ELSEVIER

Journal of Controlled Release 44 (1997) 283–293

journal of controlled release

Double-walled microparticles for single shot vaccine

Hyeon Kook Lee*, Jung Hwan Park, Kyu Chan Kwon

Biotech Research II, LG Chem Research Park, LG Chem Ltd., 104-1 Moonji-dong, Yusung-gu, Daejeon, South Korea 305-380

Abstract

It is well known that an antigen is damaged by a solvent during the microencapsulation process resulting in activity loss and poor reproducibility. In this study, novel double-walled microparticles containing hepatitis B surface antigen (HBsAg) were produced through a spray drying process. HBsAg was first encapsulated with hydroxypropyl cellulose (core microparticle). The core microparticles were then encapsulated again by poly [lactide-co-glycolide] resulting in the double-walled microparticles. By encapsulating HBsAg with hydroxypropyl cellulose in advance, it was found that HBsAg was protected from an organic solvent during the second encapsulation process with poly[lactide-co-glycolide]. Furthermore, the double-walled microparticle produced higher antibody titer than the single-walled microparticle when injected into guinea pigs. Finally the double-walled microparticle was compared with the alum formulation for feasibility as a hepatitis B single shot vaccine. The single shot of the double-walled microparticle was at least comparable with the two shots of the alum formulation in producing antibody. It resulted mainly from the HBsAg protective ability of hydroxypropyl cellulose against a solvent during the encapsulation process and the natural infection mimetic type of HBsAg release from the double-walled microparticles.

Keywords: Single shot vaccine; Double-walled microparticle; Hepatitis B surface antigen; Poly[lactide-co-glycolide]; Spray drying

1. Introduction

Great efforts have been made to utilize microparticles, containing an antigen, as a single shot vaccine [1–6]. The microparticle was found to release antigen in vitro and in vivo in a pre-programmed manner such as a pulsatile or a continuous mode for several months eliminating the necessity of multiple shots for complete immunization. Nevertheless, obstacles still exist and need to be overcome for its practical application. One such obstacle is the degradation of antigen due to its inevitable contact with an organic solvent for biodegradable polymer during the microencapsulation process [7–15]. Particularly, the hepatitis B surface antigen (HBsAg) is susceptible to an organic solvent. Upon contact with an organic solvent, lipids in HBsAg are extracted resulting in the decrease of lipids in HBsAg. This causes a conformational change and decrease in the antigenicity of HBsAg [16].

In this study, we propose the novel double-walled microparticle system in which HBsAg can be kept intact from an organic solvent during the encapsulation process. It should be noted that the double-walled microparticle in this study is different from the double-walled microparticles reported in previous studies [17–19]. First, our combination of two polymers for the double walls consisted of a hydrophilic and a hydrophobic polymer. In contrast, their

*Corresponding author. Tel: +82 42 8662253; fax: +82 42 8620331.

PII S0168-3659(96)01534-9

combinations consisted of two hydrophobic polymers soluble only in organic solvents. Most importantly, antigens in our double-walled microparticle do not come in contact with the organic solvent during the encapsulation process while antigens or drugs in their double-walled microparticles come in contact with the organic solvent. The foregoing differences will become clear from the concept of our double-walled microparticle explained below.

The proposed concept is as follows: Instead of using HBsAg itself, HBsAg is first encapsulated with the hydrophilic polymer that is insoluble in an organic solvent for a later encapsulation process with poly[lactide-co-glycolide] (PLGA). HBsAg encapsulated with the hydrophilic polymer (core microparticle) is then dispersed in an organic solvent containing PLGA for further encapsulation with PLGA. Since HBsAg is already encapsulated with the hydrophilic polymer, it can avoid contact with the organic solvent during the entire encapsulation process, and its antigenicity can be kept intact. In order to examine the proposed concept, we produced and characterized the core and the double-walled microparticles containing HBsAg. Whether the antigenicity of HBsAg could be kept intact after encapsulation was first examined in vitro. Furthermore, the double-walled microparticle was compared with a single-walled microparticle through in vitro and in vivo experiments. The single-walled microparticle means the conventional microparticle in which HBsAg was encapsulated only with PLGA. It is the microparticle that has been produced and studied as a drug delivery system which includes a single shot vaccine mentioned in previous studies [11–15,20–27]. Finally the double-walled microparticle was compared with alum formulation to see if the double-walled microparticle could function as a hepatitis B single shot vaccine.

2. Materials and methods

2.1. Materials

Two bulks of HBsAg (HBsAg in PBS buffer) were obtained from LG Chemical Ltd., Korea. They were from portions of commercial batches (batch numbers UP048 and UB054) for the hepatitis B vaccine currently sold as EUVAX B® or RECOMVAX B® in Korea, South East Asia, and South America. Protein contents in the batches were 720 mg/l for UP048 and 700 mg/l for UB054. The production method and the characteristics of the HBsAg were well documented [28]. Briefly, S-HBsAg was produced from recombinant yeast (*Saccaromyces cerevisiae*). The purity was better than 98% with 40–70% of the lipids and 2–4% of the sugars based on protein content. PLGA with a molecular weight of 56 100 Da was from Birmingham Polymer Inc, USA. Its monomer ratio of lactide/glycolide was 50:50. Hydroxypropyl cellulose (HPC) was NF grade from Hongsung, Korea. It was a low-substitute HPC (5–16% of hydroxypropoxy groups) with a low viscosity. Aluminium hydroxide as alum adjuvant was obtained from Superfos Biosector, Vedbaek, Denmark. Solvents such as carbon tetrachloride, chloroform, methylene chloride, ethylacetate, benzene, dioxane, acetone, and acetonitrile were from Sigma. Salts for the preparation of PBS buffer were composed of sodium phosphate dibasic, potassium phosphate monobasic, and sodium chloride. Those were analytical grades from Sigma. Nonionic surfactant, Tween80 was also supplied by Sigma and was used to disperse the microparticles for injection. Deionized (DI) water was further filtered through a 0.22-μm membrane filter before use.

