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Vaccine properties of antigens entrapped in microparticles produced by spray-drying technique and using various polyester polymers

Benoît Baras[a, b,*], Marie-Ange Benoit[b], Odile Poulain-Godefroy[a], Anne-Marie Schacht[a], André Capron[a], Jean Gillard[b], Gilles Riveau[a]

[a]*Laboratoire des Relations Hôte-Parasite et Stratégies Vaccinales, INSERM U167, Institut Pasteur de Lille, 1 rue du Professeur Calmette, BP-245, F-59019 Lille Cedex, France*

[b]*Laboratoire de Pharmacie Galénique, Industrielle et Officinale, Ecole de Pharmacie, Université Catholique de Louvain, avenue Emmanuel Mounier 73.20, B-1200 Brussels, Belgium*

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Abstract

The present study investigated the suitability of various microparticles produced by spray-drying technique to entrap and preserve the physiochemical and biological properties of an antigen. These microparticles were constituted either by poly(lactide) polymers characterized by various molecular weight or poly(lactide-co-glycolide) polymers. The recombinant 28 kDa glutathione S-transferase of *Schistosoma mansoni* (rSm28GST) characterized by major epitopes involved in the active site of this enzyme was selected as model antigen. The microparticles were characterized by a mean size ≤ 5 μm and an antigen loading of approximately 2% (w/w). The analysis by SDS-PAGE electrophoresis of the rSm28GST released from microparticles confirmed the conservation of its physicochemical characteristics. The conservation of the native structure of the entrapped antigen was confirmed by detecting its enzymatic activity after release from microparticles. A single intraperitoneal immunization of mice with rSm28GST entrapped in microparticles resulted in a specific antibody response, which remained high for at least 7 months. The analysis of the isotype profile indicated that immunized mice primarily produced anti-rSm28GST immunoglobulin (Ig) G1 with the coexistence of lower IgG2a and IgG2b levels. Finally, the recognition of the major epitopic regions and the neutralization of the enzymatic activity of the rSm28GST by the antisera confirmed the specificity of the response against the native structure of the antigen. These results confirmed the integrity of the entrapped antigen. Moreover, our results supported the hypothesis that the duration of antigen release is the limiting factor for the duration of antibody production. Indeed, the use of polymers characterized by different molecular weights allowed us to modify the duration of the immune response. Together, these results demonstrated that microencapsulation of an antigen by spray-drying preserved its crucial characteristics required to generate an effective humoral immune response after a single-dose administration.

Keywords: Spray-drying; Microparticles; Antigen stabilization

1. Introduction

Recent scientific advances have provided information relevant to the design of vaccines against a wide variety of infectious agents. As a result, numerous vaccine antigens have been identified and produced in the form of subunits, synthetic peptides and proteins expressed in a variety of vectors through recombinant genetics. Although these new antigens offer advantages in specificity and safety, they are in many cases weakly immunogenic. This lack of immunogenicity has created an acute need to identify pharmaceutically acceptable delivery systems or adjuvants [1]. The microencapsulation of proteins in biodegradable polymers is now well

* Corresponding author. Tel.: +33-3-2087-7781; fax: +33-3-2087-7888.

E-mail address: ipv@pasteur-lille.fr (B. Baras).

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recognized for controlled-release vaccines requiring only a single administration [2].

Polylactide (PLA) and poly(lactide-co-glycolide) (PLG) are the most commonly used polymers. These polyester polymers have several advantages, including biocompatibility and biodegradability, extensive knowledge of their in vitro and in vivo degradation rates and Food and Drug Administration approval for a number of clinical applications in humans [3]. The molecular weight of the polymer is an important parameter for controlling the release profile of the entrapped antigen as it modifies the microparticle degradation rate, the microparticle size and the antigen loading in the microparticles [4,5]. These results indicate that the use of various polymers would allow the modulation of the immune response. The production technology mainly used in the manufacture of microparticles has been solvent evaporation or solvent extraction from emulsion systems. Problems encountered with these methods of production include the use of high shearing forces, long exposure time of the antigen to organic solvents and the necessity for lyophilization to obtain a stable powder. These problems may result in the degradation of a sensitive antigen.

Preparation of microparticles by spray-drying seems to be an attractive alternative to conventional microencapsulation techniques. This method aerosolizes an emulsion of polymer and antigen to create microdroplets that solidify in a brief blast of heated air. From a manufacturing viewpoint, this technique offers the advantage of being a single-step process which can readily be scaled up [6]. A stable powder is rapidly obtained without lyophilization. Due to the rapid evaporation of the solvent, the temperature of the droplets can be kept below the drying air temperature [7], and for this reason spray-drying can be applied to heat sensitive materials [8]. As polypeptides are water soluble, while the selected polymers are soluble only in organic solvent, preparation methods of microparticles that combines both organic and water phases have been searched. The initial stages in the microencapsulation of proteins involved emulsification of aqueous solutions of proteins with polymers dissolved in an organic solvent. Nevertheless, and contrary to other microencapsulation techniques, the exposure time of the antigen to organic solvent is significantly reduced with spray-drying.

