the mean ± SD (n = 4-6).

types of nanoparticles displayed a high tropism for the terminal jejunum and ileum regions of the gut.

Figures 4-6 shows fluorescence microscopy images of ileum samples from animals treated with 10 mg of nanoparticle formulations, characterized by red fluorescent spots (due to RBITC). Control nanoparticles (NP) displayed a restricted localization in the mucosa, mainly in the outer layer of the ileum (mucus layer) and a low ability to both enter in the enterocytes (Figure 4a) and interact with Peyer`s patches (Figure 4c).

On the contrary, mannosylated nanoparticles were found broadly distributed within the ileum (Figure 4b) and demonstrated a strong capacity to adhere and penetrate Peyer's patches (Figure 4d). This strong tissue affinity to the GALT may be related to the high binding affinity of mannose residues to the so-called mannose-binding lectins (MBL) which are expressed on the lymphoid and non-lymphoid cells of the gut (62), including the professional antigen presenting cells like dendritic cells (DCs) (64, 65). On the other hand, it is well known that glycoconjugates enriched in mannose moieties are used by microorganisms as adhesion factors, promoting their interaction with the mucosal tissue of the gastrointestinal tract (61, 66).

For these reasons, mannosylation of either free antigen or carriers (i.e. nanoparticles or liposomes) loaded with antigen has been considered as a promising adjuvant strategy to enhance antigen presentation (57, 67-69).

This intense interaction with the ileum mucosa and uptake for Peyer's patches was also observed for flagellin-coated nanoparticles (Figure 5), which was also corroborated by immunofluorescence after incubation of the samples with rabbit serum containing polyclonal anti-*Salmonella* Enteritidis antibodies and, subsequent, detection with GAR/Igs/FITC antibodies (56). More interesting, the bioadhesive profile of F-NP was quite similar to the colonization profile described for *Salmonella* Enteritidis in the gastrointestinal tract of rats (56, 60). In mice, *Salmonella* cells appear to preferentially adhere to and enter the M cells of the follicle-associated epithelium of the intestine, although invasion of normally non-phagocytic enterocytes also occurs (60). In order to confirm the ability of flagellin-coated nanoparticles to develop similar tropism to that for the whole bacteria, competitive studies between both vectors were performed. When F-NP and *Salmonella* Enteritidis cells (fluorescently labeled with fluoresceine isothioacyanate, FITC) were administered together (56), both of them displayed a similar distribution within the gut, including a similar ability to target Peyer's patches. However, *Salmonella* cells appeared to be able to interact and penetrate in Peyer's patches more rapidly that F-NP (Figure 6).

Nevertheless, from all of these results it resulted far from clear if ligand-coated nanoparticles were capable, after bioadhesive interaction, to cross the mucosa and be absorbed. In order to try to elucidate this paradigm, two sets of experiments were carried out. In the former,