2.2. Microencapsulation

The core microparticles were produced by spray-drying the mixture of bulk HBsAg and HPC with the Mini Spray Dryer B-191 from Buchi. The bulk HBsAg was diluted to a protein content of 600 mg/l with PBS buffer before encapsulation. The ratios of HPC/HBsAg were 5 for the bulk HBsAg, UB054 and 15 for the bulk HBsAg, UP048. Inlet and outlet temperatures for spray drying were 70 and 51°C, respectively. Flow and feeding rates were 600 l/min and 3 ml/min, respectively. The core microparticles produced were further dried in a vacuum at 25°C for 24 h before the second encapsulation process when PLGA was applied. The dried core microparticles were then dispersed in ethylacetate in which PLGA had been dissolved. The concentrations of PLGA in

ethylacetate were 1.5% for the bulk HBsAg UB054 and 1.0% for UP048. The concentration of the core microparticle in ethylacetate was 5 in the weight ratio of PLGA/HPC. The double-walled microparticles were finally produced by spray-drying the suspension of the core microparticles in ethylacetate containing PLGA. Inlet and outlet temperatures for the second encapsulation were 60 and 41°C, respectively. Flow and feeding rates were the same as those in the first encapsulation process with HPC. The double-walled microparticles were further dried in a vacuum at 25°C for 24 h. After vacuum drying, the microparticles were sealed in vials and stored at 4°C until use. For comparison, the single-walled microparticles were produced using the same process as for the double-walled microparticles except that the first encapsulation with HPC was omitted. Briefly, the bulk HBsAg was diluted to a protein content of 60 μg/ml. The diluted bulk HBsAg was emulsified in ethylacetate containing 1.5% of PLGA by sonication. The ratio of PLGA/HBsAg was 5. The final resulting W/O emulsion was spray-dried. Therefore, in the case of the single-walled microparticles, HBsAg was encapsulated only with the one layer of PLGA and inevitably contacted ethylacetate during encapsulation. In addition to the single-walled microparticles, HBsAg alum formulations were prepared by mixing the bulk HBsAg and aluminium hydroxide. The mixing ratio was 20 μg HBsAg/1.5 mg aluminium hydroxide.

2.3. Characterization of microparticles

The particle size and the distribution of the core and the double-walled microparticles were obtained by the Malvern laser particle size analyzer, Master-Sizer MS20. The shape and the morphology of the microparticles were studied using the Jeol scanning electron microscope, JSM840A. The surface area of the double-walled microparticles was obtained by BET method using the Coulter Omnisorb100 [29]. HBsAg loading in the microparticles was analyzed by determining the amounts of proteins in the microparticles by Lowry method [30]. Before determining the HBsAg loading, PLGA was removed by 0.1 N NaOH digestion at 37°C and by centrifugation [3].

2.4. Screening organic solvents for encapsulation

As mentioned previously, a hydrophilic polymer (HPC) should not be dissolved in a solvent for PLGA in order to protect HBsAg during the second encapsulation process. Therefore, the ideal type of solvent would be one which does not dissolve HPC but PLGA. Small amounts of HPC (10 mg) or PLGA (100 mg) were added to one of the solvents (10 ml): carbon tetrachloride, methylene chloride, ethylacetate, benzene, dioxane, acetone, acetonitrile, and chloroform. Each mixture was then stirred using a magnetic stirrer for 6 h. This was much longer than what was required for the second encapsulation. The solubility of PLGA or HPC was qualitatively determined by observing if the polymer was easily dissolved, swollen, or hardly dissolved into the solvent. This method was good enough for this study as can later be seen in in vitro and in vivo tests. PLGA was easily dissolved in all of the solvents except benzene in which it was swollen. HPC was easily soluble in all of the solvents except ethylacetate and acetonitrile. HPC was hardly soluble in ethylacetate and acetonitrile. Further study led us to choose ethylacetate as the best solvent for this study. Even though most of the solvent in microparticles can be removed during the additional drying process, using ethylacetate is an advantage because it is not a chloride compound. It is actually used as an inactive ingredient for ophthalmic, suppository, and controlled release systems as well as food [31,32].

2.5. Protection tests

In order to verify the concept of this study as well as the results in the previous section, the core microparticles (LB05Z03-H) were contacted with solvents (Table 1). The core microparticles (3 mg of HBsAg) were added to solvents such as ethylacetate, acetonitrile, and methylene chloride. These are the solvents frequently used for the production of microparticles with PLGA. The mixture was stirred for 6 h. Then, it was filtered through a 0.22-μm membrane filter. The core microparticles obtained were dried in a vacuum to remove any residual solvent. The dried core microparticles were then dissolved in DI water to determine the amounts of proteins and the antigenicity of HBsAg. For com-

Table 1
Characteristics of the microparticles

Batch number	Type of microparticle	PLGA/HPC (w/w)	Diameter (μm)[d]	HBsAg (μg/mg)[e]	BET area (m^2/g)	Area/volume (m^2/ml)[f]	Batch number of HBsAg
LB05Z03-H	Core[a]		4.9	34.00		1.41	UB054
LB05508-H	Double walls	5	12.2	5.50	1.26	1.28	UB054
LB05511-Z	Single wall[b]		11.6	5.40		1.33	UB054
LB05Z02-H	Core[c]		3.9	21.00	2.20	2.95	UP048
LB05505-H	Double walls	5	8.5	4.08	1.82	1.91	UP048