The microencapsulation of hydrophilic macromolecules by spray-drying techniques has been mostly limited to model proteins, such as bovine serum albumin [9]. In this study, the recombinant 28 kDa glutathione S-transferase (rSm28GST, 211 amino acids, a.a.) [10] was used as the model antigen. This enzyme is one of the candidate antigens selected by the World Health Organization for vaccine evaluation against schistosomiasis [11], a chronic and debilitating parasite disease. GST from schistosomes is thought to play a key role in the detoxification processes involved in their defense against host immune systems. rSm28GST has proven vaccinal efficacy in several animal models, including primates [12,13]. Epitopic mapping of rSm28GST has identified two regions of the protein that have a relevant role in the protection [14,15]. The immune responses to both the C-terminal (a.a. 190–211) and N-terminal (a.a. 24–43) regions mediate a reduction in both female parasite fecundity and egg viability. Moreover, C-terminal and N-terminal peptides were shown to be associated with the enzymatic site of the protein.

This study shows that the spray-drying technique is definitively adapted to entrap highly conserved antigens capable of inducing a long-lasting antibody response endowed with biological activity. Other authors have already used spray-drying for encapsulation of antigens to elicit immune responses after a single dose [16–18] and some have shown that some peptides can be degraded during spray-drying [19,20]. Indeed, during the spray-drying process, the protein is exposed to physical stresses (organic solvents, aqueous/organic interface and relatively adverse temperature and emulsion conditions) that may produce structural changes of the protein and thus may result in partial or total loss of enzymatic activity or antigenicity [9]. However, following the process used, spray-drying can be used to entrap antigens, which conserve their native properties. To study our method of encapsulation, we have used as an antigen, an enzyme characterized by major epitopic regions involved in the enzymatic site. These results showed that microencapsulation by spray-drying can be adapted to entrap antigens whilst conserving their biological activity. Moreover, the use of various polylactide, characterized by various molecular weights, to produce microparticles, allowed the modification of both the duration of the immune response and the antigen presentation to the immune system.

2. 2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial suppliers: poly-D,L-lactide R 203 (molecular weight, 16,000), poly-D,L-lactide R 206 (molecular weight, 109,000), poly-D,L-lactide R 207 (molecular weight, 209,000) and polylactide-co-glycolide RG 756 (molecular weight, 49,000; 75:25 ratio of lactide/glycolide) (Boehringer-Ingelheim); polyvinyl alcohol (PVA) (molecular weight, 13,000 to 23,000; 87–89 % hydrolyzed) (Aldrich Chemical Co., Bornem, Belgium); dichloromethane (DCM) (UCB, Braine L'Alleud, Belgium); sodium dodecyl sulfate (SDS), CDNB (1-

chloro-2,4 dinitrobenzene) and GSH (glutathione) (Sigma Chemical Co., St Louis, MO, USA); low molecular weight standard (range from 14–94 kDa) was supplied by Bio-Rad (Nazareth, Belgium); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG isotypes (Southern Biotechnology Associates Inc., Birmingham, AL, USA); biotinylated goat anti-mouse IgA and IgG(H + L) antibodies and streptavidin-horseradish peroxidase (Amersham, Les Ulis, France); ABTS (2,2′-azino-di-[3–ethyl–benzothiozolin–sulfonate]) (Boehringer, Mannheim, Germany); Tween, gelatin and Folin-Ciolcateu's phenol reagent (Merck, Darmstadt, Germany). All substances used were of analytical grade whereas all solvents were of HPLC grade.

2.2. Antigen

Recombinant Sm28GST (rSm28GST) was produced in *Saccharomyces cerevisae* (kindly provided by Transgène S.A., France) and was affinity purified as previously described [21].

2.3. Preparation of rSm28GST-loaded spray-dried microparticles

A solution of rSm28GST (10 mg/ml) containing PVA (10 mg/ml) in 1 ml of ultrapure water was emulsified with a solution of polymer (0.5%, w/v) in 100 ml of DCM using an Ultraturrax model T 25 (IKA Laboratory Technology, Staufen, Germany) at 8,000 rpm for 10 min at room temperature.

Microparticles were obtained by spraying the emulsion through the nozzle (0.5 mm) of a Büchi Mini Spray Dryer Model 190 (Büchi Laboratoriums-Technik AG, Flawil, Switzerland).

The processing parameters were set as follows: inlet temperature ($45 \pm 2^{\circ}C$), outlet temperature ($34 \pm 2^{\circ}C$), aspirator (setting 15), pump rate (4.5–5.5 ml/min), air flow rate (500 l/h) and spray flow pressure (6 bar).

2.4. Microparticle characterization

The microparticles dispersed in a filtered (0.1 μm) saline solution of 0.9% (w/v) NaCl were sized using a Coulter Multisizer (Coulter Electronics Ltd., Luton, UK) equipped with a sieve of 100 μm aperture and under continuous stirring. Particle size was expressed as volume mean diameter (VMD) in micrometers ± standard error of the mean (S.E.M.) ($n = 3$).

For evaluation of the rSm28GST loading in microparticles, 30 to 50 milligrams of spray-dried microparticles, accurately weighed, were dissolved in 3.0 ml of 1 M NaOH containing 5% (w/v) SDS over 24 h at room temperature [22]. After centrifugation ($4000 \times g$ for 10 min at room temperature), the supernatant was assayed following the method of Lowry et al. [23] to determine the antigen concentration. The percentage (w/w) of entrapped antigen per dry weight of spray-dried microparticles was determined using a standard curve of BSA (bovine serum albumine). These results were then compared with those obtained from known quantities of nonentrapped purified rSm28GST and with same quantities of empty microparticles. All standards were treated in the same manner than entrapped rSm28GST. Each sample was assayed in triplicate.