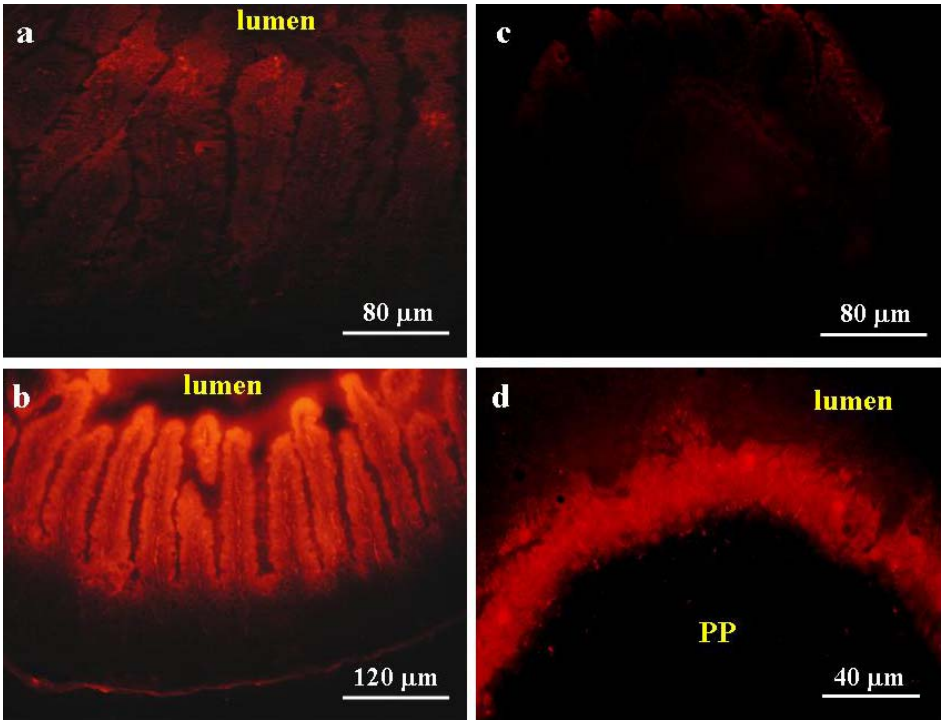

Figure 4. Visualization of the nanoparticles in the normal mucosa of the ileum and the follicle-associated epithelium of Peyer's patches (PP) by fluorescence microscopy. (a) Conventional nanoparticles (NP) in normal mucosa; (b) mannosylated nanoparticles (M-NP) in normal mucosa; (c) NP in Peyer's patches; (d) M-NP in Peyer's patches. Animals received orally 10 mg of RBITC-labelled nanoparticles and were sacrificed 2 hours later.

nanoparticles were labeled with ^{99m}Technetium. Radiolabeled nanoparticles (1 mCi, 10 mg) were dispersed in 1 ml water, filtered and given by oral administration to laboratory animals (70). Animals were anesthetized with 2% isofluorane and placed in prone position on a gamma camera for 24 hours. During the first hour, the radiolabeled nanoparticles mainly remained in the stomach, while the activity slowly moved to distal parts of the gut at later times (Figure 7). In any case, no evidences of nanoparticle translocation were found. In fact, all the organs of animals (with the exception of the gastrointestinal tract) appeared to be free of radioactivity which was mainly eliminated by faeces.

In the latter, toxicological studies were carried out. In this case, the LD50 of nanoparticles after a single oral dose was found to be higher than 2000 mg/kg (unpublished data). More important, the oral administration of daily doses of either 30 mg or 300 mg of nanoparticles did not induce any sign or evidence of chronic toxicity.

All of these results appear to indicate that these nanoparticles would be able to strongly interact with components of the gut mucosa (including enterocytes and Peyer's patches) and, thus, increase the time of residence of nanoparticles in close contact with the mucosa. However, after a period of time, nanoparticles would be detached and eliminated. This hypothesis is also supported by previous results of transgene expression of intestine with DNA-chitosan nanoparticles (71). In fact, these nanoparticles were able to induce a maximum of transgene expression 24 h post administration followed by a quick decrease of this phenomenon which became hardly detectable after 72h. These results correlate with the enterocytes' lifetime determined by Ferraris and collaborators (72).

In summary, it seems that both ligands (mannosamine and flagellin) may facilitate the interaction and penetration of the nanoparticles to both normal and lymphoid tissues which gave them the possibility to be used as non-live microorganisms like polymeric vector in oral delivery systems.

3.3. Immune Response

In order to evaluate the ability of the different nanoparticle formulations as adjuvants, an immunization study in Balb/c mice was performed using ovalbumin (OVA) as antigen model. For this purpose, nanoparticles containing OVA were administered as a single dose either by the subcutaneous or the oral route. Conventional OVA-loaded nanoparticles (OVA-NP) were used as control. For subcutaneous administration, the animals were injected with 50 µL PBS containing 20 µg OVA in the form of OVA-F-NP, OVA-M-NP, OVA-NP or free OVA-solution. For oral administration, formulations containing 100 µg