[a]HPC/HBsAg=5.
[b]PLGA/HBsAg=5.
[c]HPC/HBsAg=15.
[d]Volume average from the laser particle size analyzer.
[e]HBsAg in mg microparticles.
[f]Theoretical area from the laser particle size analyzer.

parison, the same experiments as above were performed with the bulk HBsAg itself. Antigenicity was determined by the EIA method using the Auzyme kit from Abbott [33], and was expressed as a relative antigenicity. It is defined as the percent ratio of the absorbance of a sample to that of the fresh bulk HBsAg containing the same amounts of HBsAg.

2.6. In vivo animal tests

Throughout this study, female guinea pigs weighing 350–400 g were used. Each group consisted of 11 guinea pigs. Microparticle formulations were prepared by dispersing the microparticles in PBS buffer containing Tween80 (0.01%, w/w) before injection. One millilitre of dosage volume was injected subcutaneously on the back of the guinea pig using a 23G needle syringe. At predetermined intervals, blood was collected from each guinea pig by a cardiac puncture technique [24].

The concentration of HBsAg antibody in the collected blood was then determined using the solid phase enzyme linked immunoassay (Ausab kit from Abbott) [20,34]. To express actual antibody concentration (antibody titer) in mIU/ml, a standard curve was generated using the calibrated anti-hepatitis B panel provided by Abbott [20]. Average antibody titer was obtained from the geometric mean of antibody titers from the 11 guinea pigs.

Two sets of guinea pig tests were performed in this study. Each set used the same batch of bulk HBsAg. Among the two sets, the first was performed using the double- (LB05508-H) and the single- (LB05511-Z) walled microparticle formulations with two groups of the guinea pigs (Table 1). Eleven guinea pigs in each group were injected once with one of the two microparticle formulations on day 0. Each microparticle formulation contained 20 μg of HBsAg. Blood was collected to determine antibody titer at intervals of 28 days for 112 days. The goal was to see if the double-walled microparticle was indeed superior to the single-walled microparticle in vivo since HPC in the double-walled microparticle was expected to protect HBsAg from ethylacetate during the encapsulation process with PLGA.

The second set was performed with the double-walled microparticle formulation (LB05505-H) and the two alum formulations. Three groups of guinea pigs were used. One group was injected only once with the microparticle formulation containing 20 μg of HBsAg. The other two groups of guinea pigs were injected with the alum formulations. One was injected only once with the alum formulation containing 20 μg of HBsAg as with the double-walled microparticle formulation. In contrast, the other group was injected twice on days 0 and 15 with the alum formulation containing 10 μg of HBsAg (total 20 μg of HBsAg). Blood was collected at intervals of 15 days or once a month for 160 days. The goal of the second set was to find out if the one shot of the double-walled microparticle could work as the single

shot vaccine in vivo compared with the one or two shot(s) of the alum formulation.

2.7. In vitro release tests

The double-walled (LB05508-H) and the single-walled (LB05511-Z) microparticles were subjected to in vitro release tests (Table 1). These were the same microparticles used in the first set of guinea pig test. The microparticles containing 750 μg of HBsAg were dispersed in 15 ml of PBS buffer containing Tween80 (0.01%, w/w). Bottles containing the dispersed microparticles were rotated at 37°C for 120 days. At predetermined time intervals, the bottle was centrifuged and supernatant was taken for the analysis of released HBsAg. The amounts of HBsAg in the supernatant were determined using the same method as for HBsAg loading in the microparticle. Results were reported as an average from three independent experiments for each microparticle.

3. Results and discussion

3.1. Characteristics of the microparticles

The characteristics of the microparticles produced in this study are summarized in Table 1 and Fig. 1. Volume average particle sizes and unimodal distributions are comparable with those of the microparticles produced by spray drying in previous studies [6,21–23]. This size is known to be easily phagocytosed by antigen presenting cells such as

