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Short communication

In vivo fate and immune pulmonary response after nasal administration of microspheres loaded with phosphorylcholine-thyroglobulin

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Abstract

Phosphorylcholine is a widely occurring hapten which is present in the cell wall of many prokaryotes. It is, therefore, an attractive candidate for the development of a vaccine against many bacterial diseases. Poly(D,L-lactide-co-glycolide) microspheres loaded with phosphorylcholine linked to thyroglobulin (PC-Thyr) as protein carrier were prepared. The effect of the protein concentration on antigen encapsulation and release as well as on microsphere morphology has been investigated. When administered intranasally, PC-Thyr-loaded microspheres were taken up by epithelial cells of the nasopharyngeal associated lymphoid tissue and induced a specific IgA and IgG response in pulmonary secretions as well as a strong systemic immune response in BALB/c mice.

Keywords: Phosphorylcholine; Microspheres; Nasal administration

1. Introduction

When establishing priorities for vaccine research, the World Health Organization (WHO) stressed the need to develop safe, easy to use, and highly stable vaccine (World Health Organization, 1997). In that respect, mucosal vaccination has several advantages over parenteral vaccination, as

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it is safe, induces minimal adverse effects, and can be administered at home (Walker, 1994). The use of antigen delivery systems consisting of biodegradable microspheres are designed to increase antigen uptake by the M cells (Eldridge et al., 1990), to enhance the mucosal immune response (Eldridge et al., 1991) and to induce long and slow antigen release (O'Hagan et al., 1991). The use of poly(D,L-lactide-co-glycolide) (DL-PLG) microspheres is particularly promising (Shahin et al., 1995). We further validated this approach by showing that intragastric immunization of mice with phosphorylcholine (PC) coupled to thyroglobulin and entrapped in DL-PLG microspheres afforded protection against a lethal oral challenge by the *Salmonella typhimurium* strain C5 (Allaoui-Attarki et al., 1997). We chose PC as antigen because it is a ubiquitous hapten present on different pathogenic micro-organisms which colonize or invade host mucosa at different epithelial sites (Pecquet et al., 1992; Gillepsie et al., 1996; Kolberg et al., 1997). Parenterally administered PC has been known to be a protective antigen against parenteral inoculation of *Streptococcus pneumoniae* (Wallick et al., 1983; Fisher et al., 1995). However, whether anti-PC pulmonary immunity also protects against pneumococcal pneumonia is not known. Because of the compartmentalization of the common mucosal immune system (Moldoveanu et al., 1995), intranasal immunization is the best route of administration to induce a specific immune response in the respiratory system. This study investigate the possibility of using PC-Thyr loaded microspheres by the intranasal route to stimulate the pulmonary anti-PC immunity.

2. Materials and methods

Phosphorylcholine (PC) was conjugated to porcine thyroglobulin (Sigma, St. Louis, MO) and the resulting conjugate (PC-Thyr) was entrapped in DL-PLG (ratio 75/25; molecular size, 128 kDa; Birmingham polymers, Birmingham, AL) microspheres by multiple-solvent evaporation emulsion (Blanco-Prieto et al., 1994). Concentrations of 40, 120 and 200 mg/ml of PC-Thyr were tested. Unloaded microspheres were prepared identically, but without PC-Thyr. Fluorescent 200 mg/ml PC-Thyr-loaded microspheres were obtained by the addition of 100 μl/ml of perylen (Aldrich Chemical, Saint Quentin Fallavier, France) in the organic phase. Particle size distributions of freeze-dried microspheres were determined using a Coulter Multisizer II. A scanning electron microscope (SEM) (XL-40, Philips, The Netherlands) was used to evaluate both the morphology and surface characteristics of the microspheres.

For determining the encapsulation efficiency, the conjugate was released from 30 mg of microspheres by overnight incubation in 3 ml of 0.1 N NaOH with stirring. The amount of encapsulated thyroglobulin was determined by a BCA protein assay. Encapsulation efficiency was expressed as the ratio of the amount of thyroglobulin entrapped to the initial amount introduced into the polymerization medium. For in vitro release study, an amount of microspheres corresponding to 0.7 mg of thyroglobulin was incubated in 4 ml of saline phosphate buffer under stirring at 37°C. At different time intervals, 350 μl of the suspension were removed and replaced with 350 μl of fresh buffer to maintain sink condition. Samples were centrifuged (4000 rpm for 10 min) and the thyroglobulin concentration was determined in the supernatant by a BCA protein assay.