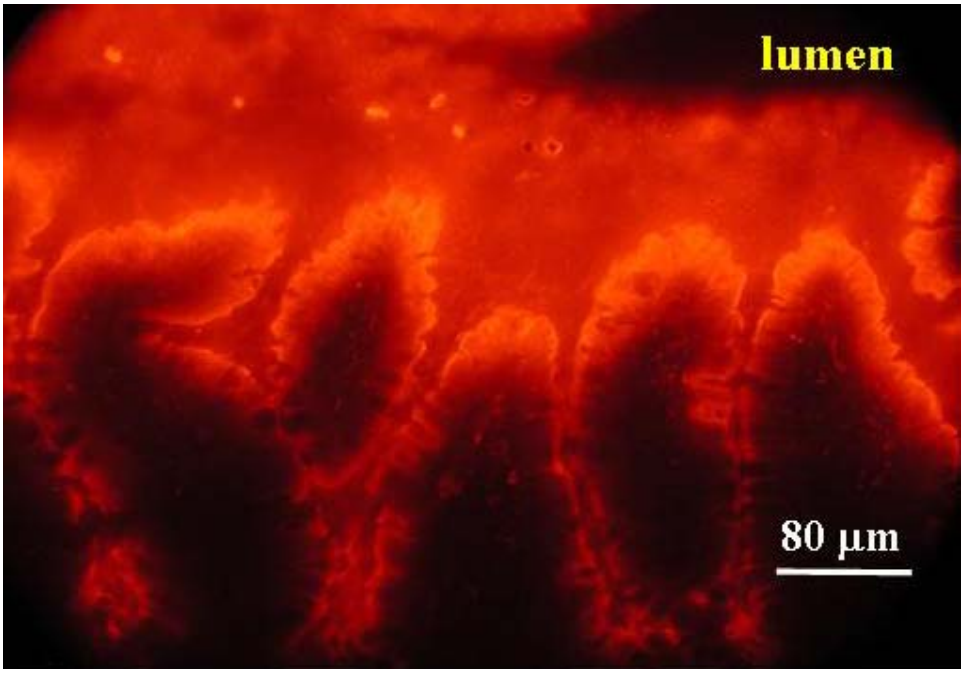

Figure 5. Visualization of the flagellin coated-nanoparticles in the normal mucosa of the ileum. Animals received orally 10 mg of RBITC-labelled nanoparticles and were sacrificed 2 hours later.

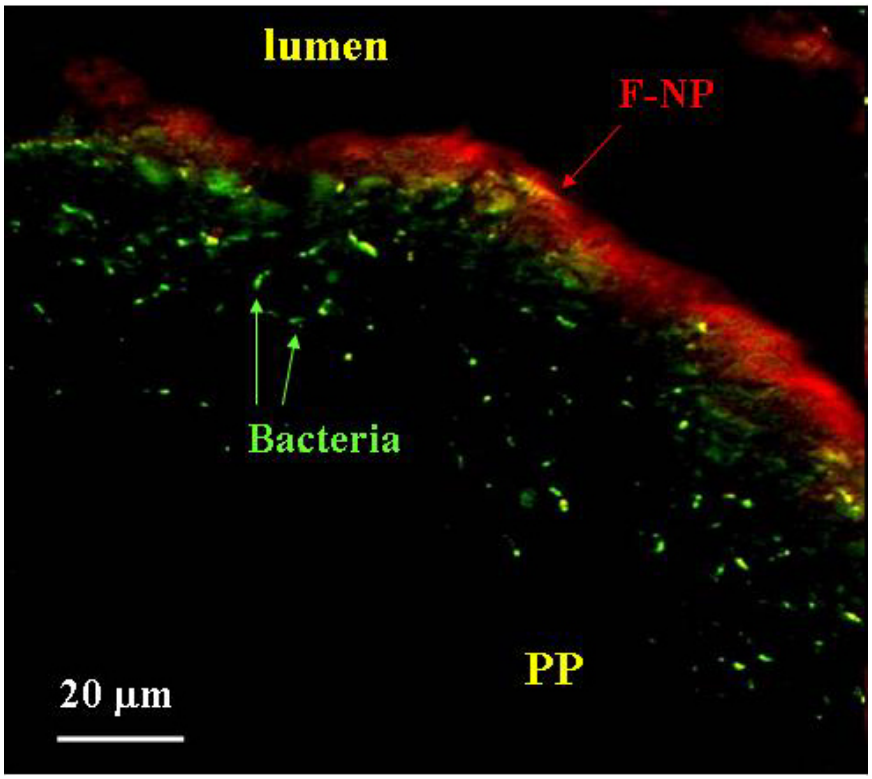

Figure 6. Distribution of both flagellin-coated nanoparticles (F-NP) and *Salmonella* Enteritis cells in the Peyer's patches of the ileum of rats visualized by confocal laser scanning microscopy. Nanoparticles and *Salmonella* cells were orally administered at the same time and animals were sacrificed 2 hours later.