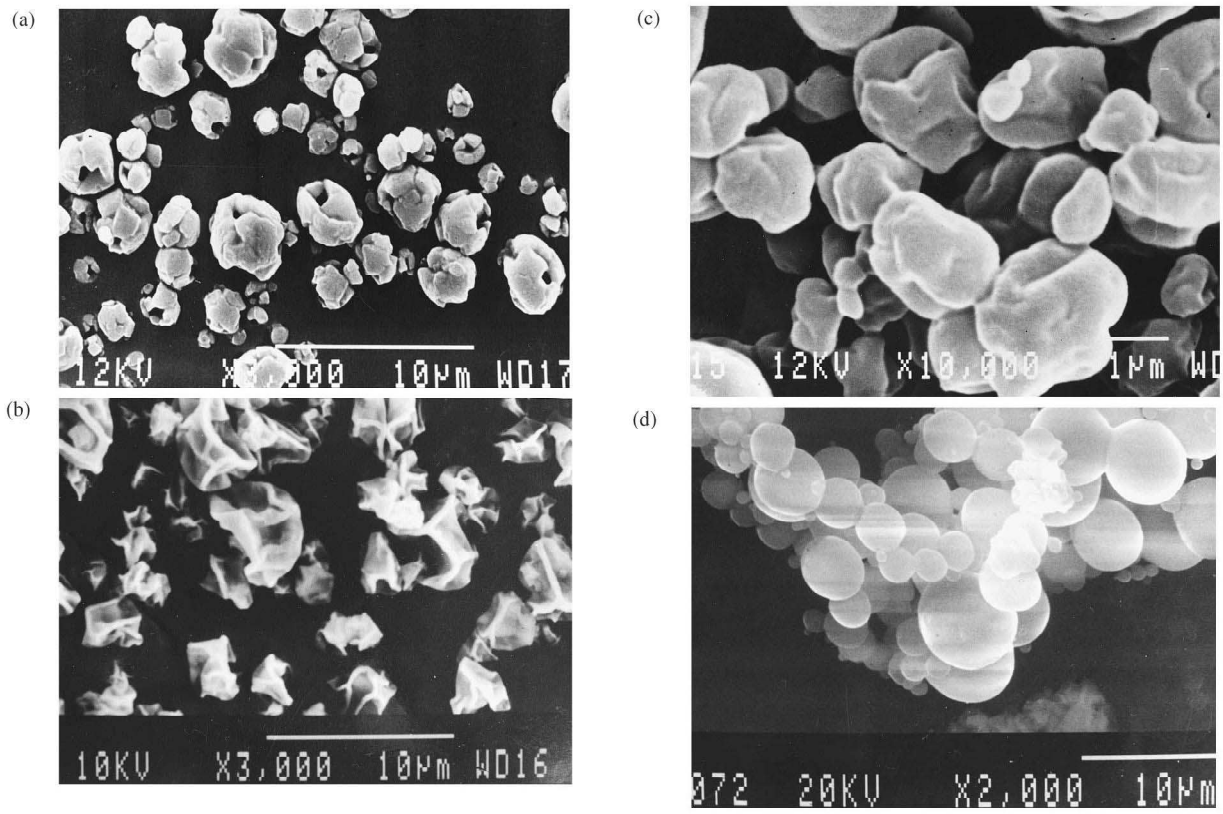

Fig. 1. Morphologies of the bulk HBsAg, and the microparticles after spray drying. Bulk HBsAg UP048 was used (Table 1): (a) bulk HBsAg; (b) core microparticles; (c) double-walled microparticles; (d) double-walled microparticles with improved encapsulation conditions.

macrophages [13,35–37]. The ability to be phagocytosed by macrophages was suggested to be important for the effective induction of potent antibody response [38,39]. It should be noted that surface areas from the BET measurement and the particle size analyzer are comparable. Surface area from the particle size analyzer is the theoretical surface area per unit volume of the microparticles which is deduced from average particle size and size distribution assuming that the microparticles are perfect spheres without any pores. Therefore, considering that the densities of the polymers ranged from 1.1 to 1.3 g/ml, the double-walled microparticles produced in this study did not seem to have many pores on their surface. Result from SEM in Fig. 1 supports this. The surface of the double-walled microparticle (Fig. 1c) is smooth and does not show many pores even though it has a few dents. Also, the size from SEM is consistent with the size from the particle size analyzer. The morphology of the spray-dried bulk HBsAg (Fig. 1a) was spherical with one large hole in the center. Also, it appears that several pieces of bent boards were assembled into a sphere. The morphology of the core microparticle (Fig. 1b) is much different from that of the bulk HBsAg indicating that HBsAg was indeed encapsulated with HPC. In Fig. 1c, the double-walled microparticles are shown to be spherical or oval with a few dents. Again, the morphology of the double-walled microparticle is different from those of the bulk HBsAg and the core microparticle. The dent had resulted from the evaporation of ethylacetate during the encapsulation. Later, with improved operating conditions for spray drying, perfect spherical double-walled microparticles without any dents could be produced (Fig. 1d). The morphological changes of the microparticles are the evidence that the final microparticles produced were the double-walled microparticles containing HBsAg.

3.2. Protection study

HBsAg in the core microparticle kept 92% of initial antigenicity (Fig. 2) after the first encapsulation process with HPC, indicating that the encapsulation process and HPC did not harm HBsAg. After contact with the solvents, the relative antigenicities

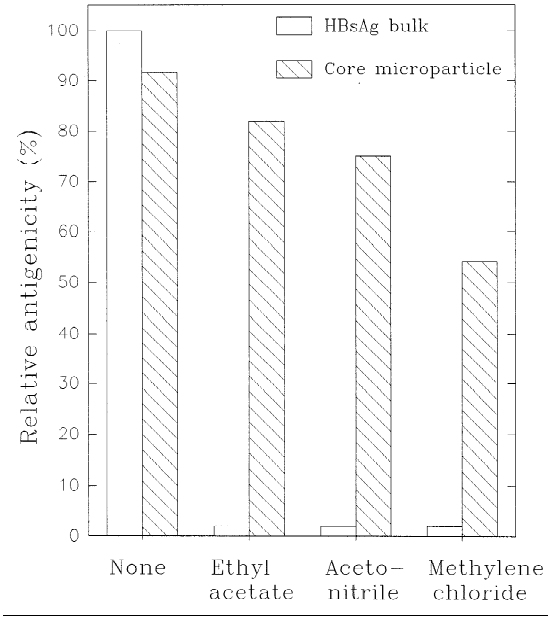

Fig. 2. Effect of organic solvents on the antigenicities of bulk HBsAg and core microparticles containing HBsAg (LB05Z03-H) (Table 1).

were 84% for ethylacetate, 78% for acetonitrile, and 58% for methylene chloride. The decrease in the antigenicity is consistent with the solubilities of HPC in the solvents. It is surprising that HPC could protect HBsAg somewhat even from methylene chloride because HPC is easily soluble in methylene chloride. Presumably, the solubilized HPC molecules in methylene chloride seemed to protect HBsAg through molecular interactions. Further study is required to elucidate its protective mechanism. In contrast, bulk HBsAg itself without HPC encapsulation lost all of its antigenicity when it came in contact with the solvents. Most of them could not even be re-dissolved in PBS buffer indicating that the conformation of HBsAg was changed due to the extraction of lipids in HBsAg into the solvents. It is consistent with the previous study which showed that the antigenicity of HBsAg decreased due to the extraction of lipids in HBsAg [16]. Now it is obvious that the antigenicity loss of HBsAg during the encapsulation process with a solvent can be avoided by preparing the core microparticle through HPC encapsulation before PLGA encapsulation.