2.5. In vitro release study

Microparticles (40–60 mg) were suspended in 3 ml of phosphate buffer saline (PBS, pH 7.4) (0.01 M phosphate buffered saline, 0.138 M NaCl and 0.0027 M KCl) containing 0.1% (w/v) sodium azide as bacteriostatic agent. Release studies were conducted for 340 h at 37°C under continuous stirring. For these studies, the medium (accurately measured) was withdrawn at each time interval and rSm28GST release was determined by the analytical method describe above, the suspension being refilled with the same volume of fresh medium. Each assay was replicated three times.

2.6. Study of the primary structure integrity of the entrapped antigen

Microparticles (30 mg) containing rSm28GST were suspended in PBS for 340 h at 37°C under continuous stirring. After centrifugation ($4000 \times g$ for 10 min at room temperature), the supernatant was harvested for analysis. The assessment of the integrity of rSm28GST released from microparticles into PBS and free rSm28GST was investigated using polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were analysed using a 15% gel with the Hoefer system (Hoefer Scientific Instruments, SE 600 Series Electrophoresis Unit, San Francisco, CA) according to the method described by Laemmli [24]. Low molecular weight standards (14 to 94 kDa) were used. The gel was fixed and stained with Coomassie brillant blue R-250 (0.1%, w/v) in a solution of water/acetic acid/methanol (50/10/40).

2.7. Immunization

Immunization experiments were performed by intraperitoneal route using 10 female BALB/c mice (6–8 week old, Iffa Credo, L'Arbresle-France). All rSm28GST-loaded microparticles were delivered in a single dose containing 100 μg of rSm28GST in 200 μl of PBS. Control groups were immunized with the same amount of free antigen or with empty microparticles. Blood samples were collected from the retro-orbital plexus and the serum harvested following coagulation

and centrifugation (4000 × *g* for 10 min at 4°C). Sera were stored at −20°C until assayed for the presence of specific antibodies.

2.8. ELISA

The purified rSm28GST (10 μg/ml in PBS, 50 μl/well) was coated onto 96-well Immulon 3 microtiter plates (Dynatech Laboratory Inc., Chantilly, VA, USA) for 2 h 30 min at 37°C. Plates were washed three times with 0.1% (v/v) Tween 20 in PBS (PBS-Tween) using an automatic washer (LP35, Diagnostic Pasteur, Marne-la-Coquette, France). Sera (including nonimmunized mouse serum as a control) diluted in PBS-Tween containing 0.5% (w/w) gelatin (PBS-Tween-Gelatin), were added to the antigen-coated wells (50 μl/well for each dilution) and incubated overnight at 4°C. Plates underwent four PBS-Tween washes and were incubated for 1 h 30 min at 37°C with 50 μl of HRP-conjugated goat anti-mouse IgG1 (1/1000 dilution), IgG2a (1/500), IgG2b (1/500) and IgG3 (1/300) appropriately diluted in PBS-Tween-Gelatin (50 μl/well). After four washes, the HRP was revealed for 1 h at room temperature with 1 mg/ml ABTS in a citrate buffer (50 mM, pH 4.0) containing 0.003% H_2O_2 (50 μl/well). Absorbances were measured at 405 nm using a microplate reader (Titertek Multiscan MCC, Labsystems Group, Les Ulis, France).

For serum IgA determination in sera, the ELISA protocol included an additional step (after the incubation of sera) with biotinylated goat anti-mouse IgA antibodies diluted 1/3000 in PBS-Tween-Gelatin (50 μl/well) for 1 h 30 min at 37°C, washed six times with PBS-Tween, and incubated with streptavidin-horseradish peroxidase diluted 1/2000 in PBS-Tween-Gelatin (50 μl/well) for 30 min at 37°C. Plates were washed six times with PBS-Tween, and 50 μl of peroxidase substrate (ABTS and H_2O_2) was added.

For each assay, titers were defined as the highest dilution yielding an absorbance 3 times above the background (no serum added). The affinity was defined as $1/x$, where x is the slope of the equation: optical density $= x$ log [dilution].

2.9. Recognition of rSm28GST epitopic regions by specific sera

The recognition of rSm28GST epitopic regions by sera obtained after mice immunization with entrapped rSm28GST, free rSm28GST or empty microparticles was assessed by ELISA. Maxisorp plates (96 wells, Nunc, Intermed S.A., Denmark) were incubated for 2 h 30 min at 37°C with 10 μg/ml of a synthetic peptide corresponding to the 24–43 or 190–211 epitopic regions of the rSm28GST in 100 μl of 0.1 M sodium carbonate buffer, pH 9.6. The 190–211 synthetic peptide was a linear peptide whilst the 24–43 synthetic peptide had an octameric configuration (an eight fold branched polylysine core attached to eight peptide residues). After a single washing, wells were saturated with 200 μl of PBS containing 0.5% (w/v) gelatin for 30 min at room temperature to block nonspecific antibody binding. Plates were washed three times with PBS-Tween using an automatic washer as described above. Sera (including nonimmunized mouse serum as a control) diluted in PBS-Tween, were added to the antigen-coated wells (100 μl/well for each dilution) and incubated overnight at 4°C. Plates underwent four PBS-Tween washings and were incubated for 1 h 30 min at 37°C with 100 μl of with biotinylated goat anti-mouse IgG(H + L) antibodies diluted 1/40,000 in PBS-Tween-Gelatin. After five washings with PBS-Tween, plates were incubated with 100 μl of streptavidin-HRP diluted 1/1000 in PBS-Tween-Gelatin for 30 min at 37°C. After six washings, the HRP was revealed for 1 h at room temperature with 100 μl of 1 mg/ml ABTS in a citrate buffer (50 mM, pH 4.0) containing 0.003% H_2O_2. The optical density was measured at 405 nm on a microplate reader as described above.