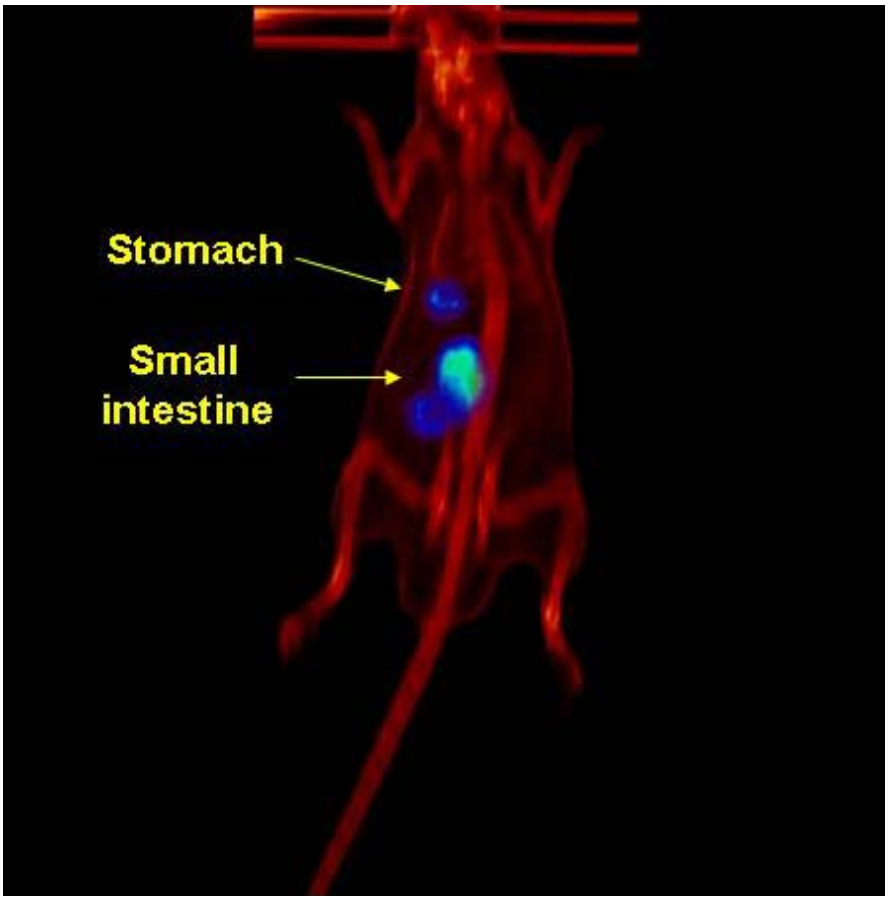

Figure 7. Study of the biodistribution of ^{99m}Technetium-F-NP obtained by gamma camera image. Animals received a single dose of 10 mg nanoparticles (1 mCi) dispersed in 1 mL water.

OVA were dispersed in water. At different times, serum and faecal samples were collected from animals and pooled. Systemic specific antibodies against OVA (IgG1 and IgG2a) were determined by ELISA. Form faeces, intestinal mucosal (IgA) antibody responses were also determined by ELISA.

Figure 8 shows the serum profiles of OVA-specific IgG1 and IgG2a in Balb/c mice for the different formulations tested. For animals immunized subcutaneously with Complete Freund's Adjuvant (CFA) the typical response was found, characterized by high levels of both IgG1 and IgG2a. Animals immunized with conventional nanoparticles (OVA-NP) showed a considerable shifting toward the Th2 subset whereas for animals immunized with ligand-coated nanoparticles a more balanced subset was observed. Comparing Th2 responses, the levels of IgG1 were high for all the ovalbumin formulations tested; however, these levels were slightly higher for CFA and conventional nanoparticles (OVA-NP) than for ligand-coated nanoparticles (OVA-F-NP and OVA-M-NP). In contrast, higher IgG2a titers were achieved when nanoparticles were decorated with mannose or flagellin. In the case of OVA-F-NP, the IgG2a titers were 5-times higher than after OVA-NP immunization or 2-times higher than after mannosamine-nanoparticles or CFA immunization.