3.3. In vivo animal tests

The protective ability of HPC was further examined in vivo using the guinea pigs. Indeed, the antibody titer of the double-walled microparticle became higher than that of the single-walled microparticle starting from 28 days after the injection even though the statistical analysis by student *t*-test indicated no significant difference at $P<0.05$ (Fig. 3). The difference in the antibody titers became larger as time passed. At day 112, the antibody titer of the double-walled microparticle was 3–4 times as much as that of the single-walled microparticle yielding a statistically significant difference at $P<0.05$. This indirectly supports the view that an organic solvent for encapsulation with PLGA is really harmful to HBsAg and that the concept of a HPC layer in the double-walled microparticle works for the protection of HBsAg from an organic solvent.

Currently, alum is the only adjuvant approved for human vaccines including multiple HBsAg vaccine by the FDA. Therefore, the effectiveness of the double-walled microparticle as the single shot vaccine was further compared with those of the two alum formulations. The results in Fig. 4 show that the alum formulation cannot be used as the single shot vaccine. The antibody titer produced by the single shot of the alum formulation was not significantly high, which is consistent with the previous study [20]. Actually, the seroconversion rate was 82% even at day 160. In contrast, the seroconversion rate for the double-walled microparticle already reached 100% at day 31. The difference in the antibody titers by the two formulations became significant at $P<0.05$ from day 46. It is not surprising because aluminium hydroxide disappears in a few days after injection and cannot release HBsAg in the body for that length of time. However, the two shots of the alum formulation boosted on day 15 showed that the boosting increased the antibody titer.

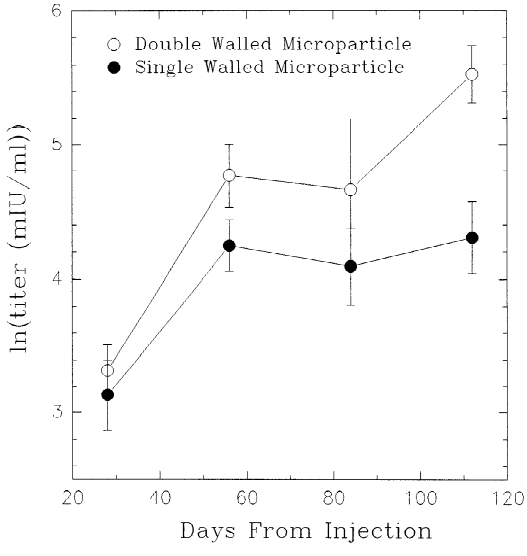

Fig. 3. Comparision of antibody response in vivo between the single- (LB05511-Z) and the double- (LB05508-H) walled microparticles (Table 1). Each microparticle formulation contained 20 μg of HBsAg and was injected only once. The error bars represent standard errors.

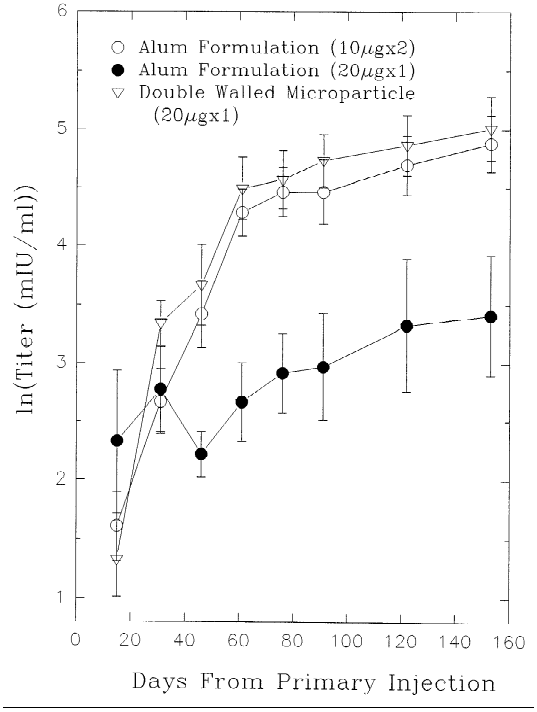

Fig. 4. Comparision of antibody response in vivo between the alum formulations and the double-walled microparticle (LB05505-H) (Table 1). One of the two alum formulations was injected twice on days 0 and 15. The other alum and the double-walled microparticle formulation were injected only once on day 0. The error bars represent standard errors.

The seroconversion rate reached 100% at day 46 yielding a significant difference at $P<0.05$ from the single shot of the alum formulation. Nevertheless, the titer was still lower than the titer produced by the single shot of the double-walled microparticle even though the statistical analysis showed no significant difference at $P<0.05$. This is the indirect evidence that in situ boosting was provided in the body with the single shot of the double-walled microparticle. In vitro release tests will be discussed below to support this.