2.10. Enzymatic activity of the rSm28GST

Glutathione S-transferases are enzymes catalyzing conjugation reactions in which GSH acts as a nucleophile. The in vitro addition of GSH to the substrate bearing an electrophilic carbon atom (e.g. CDNB) results in the formation of a thioether, thus causing a direct change in the absorbance of the substrate which is measured by spectrophotometry [25].

The enzymatic activity of both free rSm28GST and rSm28GST released from microparticles (as described above at the point 2.6.) was measured in 820 μl of reaction buffer (50 mM potassium phosphate, pH 6.5, containing 0.36 mM CNDB and 4.76 mM GSH). The enzymatic reaction was started when 10 μl of rSm28GST (3.5 pmoles/well) was added and monitored spectrophotometrically at 340 nm for 80 min.

The neutralizing activity of an anti-rSm28GST serum was analyzed in 96-well flat-bottomed standard ELISA plates as described by Grzych et al. [26], with the following modifications. Enzymatic mix (30 μl) was added to 400 μl of reaction buffer to make up a total reaction volume of 430 μl/well. This enzyme mix contained 10 μl of rSm28GST (3.5 pmoles/well) incubated with 20 μl of 50 mM potassium phosphate (pH 6.5), or the studied antisera at different dilutions in 50 mM potassium phosphate (pH 6.5), for 1 h at 37°C followed by 1 h at 4°C. The reaction was started by the addition of the reaction buffer which was composed of 50 mM potassium phosphate (pH 6.5) containing 0.36 mM CDNB and 4.76 mM GSH. The absorbance at

Table 1
Characteristics of microparticles from various polymers entrapping rSm28GST[a]

Polymer	Molecular weight (Da)	Mean particle size (μm) ± S.E.M.	90% of the volume ≤ (μm)	Antigen loading (%)
R 203	16,000	2.66 ± 0.09	4.08 ± 0.15	1.78 ± 0.14
R 206	109,000	3.48 ± 0.09	5.49 ± 0.14	1.84 ± 0.18
R 207	209,000	4.43 ± 0.11	8.49 ± 0.41	1.84 ± 0.20
RG 756	49,000	3.39 ± 0.08	5.33 ± 0.22	2.19 ± 0.17

[a] Each sample was assayed in triplicate.

340 nm was recorded at 15 s intervals for a period of 2 min. The enzymatic activity value recorded for the rSm28GST in the presence of antisera was related to that measured for the sera of nonimmunized mice, which was assigned as the 100% value for specific activity.

3. Results

3.1. Microparticle characterization and antigen loading

All microparticle preparations were homogeneous and characterized by irregular forms with a crumpled porous surface as previously described [4]. Results presented in Table 1 indicate the particle size distributions and the antigen loading for microparticles produced from the various polymers. Microparticles were characterized by a mean particle size ≤5 μm. All preparations resulted in a particle size distribution where 90% of particles were ≤10 μm. The particle size distribution was affected by the polymer molecular weight. An increase in molecular weight (from 16,000 for R 203 to 209,000 for R 207) resulted in an increase in the microparticle mean and maximal size (Table 1). No differences in antigen loading (approximately 2%, w/w) were observed when the molecular weight was modified (Table 1).

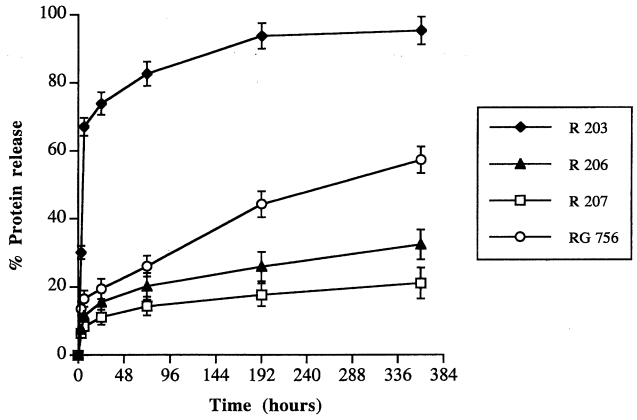

Fig. 1. In vitro release profile of the entrapped antigen from R 203, R 206, R 207 and RG 756 microparticles.