Overall, the orally elicited antibodies response was lower than that described after subcutaneous administration (mainly the Th2 response; see Figure 8). On the contrary, oral immunization with either OVA-M-NP or OVA-F-NP elicited a stronger Th1 and Th2 response compared to the control OVA-NP. Furthermore, in comparison with OVA-NP, a Th1/Th2 balance was observed after oral immunization with ligand-coated nanoparticle formulations.

On the other hand, oral or subcutaneous immunizations with the different types of nanoparticles were able to elicit high levels of intestinal IgA compared to free OVA (Figure 9). Moreover, the oral immunization with flagellin or mannosylated nanoparticles induced the highest differences with respect standard nanoparticles. Interestingly, at 6 weeks post-oral administration, this mucosal immune response was about 4-5 titers higher than that noted for OVA-NP. In addition, for both types of ligand-nanoparticles, the anti-OVA specific intestinal IgA levels were higher when animals were immunized by the oral route than subcutaneously.

From a general point of view, all nanoparticle formulations showed an important enhancement in the antibody IgG2a and IgG1 responses compared to free OVA. Interestingly, subcutaneous immunization with conventional nanoparticles (OVA-NP) showed considerable shifting toward Th2 subset; in contrast, a considerable shifting toward the Th1 subset was noted in the ligand-coated nanoparticles, especially when using flagellin. This finding appear to be directly related with the high tropism of flagellin or mannosamine nanoparticles to the distal regions of the intestine (ileum) and uptake by Peyer's patches (see above). This may be correlated with previous study indicating that the delivery of OVA-loaded microbeads to lower intestine region enhances Th1 response (73).

For OVA-loaded flagellin nanoparticles (OVA-F-NP), Th1 enhancement would be a consequence of their effective uptake and subsequent activation of antigen presenting cells via TLR-5. Most TLR agonists function as adjuvants by stimulating the production of cytokines and the maturation of dendritic cells, thereby linking innate and adaptive immunity. Thus, for example, flagellin from gram-negative organisms signals, via interaction with TLR5, has effects on both innate and adaptive immune responses (74, 75), being described to activate dendritic cells releasing cytokines to produce a Th1 response (76).

OVA-loaded mannosylated nanoparticles (OVA-M-NP) also induced high IgG2a levels (Th1 response) after either subcutaneous or oral administration. This response may be related to effective uptake of mannosylated nanoparticles by APCs. In fact, successful enhancement of Th1 cytokine (IL-12 and INF-γ) secretion after intravenous administration of mannosylated liposomes (Man liposome/pCMV-OVA) (77) or mannan-coated liposome-protamine-DNA (LPD) nanoparticles (78) have been reported. In our case, the IgG2a anti OVA response elicited after mannosylated-nanoparticles immunization was lower than when using flagellin-nanoparticles. Uptake of antigens by C-type lectin receptors (calcium dependent lectin that recognizes mannose residues (79, 80)) does not necessarily result in the induction of potent effector T-cells, although facilitates the antigen-presentation capacity of dendritic cells (81).

In contrast, TLR ligation usually leads to DCs maturation and activation result in robust activation of immune responses and the induction of effector T-cells (75).

Dendritic cells residing in different tissues induce distinct immune responses from T cells. Thus, intestinal immune system has a predisposition towards Th2-cell responses since antigen presentation by Peyer´s patches DCs are characterized by the production of IL-4, IL-6, and IL-10, which inhibit a Th1 response (82). Therefore, under these circumstances, the role of the adjuvant becomes critical.

Interestingly, the oral immunization with flagellin or mannosamine coated-nanoparticles induced a higher mucosal IgA response than that obtained after subcutaneous administration, and always higher than control nanoparticles (OVA-NP). This phenomenon may be related again to the effective uptake of ligand-coated nanoparticles by gut Peyer's patches, since, as it was stated before, dendritic cells from murine Peyer's patches produce high levels of IL-4, IL-6, and IL-10, involved in IgA class switch (83, 84).