Microsphere capture by the Nasal Associated Lymphoid Tissue (NALT) was studied in mice. Under light anesthesia with sodium pentobarbital (Sanofi Santé Animale, Libourne, France), BALB/c mice were administered intranasally with 7.5 mg of fluorescent PC-Thyr loaded microspheres. Thirty-five min after inoculation, mice were sacrificed. Sections of rhinopharynx have been realized as described (Pereswetoff-Morath et al., 1996). The samples were embedded in paraffin, sectioned, stained with 2% Evans blue (w/v) and examined by fluorescent microscopy.

Groups of BALB/c mice were immunized intranasally under light anesthesia with sodium pentobarbital. On 3 consecutive days, a dose comprising 7.5 mg of either blank or PC-Thyr-loaded microspheres, or of 15 μg of PC in free PC-Thyr in 50 μl sterile saline, was deposited in the nostrils. Mice were boosted with identical

doses 29, 30 and 31 days later. The anti-PC IgA and IgG responses were assessed by ELISA, 45 days after the first immunization dose, at the time of peak antibody responses to PC (Allaoui-Attarki et al., 1997). Blood samples were collected by retro-orbital puncture. Sera were diluted 1:40 in bicarbonate buffer. Lung washes were collected immediately after death, as described (Guzman et al., 1993). We considered that sample dilutions were 1:10 (Guzman et al., 1993). All samples were immediately frozen at −80°C until ELISA was performed as described (Allaoui-Attarki et al., 1998). Antibodies titers, expressed as $\log_2$ of the reciprocal of the dilution, were compared by Student's *t*-test.

3. Results and discussion

The size distribution of PC-Thyr-loaded microspheres was determined for three different concentrations of PC-Thyr conjugate: 40, 120 and 200 mg/ml. No effect of conjugate concentration on microspheres mean diameter was found (Table 1). However, microspheres prepared without PC-Thyr were found to be smaller with a mean diameter of 2.5 μm (data not shown). Loading efficiency of PC-Thyr in microspheres was studied as a function of the PC-Thyr concentration in the internal aqueous phase. For 40 and 120 mg/ml concentrations values, it was found that the encapsulation efficiency was stable. However, the encapsulation efficiency decreased when the initial amount of PC-Thyr was of 200 mg/ml (Table 1). Theoretically, during the formation of microspheres by the multiple emulsion/evaporation method, the polymer solution separates the internal aqueous phase from the external aqueous phase, thus preventing the diffusion of the protein into the outer water phase. Nevertheless, an exchange between the two aqueous phases (external and internal) might occur as a consequence of the adsorption of the protein at the microsphere surface. It was previously shown that the accumulation of the protein on the microsphere surface and their rapid elution formed pores or channels on the particle surface (Blanco-Prieto et al., 1996). Therefore, the morphological appearance of the microspheres obtained for different PC-Thyr amount should be modified. Indeed, with 120 mg/ml of conjugate, microspheres were smooth with no apparent pores (Fig. 1a) whereas the surface of particles prepared with 200 mg/ml of PC-Thyr appeared highly porous (Fig. 1b). Therefore, it is assumed that during the microencapsulation process, PC-Thyr was adsorbed at the interface of internal aqueous globules and polymeric phase. When the conjugate concentration was elevated, the interface of internal globules was saturated and proteins embedded in regions close to the microsphere surface. The resulting PC-Thyr elution explained the formation of pores and might be responsible for the decreased of the encapsulation efficiency.

Our in vitro released experiments showed that in any experimental condition, only a poor amount of PC-Thyr was released after incubation (Table 1). This was not the case in the presence of proteolytic enzymes where about 20% of PC-Thyr was released after the same time of incubation (Allaoui-Attarki et al., 1998). These release studies suggest that the protein adsorbed on the surface of microspheres is tightly bound due to possible conformational changes. However, this adsorption did not modify the immunogenicity of PC-Thyr since neither the absorbance spectrum of

Table 1
Effect of the PC-Thyr concentration on particle size and encapsulation efficiency and burst release[a]

PC-Thyr concentration (mg/ml)	Average size (μm)	Encapsulation efficiency (%)	PC-Thyr released after 15 min (%)
40	3.2	86.2	1.6
120	3.4	87.6	0.7
200	3.2	76.5	0.9

[a] Mean of three independent measurements.