3.4. In vitro release tests

The HBsAg release pattern of the double-walled microparticles can be expected to be different from that of the single-walled microparticle due to the existence of one more layer in the double-walled microparticle [17–19]. In Fig. 5, the total released amounts of HBsAg for 90 days were similar; 90% from the double-walled microparticle and 88% from the single-walled microparticle. However, 95% of HBsAg was released from the double-walled microparticles for 120 days while HBsAg in the single-walled microparticles was not released any more after 66 days. This result is consistent with the in vivo result in Fig. 3. The antibody titer of the single-walled microparticle stopped increasing at day 61 while the antibody titer of the double-walled microparticle still increased. Previous studies mentioned unfavorable interaction between proteins and PLGA [7,40]. Therefore, the incomplete release of HBsAg from the single-walled microparticle might result from unfavorable interaction between PLGA and HBsAg due to direct contact. In contrast, HPC in the double-walled microparticle prevented HBsAg from its direct contact with PLGA. It was shown in the previous section that HPC did not harm the antigenicity of HBsAg during encapsulation. The release pattern of HBsAg from each microparticle showed a sudden increase around day 15 following a large initial burst. It is more evident when results are presented on the release rate (Fig. 6). The initial large burst followed by a pulse on day 15 is comparable with previous studies [24,41]. However, the magnitude of HBsAg release in the pulse from the single-walled microparticle was much larger than that from the double-walled microparticle while the initial bursts were similar. This difference obviously resulted from the existence of one more wall, HPC in the double-walled microparticle. Ada suggested that

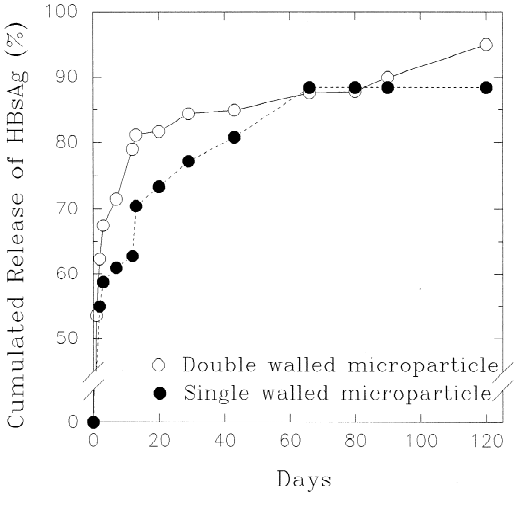

Fig. 5. Cumulated in vitro release of HBsAg from the single- (LB05511-Z) and the double- (LB05508-H) walled microparticles (Table 1).

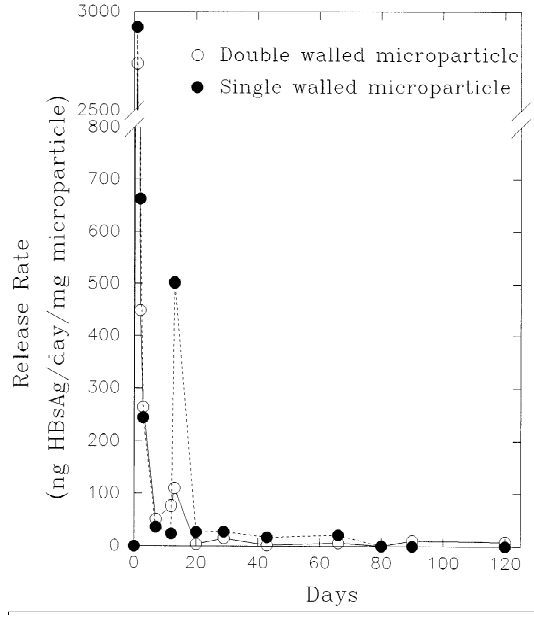

Fig. 6. In vitro release rates of HBsAg from the single (LB05511-Z) and the double (LB05508-H) walled microparticles (Table 1).

the continuous or pulsed release of an antigen should not be the best release pattern from microparticles [42]. Even though current vaccinations employ the same doses for all required shots, a better dosing schedule should contain large amounts of antigens for the first (primary) injection and smaller amounts of antigens for subsequent (boosting) injections. Since the affinity of B cells is not initially high enough for antigens, the first injection requires relatively large amounts of antigens. However, the boosting injections require only a small amount of antigens since mature B cells have higher affinity. Based on the above findings, he proposed a natural infection mimetic pattern for successful single shot vaccine: the large initial burst and the continuous release of antigen thereafter. The magnitude of the initial burst should be several orders higher than the later continuous release which can be as low as 0.001 μg to be effective [12]. As can be seen in Fig. 6, the double-walled microparticle is closer to the natural infection mimetic pattern than the single-walled microparticle. It should be noted that the released amounts of HBsAg from the microparticles were determined by measuring the released amounts of protein. Therefore, it is questionable if the released HBsAg was antigenically active HBsAg or not. Unfortunately, it is impossible with current technology to determine the antigenicity of the released HBsAg from the microparticles. First, HBsAg exposed to a solution starts to lose its antigenicity from 2 h after placement at 37°C so that the HBsAg released and accumulated in the releasing medium already loses most of its antigenicity before analysis [43]. Secondly, the release rate of HBsAg is too small (especially during a period later than day 20) to determine the antigenicity using available methods such as EIA, HPLC, and SDS-PAGE. Further the antigenicity of HBsAg remaining in the microparticles cannot be determined either since the removal of PLGA required organic solvent or high pH at 37°C which is harmful to HBsAg. However, we can at least qualitatively check if the released HBsAg is the antigenically active form or not by comparing the results from the in vitro releasing test with the results from the in vivo antibody formation. Examining Fig. 3 and Fig. 5, we find that the release patterns of HBsAg from the two microparticles match well with the patterns of the corresponding antibody formations as in the previous study with tetanus toxoid [41]. Further, in Fig. 4, the antibody titer produced by the single shot of the double-walled microparticle is much higher than the antibody titer produced by the single shot of the alum formulation and is comparable with the antibody titer produced by the two shots of alum formulation. If the released HBsAg from the double-walled microparticle was not antigenically active HBsAg, the antibody titer should have been similar to the antibody titer produced by the one shot of the alum formulation and much less than the antibody titer produced by the two shots of the alum formulation. Therefore, even though we don't know if the HBsAg released from the microparticles was 100% active HBsAg or not, we can reasonably assume that the HBsAg released from the microparticles was active enough to match with the two shots of the alum formulation in this study.