3.2. Release of the entrapped antigen from microparticles

The dissolution profiles always showed a gradual release of the entrapped antigen within 340 h after a fast release at the beginning (Fig. 1). The release was fast from microparticles made of low molecular weight polymers (R 203 and RG 756 with respectively 99.12 ± 4.12 and 57.27 ± 3.87% of rSm28GST released in 360 h) when compared with microparticles made of high molecular weight polymers (R 206 and R 207 with respectively 32.36 ± 4.40 and 21.03 ± 3.57% of rSm28GST released in 360 h) (Fig. 1). Since all polylactide microparticles almost bore the same drug content (about 2%, w/w), the differences in the release rate of the entrapped antigen depended principally on the microparticles size and the degradation rate of the polymers. Thus, the decrease of the molecular weight of PLA polymers (R 203 < R 206 < R 207) associated

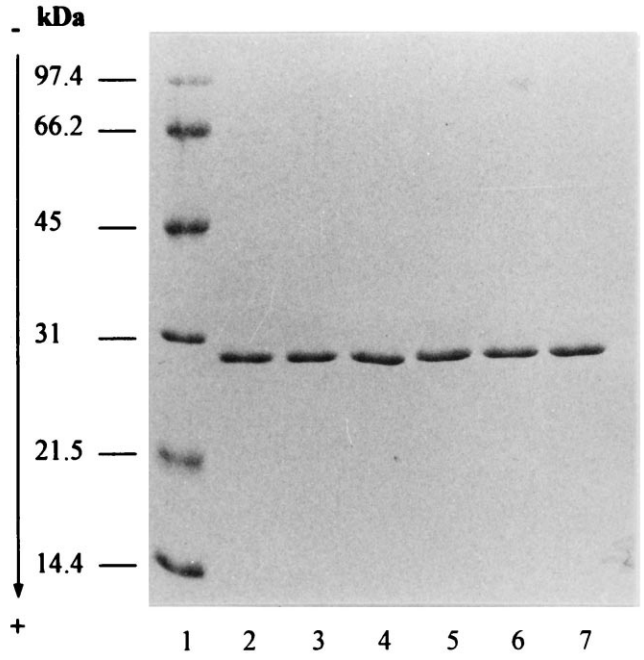

Fig. 2. Coomassie R 250-stained SDS-PAGE of rSm28GST (1.0 μg) released from spray-dried microparticles of R 203 (lane 3), R 206 (lane 4), R 207 (lane 5) and RG 756 (lane 6). Lane 1: molecular weight markers. Lanes 2 and 7: free purified rSm28GST.

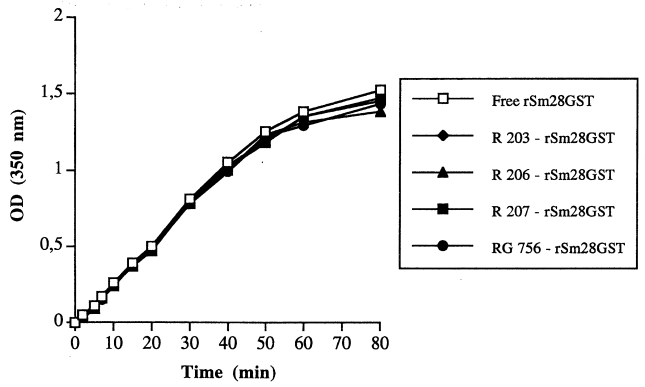

Fig. 3. Enzymatic activity of the entrapped rSm28GST released from microparticles. The glutathione S-transferase activity catalyzed by entrapped rSm28GST release from microparticles (closed symbols) was compared with that catalyzed by free rSm28GST (open symbol) in over time. The protein concentration (3.5 pmoles/well) was identical for each preparation.

to an increase of the degradation rate of the polymer and a decrease in the microparticles size (Table 1), resulted in a fast release profile of rSm28GST.

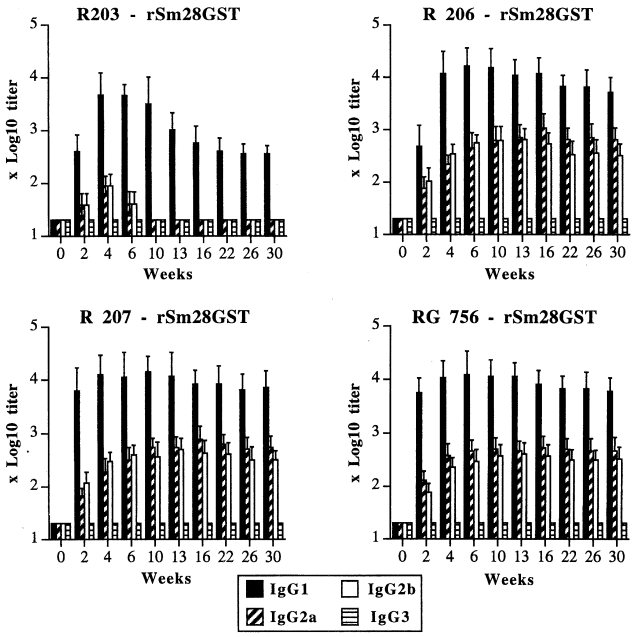

Fig. 4. Antibody isotype profiles elicited after immunization with rSm28GST entrapped in microparticles. Mice were immunized with R 203, R 206, R 207 or RG 756 microparticles. Anti-rSm28GST IgG1, IgG2a, IgG2b and IgG3 titers were determined at the indicated time points for individual serum from 10 BALB/c mice per group, before (week 0) and after immunization (from week 2). Results are expressed as titer mean values ± standard deviation of the mean (S.D.). Titers are expressed as log 10 of the maximal dilution of the antiserum which gave an absorbance reading three-fold higher than background. The results shown are from one representative experiment of three.