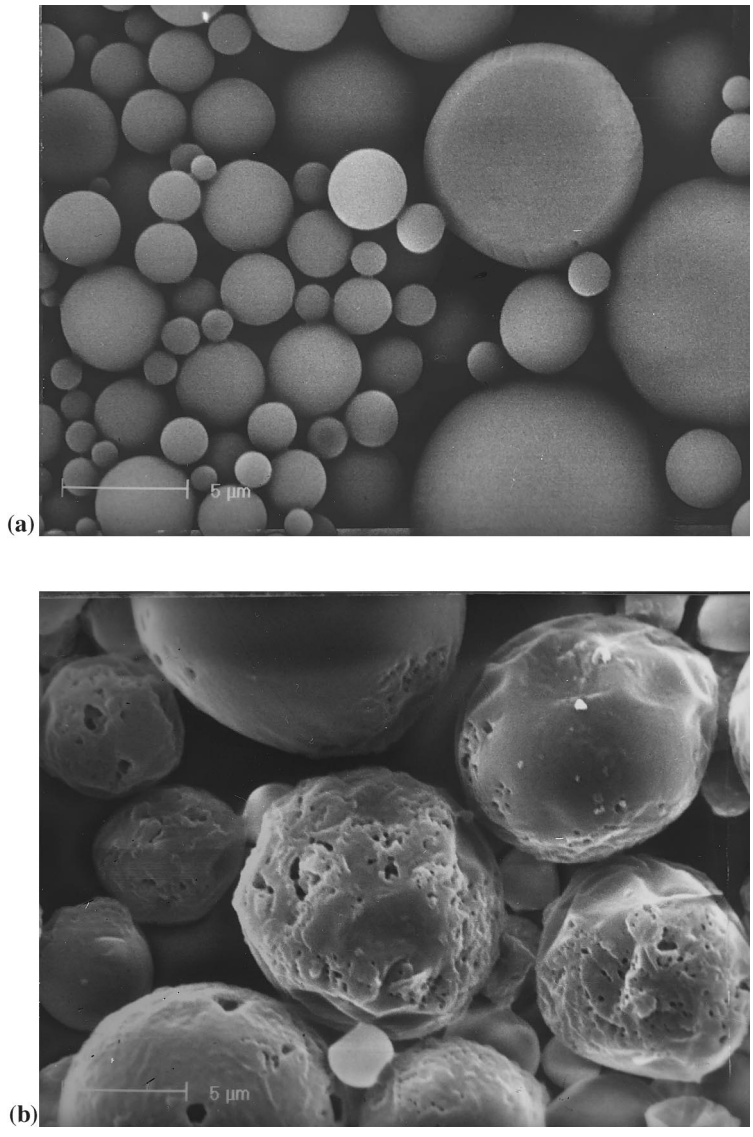

Fig. 1. Electron micrographs of microspheres formulated with 120 mg/ml (a) or 200 mg/ml (b) of the conjugate PC-Thyr.

the PC-Thyr conjugate, nor its PC-immunologic reactivity were changed by the microencapsulation process (Allaoui-Attarki et al., 1998).

We have previously shown that microspheres prepared with 200 mg/ml of PC-Thyr, administered intragastrically to mice, induced intestinal anti-PC antibody responses (Allaoui-Attarki et al., 1998). This response afforded protection against a lethal oral challenge by the *Salmonella typhimurium* strain C5 (Allaoui-Attarki et al., 1997). In order to explore the protection provided by the same vaccine preparation against pulmonary PC-bearing pathogenic bacterium such as *Streptococcus pneumoniae*, we explored the potential ability of PC-Thyr-loaded microspheres to induce anti-PC antibody response in bronchoalveolar fluids of intranasally immunized mice. Microspheres must first be able to be capture by the NALT. In the present study, we were able to show that, 35 min after intranasal administration,