In summary, oral administration of ligand-coated nanoparticles induced stronger and more balanced serum titers of IgG2a (Th1) and IgG1 (Th2) than control nanoparticles which induced a typical Th2 response. This Th1 response enhancement may be related to the high tropism of both flagellin- and mannosylated-nanoparticles to the lower intestine (ileum) and uptake by Peyer's patches rich in APCs. On the contrary, naked nanoparticles displayed a low ability to target and interact with Peyer's patches, thus eliciting a week Th2-predominant immune response. This fact that can be negative for vaccination purposes, can be of interest to induce tolerance for immunotherapy with allergens.

4. PERSPECTIVES

Vaccination is generally accepted as the most practical measure in that is easy to apply and the most economic, however, present vaccines (in general) have limited efficacy or/and show safety problems. The ideal vaccine has to satisfy different sets of requirements (26, 85-88). Firstly, a good vaccine should induce the right sort of immune response as well as to stimulate a strong, protective and long-lasting immune response. The protective immune responses against extracellular pathogens seem to be mediated by long-lived humoral immune responses through the production of antibodies. However, in the control of intracellular infection, cellular immune responses have been shown to be crucial in mediating protection. Therefore, the development of a successful vaccine against those diseases will be facilitated by a thorough understanding of how cellular immune responses are generated and maintained *in vivo*.

Secondly, a single dose of vaccine should confer robust, long-lived immunity. It is well known that the successful completion of a full regimen for a given vaccine tends to decline as required boosts increase in number and over long periods of time.

On the other hand, an ideal vaccine should be safe, including children, elderly and immunocompromised subjects. Thus, the vaccine should not lead any associated disease such as allergic responses in recipients or significant local inflammation. In addition, the vaccine should not be capable of causing disease in others, which is particularly a major problem with some live-attenuated vaccines capable to keep residual pathogenicity.

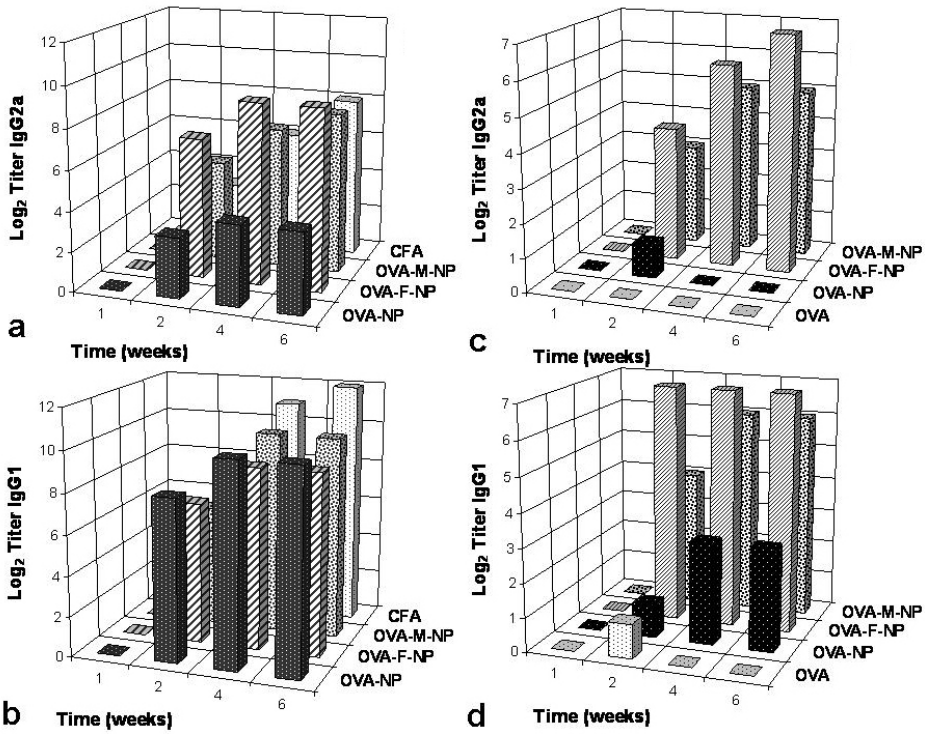

Figure 8. Serum profiles of OVA-specific IgG2a and IgG1 in BALB/c mice (n = 10). The immunization was performed at day 0 by a single dose of the formulations containing 20 μg OVA in case of subcutaneous route (a and b) and 100 μg OVA when used orally (c and d). The formulations were OVA-NP (ovalbumin-loaded poly(anhydride) nanoparticles), OVA-F-NP (OVA-loaded flagellin nanoparticles), OVA-M-NP (OVA-loaded mannosylated nanoparticles), CFA (ovalbumin dispersed in Complete Freund Adjuvant; only by subcutaneous route), and OVA (ovalbumin dissolved in PBS; only by oral route).