3.3. Conservation of the primary structure integrity and the enzymatic activity of the entrapped antigen

The effect of the preparative process on the integrity of rSm28GST was investigated by SDS-PAGE (Fig. 2). After incubation in PBS for 340 h, no aggregation or degradation of the rSm28GST released from microparticles composed of the various polymers was observed (Fig. 2, lanes 3 to 6) when compared with free rSm28GST (Fig. 2, lanes 2 and 7).

To test whether the entrapped antigen was structurally intact, the enzymatic activity of the rSm28GST was determined after incubation of microparticles in PBS for 340 h. As shown in Fig. 3, the enzymatic activity of rSm28GST release from microparticles was found to be similar to that observed with free antigen.

3.4. Systemic immune response after intraperitoneal immunization

Systemic immunization using rSm28GST-loaded microparticles was conducted on female BALB/c mice following a single intraperitoneal injection. For each polymer used, the production of rSm28GST-specific IgG antibodies was detected after 2 weeks whereas the administration of free rSm28GST or empty microparticles did not induce any immune response (data not shown). As shown in Fig. 4, a single intraperitoneal immunization with each type of rSm28GST-containing microparticles was characterized by the coexistence of IgG1, IgG2a and IgG2b isotypes without the detection of any specific IgG3 or IgA antibody response. After 6 weeks, the predominant IgG1 antibody response against rSm28GST resulted in a titer of approximately 4800 with R 203; 16,400 with R 206; 11,300 with R 207 and 12,000 with RG 756. This IgG1 antibody response decreased after 30 weeks to reach a titer of approximately 400 with R 203; 5,300 with R 206; 7,400 with R 207 and 6,000 with RG 756. The highest molecular weight polymer (R 207) showed a slower decrease of the IgG1 titer when compared with the small molecular weight polymer (R 203). The IgG2a and IgG2b antibody responses were constantly below 1000 for all of the polymers. Moreover, the polymers used were not equally efficient in generating long lasting IgG2a and IgG2b responses. Indeed, the IgG2a and IgG2b antibody responses observed after immunization with the rSm28GST entrapped in R 203 microparticles were not detectable after 10 and 16 weeks, respectively, and the immune response became essentially an IgG1 isotypic response (Fig. 4). When the polymers characterized by a high molecular weight (R 206 and R 207) were used, peaks in the anti-rSm28GST IgG1 antibody titers occurred earlier (10 weeks) than those of the IgG2a and IgG2b antibody titers (16 weeks). For rSm28GST entrapped in R 203

Table 2
Recognition and affinity of specific IgG(H + L) antibodies against the two epitopic regions of rSm28GST involved in the enzymatic site[a]

Immunization conditions	a.a. 24–43 region		a.a. 190–211 region	
	recognition[b]	affinity[c]	recognition	affinity
rSm28GST-R 203	–[d]	–	80 ± 9	5.99×10^{-3}
rSm28GST-R 206	276 ± 20	1.10×10^{-3}	152 ± 11	4.18×10^{-3}
rSm28GST-R 207	182 ± 13	5.18×10^{-3}	290 ± 24	1.96×10^{-3}
rSm28GST-RG 756	68 ± 5	8.62×10^{-3}	385 ± 27	1.16×10^{-3}

[a] Results were obtained from sera of 10 mice per group at week 8 after a single intraperitoneal immunization.
[b] Titers are defined as the highest dilution yielding an absorbance 3 times above the background (no serum added). Results were expressed as mean titer values ± S.D.
[c] Affinity = 1/slope from titration curves (optical density = [slope] log [dilution]).
[d] –, titer < 20 and thus an affinity value not detectable. For nonimmunized mice and mice immunized either with free rSm28GST or empty microparticles, titers were < 20.

microparticles, the production of IgG1 isotype was concomitant with the production of IgG2a and IgG2b isotypes.

3.5. Recognition of major epitopes of the rSm28GST by specific serum

As shown in Table 2, anti-rSm28GST sera (week 8) obtained from mice immunized with rSm28GST entrapped in microparticles recognized epitopic regions of the rSm28GST. The specificity of recognition of these regions varied according to the nature or molecular weight of the polymer used to produce the microparticles. Mice immunized with rSm28GST entrapped in R 206, R 207 or RG 756 microparticles elicited anti-rSm28GST antibody titers against the epitopic regions associated with both of the antigen enzymatic sites (regions 24–43 and 190–211). In contrast to the rSm28GST-loaded R 206 microparticles, R 207 and RG 756 microparticles both elicited higher antibody titers against the 190–211 region and higher antibody affinities against the 24–43 region. Mice immunized with rSm28GST entrapped in R 203 microparticles elicited antibodies, which recognized only the 190–211 region of the rSm28GST with a high affinity. No recognition of the epitopic regions of the rSm28GST was detected by sera obtained from mice immunized with empty microparticles or free rSm28GST (titer < 20).