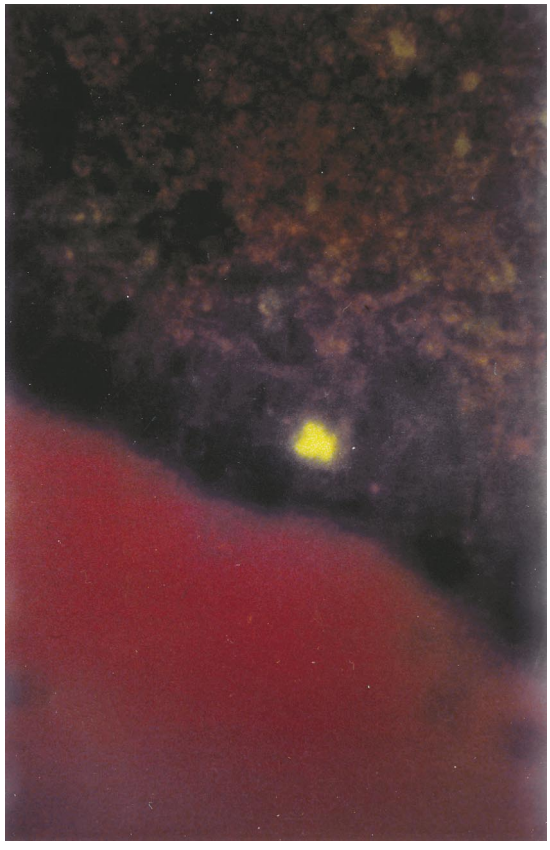

Fig. 2. Uptake of fluorescent PC-Thyr loaded microspheres by epithelial cells of nasal lymphoid follicles (× 100).

fluorescent PC-Thyr-loaded microspheres were captured by epithelial cells of nasal lymphoid follicles (Fig. 2). Intranasal immunizations with PC-Thyr induced significantly higher IgA and IgG anti-PC titers than intranasal immunization with unloaded microspheres (Table 2). High levels of serum IgG against soluble or particulate antigens following immunization via the respiratory tract have been reported by several groups (Shahin et al., 1992; Russell et al., 1996). A mechanism of transudation may explain the high levels of anti-PC IgG found in the lungs of the mice immunized either subcutaneously and intranasally (Toews et al., 1985). On the opposite, anti-PC IgA levels were lower in serum than in pulmonary secretions of immunized mice, which implies that mucosal IgA was produced by intraepithelial local B cells. The present results show that the anti-PC antibody response induced by intranasal immunization with PC-Thyr-loaded microspheres was not greater than that observed after intranasal vaccination with free PC-Thyr. Free PC-Thyr is a soluble antigen devoid of cell adhesion properties, and might therefore freely penetrate the entire nasal epithelium and reach the immune system (Kuper et al., 1992). Thus, the main advantage of using a particulate carrier for anti-PC nasal immunization might be to induce long-lasting local immunity by interacting with mucosal surfaces (Walker, 1994) and to generate a mucosal memory response (McGhee et al., 1993).

Table 2
Anti-PC antibodies in sera and secretions of immunized mice[a]

Immunization group	Route	Anti-PC titer in indicated fluid			
		IgA		IgG	
		Serum	Broncho-alveolar fluid	Serum	Broncho-alveolar fluid
PC-Thyr in microspheres	i.n.[b]	3.6 (1.1)	5.1 (2.0)	9.1 (1.3)	8.5 (2.3)
Free PC-Thyr	i.n.	2.5 (0.9)	6.3 (4.7)	9.2 (1.2)	6.1 (0.4)
Free PC-Thyr	s.c.[c]	0.8 (0.9)	0	8.4 (1.0)	4.8 (1.8)
Unloaded microspheres	i.n.	0.2 (0.6)	0	0.5 (0.5)	0

[a] Sera and secretions were collected 2 weeks after the last immunization. Means (S.E.M.) of the $\log_2$ endpoint dilution are reported. These data are results of two independent experiments.
[b] Intranasally.
[c] Subcutaneously.

4. Conclusion

In conclusion, these results strongly suggest that the potentiality of PC-Thyr-loaded microspheres to protect against pulmonary infections caused by PC-bearing micro-organisms as *Streptococcus pneumoniae* and *Haemophilus influenzae* (Kolberg et al., 1997) should be further explored. This should constitute an important step forward in the development of the concept that PC linked to a protein carrier can be used as mucosal immunogen to protect against several bacterial diseases.

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