4. Conclusion

The novel double-walled microparticles were produced by spray drying. It was demonstrated in vitro as well as in vivo that HBsAg in the double-walled microparticle was kept intact during the encapsulation process. Furthermore, the single shot of the double-walled microparticle was much better than the single shot of the alum formulation and was at least comparable to the two shots of the alum formulation in producing HBsAg antibody in the guinea pig. In conclusion, the double-walled microparticle can open the door to the practical development of the single shot vaccine including HBsAg. This comes mainly from the protective ability and the favorable release pattern of the double-walled microparticles. However, it should be mentioned that we still have lots of questions to be answered. It would interesting to find the effects of the following parameters on release pattern and immune response: the monomer ratio of PLGA, the type of hydrophilic polymer, the coating thickness of PLGA and HPC, and the different combinations of two polymers. In this way, we will be able to more accurately correlate the characteristics of the double-walled microparticle with immune response. We plan

to report some of these results in the future including comparison with the three shots of alum formulation.

Acknowledgments

The authors wish to thank Dr. Myung Jin Kim for his critical review and Mr. Soo Hyeon Kim for his assistance in performing animal experiments.

References

[1] R. Langer and J. Folkman, Polymers for the sustained release of proteins and other macromolecules, Nature 263 (1976) 797–800.
[2] R. Langer, New methods of drug delivery, Science 249 (1990) 1527–1533.
[3] D.T. O'Hagan, J.P. McGee, J. Holmgren, A.M.C.L. Mowat, A.M. Donachie, K.H.G. Mills, W. Gaisford, D. Rahman and S.J. Challacombe, Biodegradable microparticles for oral immunization, Vaccine 11 (1993) 149–154.
[4] D.T. O'Hagan, K. Palin, S.S. Davis, P. Artursson and I. Sjoholm, Microparticles as potentially orally active immunologic adjuvants, Vaccine 7 (1989) 421–424.
[5] J. Kohn, S.M. Niemi, E.C. Albert, J.C. Murphy, R. Langer and J.G. Fox, Single-step immunization using a controlled release, biodegradable polymer with sustained adjuvant activity, J. Immunol. Methods 95 (1986) 31–38.
[6] Y. Men, G. Corradin, C. Thomasin, H.P. Merkle and B. Gander, Immunopotentiation of a synthetic antigen by incorporation into biodegradable microspheres, Proc. Int. Symp. Control. Rel. Bioact. Mater. 21 (1994) 50–51.
[7] P.B. O'Donnell, M. Iwata and J.M. McGinity, Properties of multiphase microspheres of poly(DL-lactide-co-glycolic acid) prepared by a potentiometric dispersion techniques, J. Microencapsulation 12(2) (1995) 155–163.
[8] C.R. Middaugh, Biophysical approaches to the pharmaceutical development of proteins, Drug Dev. Ind. Pharm. 16 (1990) 2635–2654.
[9] R.E. Spier, Meeting on vaccine strategies for tomorrow, Vaccine 11 (1993) 1450–1452.
[10] M.T. Aguado and P.H. Lambert, Controlled-release vaccines-biodegradable polylactide/polyglycolide(PL/PG) microspheres as antigen vehicles, Immunobiology 184 (1992) 113–125.
[11] M.J. Alonso, R.K. Gupta, C. Min, G.R. Siber and R. Langer, Biodegradable microspheres as controlled-release tetanus toxoid delivery systems, Vaccine 12 (1994) 299–306.
[12] M.Z.I. Khan, J.P. Opdebeeck and I.G. Tucker, Immunopotentiation and delivery systems for antigens for single-step immunization: recent trends and progress, Pharm. Res. 11(1) (1994) 2–11.
[13] W. Morris, M.C. Steinhoff and P.K. Russell, Potential of polymer microencapsulation technology for vaccine innovation, Vaccine 12 (1994) 5–11.
[14] R.K. Gupta, G.R. Siber, M.J. Alonso and R. Langer, Development of a single-dose tetanus toxoid based on controlled release from biodegradable and biocompatible polyester microspheres. In: H.S. Ginsberg, F. Brown, R.M. Chanock and R.A. Lerner (Eds.), Vaccine 93: Modern Approaches to New Vaccines Including Prevention of Aids, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY, 1993, pp. 391–396.
[15] D.H. Jones, B.W. McBride, H. Jeffery, D.T. O'Hagan, A. Robinson and G.H. Farrar, Protection of mice from bordetella pertussis respiratory infection using microencapsulated pertussis fimbriae, Vaccine 13 (1995) 675–681.
[16] F. Gavilanes, J. Gomez-Gutierrez, M. Aracil, J.M. Gonzalez-Ros, J.A. Ferragut, E. Guerrero and D.L. Peterson, Hepatitis B surface antigen, Biochem. J. 265 (1990) 857–864.
[17] K.J. Pekarek, J.S. Jacob and E. Mathiowitz, Double-walled polymer microspheres for controlled drug release, Nature 367 (1994) 258–260.
[18] A. Gopferich, M.J. Alonso and R. Langer, Development and characterization of microencapsulated microspheres, Pharm. Res. 11(11) (1994) 1568–1574.
[19] T.G. Park, M.J. Alonso and R. Langer, Controlled release of proteins from poly(L-lactic acid) coated poly-isobutylcyanoacrylate microcapsules, J. Appl. Polym. Sci. 52 (1994) 1797–1807.
[20] R.V. Nellore, P.G. Pande, D. Young and H.R. Bhagat, Evaluation of biodegradable microspheres as vaccine adjuvant for hepatitis B Surface antigen, J. Parent. Sci. Technol. 46(5) (1992) 176–180.
[21] B. Gander, E. Wehrli, R. Alder and H.P. Merkle, Quality improvement of spray-dried, protein-loaded D,L-PLA microspheres by appropriate polymer solvent selection, J. Microencapsulation 12(1) (1995) 83–97.
[22] F. Pavanetto, I. Genta, P. Giunchedi and B. Conti, Evaluation of spray drying as a method for polylactide and polylactide-co-glycolide microsphere preparation, J. Microencapsulation 10(4) (1993) 487–497.
[23] F. Pavanetto, B. Conti, I. Genta and P. Giunchedi, Solvent evaporation, solvent extraction and spray drying for polylactide microsphere preparation, Int. J. Pharm. 84 (1992) 151–159.
[24] M.J. Alonso, S. Cohen, T.G. Park, R.K. Gupta, G.R. Siber and R. Langer, Determinants of release rate of tetanus vaccine from polyester microspheres, Pharm. Res. 10(7) (1993) 945–953.
[25] M. Singh, A. Singh and G.P. Talwar, Controlled delivery of diphtheria toxoid using biodegradable poly(D,L-lactide) microspheres, Pharm. Res. 8(7) (1991) 958–961.
[26] C. Yan, W. Rill, J. Hewetson, R. Tammariello, R. Malli and M. Kende, Persistence of immunity to ricin toxin evoked by vaccine incorporated into polylactide-co-glycolide (PLG) microparticles, Proc. Int. Symp. Control. Rel. Bioact. Mater. 21 (1994) 52–53.