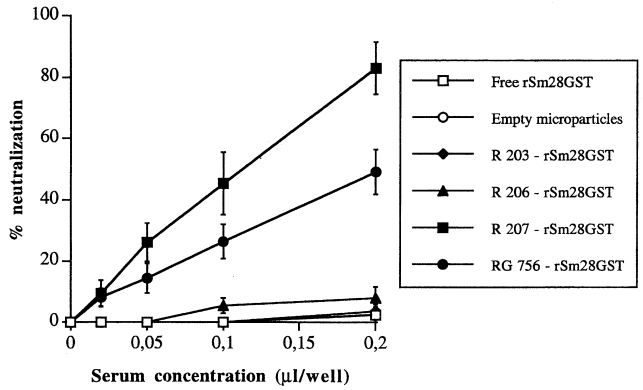

Fig. 5. Neutralization of the rSm28GST enzymatic activity by specific antiserum. The neutralizing activity was analyzed for pooled sera obtained 8 weeks after a single intraperitoneal administration of either rSm28GST-loaded R 203, R 206, R 207 or RG 756 microparticles, free rSm28GST or empty microparticles. Results are expressed as mean values ± S.D. of three separate experiments testing the neutralizing activity (%) of pooled sera for 10 mice in each group. The catalytic neutralization of rSm28GST (3.5 pmoles/well) was measured in the absence or presence of increasing concentrations of antisera. Sera expressing less than 10% neutralization at a concentration of 0.2 µl/well are considered as inefficient sera.

3.6. Antiserum-mediated neutralization of the rSm28GST enzymatic activity

The potentiality of the anti-rSm28GST sera (week 8) to neutralize the enzymatic activity of rSm28GST was also investigated. As shown in Fig. 5, antisera (0.2 µl per well) obtained after a single intraperitoneal immunization of mice with R 207- and RG 756-microparticles containing rSm28GST neutralized the enzymatic activity of purified rSm28GST. In contrast, no significant neutralization was observed with sera obtained from mice immunized either with R 203- or R 206-microparticles containing rSm28GST, empty microparticles or free antigen.

4. Discussion

The present study investigated the suitability of poly(lactide) (R 203, R 206 or R 207) or poly(lactide-co-glycolide) (RG 756) microparticles produced by a spray-drying technique to entrap and preserve the physiochemical and biological properties of an antigen. The polymers were selected on the basis of the follow-

ing properties: degradability, tissue compatibility and ease of processing [4].

In previous studies, polyacrylamide gel electrophoresis analysis indicated that the rSm28GST enzyme was not notably altered following entrapment in microparticles produced by the commonly used double emulsion-solvent evaporation technique [27]. While during the spray-drying process the protein is exposed to physical stresses (organic solvents, aqueous/organic interface and relatively adverse temperature and emulsion conditions) that may result in partial or total loss of antigenic properties such as epitope specificity, it was necessary to study the preservation of physicochemical characteristics of the antigen after its encapsulation into microparticles using spray-drying process. In this aim, an enzyme of which two major epitopes are engaged in the enzymatic site has been selected as a model antigen. Our study confirmed that the method of antigen encapsulation within polyester microparticles using the spray-drying technique also preserved the physicochemical characteristics of the entrapped protein. In addition, the enzymatic activity of the rSm28GST released from the microparticles was similar to that of free antigen. This demonstrated that the encapsulation technique of rSm28GST within the microparticles allowed full enzymatic activity to be retained, indicating that the three-dimensional structure of the antigen's active site was conserved. This study demonstrated for the first time that an antigen could preserve its enzymatic activity after microencapsulation. For other groups which used spray-drying as microencapsulation technique, this technique resulted in the partial instability of the entrapped antigen which contributed to the often observed incomplete protein release and, consequently, to the lack of booster effect observed after a single parenteral administration [28]. To avoid this problem, it seems important to stabilize the antigen in microparticles to enhance prolonged antigen delivery. Thus, recent studies have described the coencapsulation of stabilizing additives that would permit a reduction of the antigen dose and optimization of single-dose vaccine formulations [18,20]. Preservation of the integrity and stabilization of our model antigen during process could be explained by the use of an Ultraturrax instead of ultrasonication to produce the water-in-oil emulsion and by the presence of PVA associated with antigen in the aqueous phase. Indeed, we have recently observed that PVA was required to stabilize the emulsion. This stabilizer acts as a protective polymer by being adsorbed at the oil/water interface of droplets to produce a steric barrier, which protected the antigen against the external organic solvent. PVA also allowed to decrease the coalescence of the microparticles, increase the antigen loading within microparticles, decrease the microparticle size and decrease the porosity of microparticles to allow a slow antigen release from microparticles [29]. Thus, our study consisted to produce microparticles by a specific microencapsulation process, which included the use of PVA to stabilize the emulsion and to obtain a continuous antigen release.

To study the immunogenicity of our formulations on comparative basis, microparticles were administered by intraperitoneal route. Nakaoda et al. [30] demonstrated that the microparticles injected intraperitoneally were distributed over the whole peritoneal cavity, mainly localized on the surface of the serosa membranes of the mesentery, internal organs or the peritoneum without large aggregation. Contrary to intravenous or subcutaneous administration of microencapsulated antigen, the disseminated localization of the microparticles in the peritoneal cavity can enhance the chance of their interaction with immunological cells, leading to an efficient activation of the cells to promote the antibody production [30]. In our experiments, intraperitoneal administration of antigen entrapped within microparticles elicited a high humoral immune response whereas free antigen was ineffective. The results indicated that the systemic immune response consisted of IgG1, IgG2a and IgG2b isotypes without IgG3 and IgA antibody production. Other groups have also shown that antigens such as ovalbumin [31], staphylococcal enterotoxin B [32] or tetanus toxoid [33] entrapped in PLG microparticles and systemically administered into mice in a single dose elicited a greater serum IgG antibody response than the response to soluble antigen. In addition, we observed the induction of a long-lasting IgG antibody production following a single immunization with antigen entrapped in microparticles prepared by spray-drying.