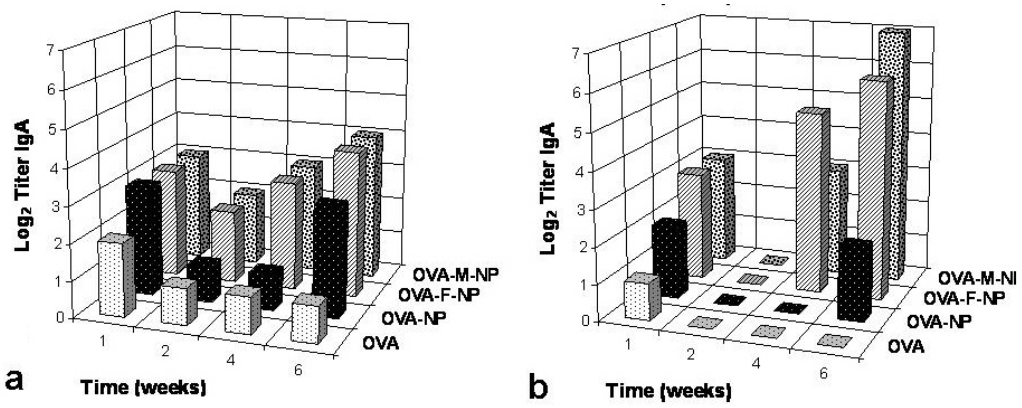

Figure 9. Faecal profile of secretory OVA-specific IgA in BALB/c mice (n = 10). The immunization was performed at day 0 by a single dose of the formulations containing 20 μg OVA in case of subcutaneous route (a) and 100 μg OVA when used orally (b). The formulations were OVA-NP (ovalbumin-loaded poly(anhydride) nanoparticles), OVA-F-NP (OVA-loaded flagellin nanoparticles), OVA-M-NP (OVA-loaded mannosylated nanoparticles), and OVA (ovalbumin dissolved in PBS).

Finally, an ideal vaccine should be affordable by the population at which they are aimed and should be formulated to resist high and low temperatures to facilitate distribution. This is a main problem for attenuated vaccines.

In consequence, the actual tendencies are oriented towards the development of new vaccines containing perfectly characterized antigens, rigorously controlled during all the steps concerning their preparation and safe. However, the new vaccines of the biotechnology era suffer in general of immunogenicity, requiring the use of adequate adjuvants. This is particularly challenging for mucosal vaccination. In fact, the mucosa is a door of entry for many pathogens. Although it is very difficult to generate mucosal

antibodies through parenteral vaccination, it is possible to obtain mucosal as well as parenteral immunity by inoculating antigen by the mucosal route (89, 90). For pathogens colonizing mucosal surfaces or those having a mucosal route of entry, protection correlates well with a strong local mucosal response (35, 91).

In this context the use of a biomimetic approach can be of interest. Thus, the adjuvant effect of particulate delivery systems would be improved by their association with compounds or molecules able to closely imitate the bacteria and virus behavior, concerning the strategies developed by these microorganisms along their evolution to interact with and invade the host.

In this report, we have demonstrate the viability of trying to imitate the strategies of micro-organisms to adhere to the surface of the gut mucosa and thus to improve the mucosal (oral) adjuvant effect of nanoparticles. The bioadhesive capacity and ileal tropism of the orally administered flagellin- or mannosamine-coated nanoparticles appeared to be instrumental for the effective elicitation of both systemic and mucosal immune responses. Further efforts should be focused on exploring the real potential of these new adjuvants and, currently, the application of flagellin-coated and mannosylated nanoparticles in oral vaccination and immunotherapy strategies is now under the investigation.

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