[27] T. Uchida, S. Martin, T.P. Foster, R.C. Wardley and S. Grimm, Dose and load studies for subcutaneous and oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin, Pharm. Res. 11(7) (1994) 1009–1015.
[28] I.H. Kim, K.J. Lim, S.M. Lee, Y.S. Kwon and H.H. Chung, Purification method of hepatitis B surface antigen, KR Patent 65305, Sept. 8, 1993.
[29] J. Kreuter, Physicochemical characterization of polyacrylic nanoparticles, Int. J. Pharm. 14 (1983) 43–58.
[30] O.H. Lowry, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[31] Inactive Ingredients for Currently Marketed Products, Interpharm, Buffalo Grove, Ill., 1991.
[32] R.J. Lewis, Sr., Food Additive Handbook, Van Nostrand Reinhold, New York, NY, 1989.
[33] Auzyme EIA, Abbott Laboratories, Production literature.
[34] Ausab EIA, Abbott Laboratories, Production literature.
[35] K. Yamaguchi and J.M. Anderson, In vivo biocompatibility studies of medisorb 65/35 D,L-lactide/glycolide copolymer microspheres, J. Control. Rel. 24 (1993) 81–93.
[36] M. Kanke, R. Geissler, D. Powell, A. Kaplan and P. DeLuca, Interaction of microspheres with blood constituents. III. Macrophage phagocytosis of various types of polymeric drug carriers, J. Parent. Sci. 42 (1988) 157–165.
[37] M. Kanke, E. Morlier, R. Geissler, D. Powell, A. Kaplan and P. DeLuca, Interaction of microspheres with blood constituents. II. Uptake and biodegradable particles by macrophages, J. Parent. Sci. 40 (1986) 114–118.
[38] D.T. O'Hagan, H. Jeffery and S.S. Davis, Long-term antibody response in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microparticles, Vaccine 11 (1993) 965–969.
[39] J.H. Eldridge, J.K. Staas, J.A. Meulbroek, T.P. Tice and R.M. Gilley, Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralising antibodies, Infect. Immun. 59 (1991) 2978–2986.
[40] R.E. Johnson, L.A. Lanaski, V. Gupta, M.J. Griffin, H.T. Guad, T.E. Needham and H. Zia, Stability of atriopeptin III in poly(DL-lactic-co-glycolide) microspheres, J. Control. Rel. 17 (1991) 61–68.
[41] B. Gander, C. Thomasin, H.P. Merkle, Y. Men and G. Corradin, Pulsed tetanus toxoid release from PLGA-microspheres and its relevance for immunogenicity in mice, Proc. Int. Symp. Control. Rel. Bioact. Mater. 20 (1993) 65–66.
[42] G. Ada, Strategies for exploiting the immune system in the design of vaccines, Mol. Immunol. 28(3) (1991) 225–230.
[43] J. Gomez-Gutierrez, I. Rodriguez-Crespo, J.M. Gonzalez-Ros, J.A. Ferragut, D.A. Paul, D.L. Peterson and F. Gavilanes, Thermal stability of hepatitis B surface antigen S protein, Biochim. Biophys. Acta 1119 (1992) 225–231.