The results showed that depending on the quality of the various polymers used, the duration of the systemic immune response could be modified. This could be explained by the variation of the release rate of the entrapped antigen in relation to the degradation rate of the polymer and the size of the microparticle. Protein release from bioerodible polymer matrix can occur by two simultaneous main events: by diffusion through a tortuous water-filled path in the polymer matrix and by the erosion of the polymer surface and/or bulk matrix [34,35]. Thus, the release rate of proteins from microparticles is dependent on the physicochemical properties of the polymer used (e.g. molecular weight, lactide/glycolide ratio, crystallinity), the protein loading level, the microparticle size, the protein distribution into the matrix and on the porosity of the microparticles [36]. In this study, we used polymers that degraded relatively rapidly (low molecular weight polymers such as R 203) for which a fast release of the entrapped antigen is obtained and others that

degraded slowly (high molecular weight polymers such as R 206 or R 207) which have a long-lasting antigen release [5,22,36]. Furthermore, with the reduction in polymer molecular weight, the matrix becomes more and more hydrophilic, allowing more water to penetrate, thereby enhancing the degradation of polymer and thus, the protein release. Finally, the low molecular weight polymers gave low-viscosity solutions when dissolved in dichloromethane [37]. Accordingly, their dispersion within the aqueous phase resulted in small emulsion droplet size and therefore, in small microparticle size. Thus, the use of various polymers with different molecular weights allowed us to modify the duration of the humoral immune response by modification of both microparticle size and degradation rate of the polymer. Our results support the hypothesis that the duration of antigen release is the limiting factor for both the duration and intensity of the antibody production. The activation state of the immune response machinery induced by continuous exposure to the antigen through the sustained release from microparticles may be different from that produced from a single exposure to the antigen.

The microparticles produced from the low molecular weight polymer (R 203) resulted in a short-lasting release of antigen, which did not allow the induction of both high and long-term immune responses and where the production of IgG1 isotype was concomitant with the production of IgG2a and IgG2b isotypes. In contrast, when the polymers characterized by a high molecular weight (R 206 and R 207) were used, a high and long-lasting immune response was obtained and the peak in the anti-rSm28GST IgG1 antibodies occurred earlier than those of the IgG2a and IgG2b antibodies. These observations may be linked to the preferential uptake of smallest size microparticles by macrophages and/or the difference in rate of antigen release among the various quality microparticles.

Anti-rSm28GST antibodies elicited after intraperitoneal immunization of mice with R 207- and RG 756-microparticles containing rSm28GST were capable of neutralizing the enzymatic activity of the protein. In the model of *Schistosoma mansoni* infection of rats and mice, it has been shown that a monoclonal antibody which neutralized the enzymatic activity conferred significant protection against challenges and induced additional dramatic reduction in egg laying and egg viability (anti-fecundity effect) [14]. The mapping of the major epitopes of rSm28GST has led to the identification of the role played by the N-terminal (a.a. 24–43) and C-terminal (a.a. 190–211) domains in the conformational site of the enzymatic activity [15]. Although the enzymatic activity was preserved after encapsulation by spray-drying technique, this current investigation indicated that the recognition of the two epitopes associated with the enzymatic site was variable depending on the type of polymer used for the microparticle preparation. This is probably due to differences in antigen distribution and presentation. In this respect, rSm28GST entrapped in R 206, R 207 and RG 756 microparticles elicited anti-rSm28GST antibodies, which recognized both amino-acid 24–43 and 190–211 regions. However, only mice immunized with rSm28GST-R 207 and RG 756 microparticles elicited high antibody titers capable of neutralizing the rSm28GST enzymatic activity. These results seemed to indicate that the recognition of the two epitopic regions by specific antibodies was necessary but not sufficient to induce neutralization. Indeed, the presence of high affinity antibodies to the a.a. 24–43 region, in association with antibodies to the a.a. 190–211 region, were required to neutralize the rSm28GST enzymatic activity. Furthermore, mice immunized with rSm28GST entrapped in R 203 microparticles elicited antibodies, which recognized only the a.a. 190–211 region with a high affinity and did not neutralize enzymatic activity.

Thus, the selection of polymers used to prepare microparticles by spray-drying will influence the intensity and the duration of the immune response but can also influence both the epitopic specificity and affinity of the produced specific antibodies.

In addition to other studies which investigated the conservation of the physicochemical characteristics and immunogenicity of the entrapped antigen [22,38,39], our model antigen allowed to evaluate additional parameters confirming that the method of microparticle preparation by spray-drying preserved the integrity of the entrapped antigen. Indeed, we have demonstrated the conservation of the enzymatic activity of an antigen and the induction of antibodies endowed with biological activity.

Finally, the preparation by the spray-drying technique is the first step for the manufacturing scale-up of microparticles for vaccine production. Indeed, spray-drying appears to be one of the most interesting processes for the reproducible larger-scale production of vaccine using synthetic vectors. Whereas many attractive features of the spray-drying process, such as reproducibility, continuous operation, high yields, defined residual moisture and control over the micrometric properties have not been completely attained with small-scale equipment used in this study, this could be reached using larger industrial equipment [40]. Nevertheless, results obtained in the present study illustrate the potential of microparticles produced from biodegradable polymers by suitable method as a controlled-release antigen delivery system to induce a long-lasting immune response which would obviate the need for booster doses of vaccines.

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