Microencapsulation of Hepatitis B Core Antigen for Vaccine Preparation

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Purpose. To prepare poly(lactide-co-glycolide)(PLGA) microspheres containing recombinant hepatitis B core antigen (HBcAg; Mw = 3,600,000) by a w/o/w emulsion/solvent evaporation method and evaluate the possibility of this system as a potent long-acting carrier for hepatitis B core antigen in mice.

Methods. Various additives had been incorporated in the internal aqueous phase during the process of microencapsulating HBcAg, HBcAg antigenicity in the medium extracted from the prepared microspheres were measured by ELISA. Shape confirmation of the HBcAg antigen was performed by a sucrose gradient velocity centrifugal technique. For *in vivo* study, prepared microspheres were administered subcutaneously to Balb/C mice, and the serum IgG level was determined by ELISA.

Results. The inactivation of HBcAg by methylene chloride was dramatically reduced by the addition of gelatin (4–8% (w/v)) to the internal aqueous phase during the preparation. Further improvement of the loading efficiency to almost 61% resulted with cooling (4°C). The prepared microspheres (4.27 μm ± 1.23 μm) containing 0.15% HBcAg displayed burst release (50–60% within 2 days). In subcutaneous inoculation, the adjuvant effect of PLGA microspheres was almost the same as that of the complete Freund's adjuvant. Whereas oral inoculation using the microspheres was not effective.

Conclusions. The pH of the added gelatin seemed to be the key to the stabilization of HBcAg from various stability tests and CD spectrum study. Finally, the possibility of using this system as a potent long-acting hepatitis B vaccine was demonstrated.

KEY WORDS: hepatitis B core antigen; microspheres; poly(lactide-co-glycolide); ELISA; vaccine.

INTRODUCTION

Commercially available vaccines for hepatitis B virus, based on the recombinant DNA technique, are safe and immunogenic but must be injected three times at 0, 1 and 6 months to provide protective antibody levels. Oil and mineral adjuvants currently available, such as complete Freund's adjuvant (CFA), can cause adverse reactions including local granulomas, pain, fever, and possibly malignancies (1–2), making them unacceptable for human use.

To overcome these problems, the usefulness of sustained-antigen-releasing microspheres has been suggested for subcutaneously or orally delivered vaccines (3–5). We have examined the effect of the inoculation route (5) and particle size (6–7) on the antibody level following administration of microspheres made of a biodegradable polymer, poly(lactide-co-glycolide) (PLGA), using ovalbumin (OVA) as a model antigen.

In the present study, we encapsulated hepatitis B core antigen (HBcAg) having a molecular weight of almost 3,600,000 into the PLGA polymer and conducted an *in vitro* characterization. Little work (8) has been done using the hepatitis B antigen due to its instability in microencapsulation. Usually the hepatitis B core antigen exists as a particle composed of 180 subunit proteins (Mw of one subunit is almost 20,000). Conformational change readily occurs on contact with a low concentration of surfactant or organic solvent, resulting in dramatic reduction of immunogenicity (9–10). Therefore we screened water-soluble additives for those which could protect the immunogenicity of HBcAg from the methylene chloride used in the preparation process and used them for the microencapsulation. We also evaluated the possibility of using this system as a potent long-acting carrier for hepatitis B core antigen.

EXPERIMENTS

Materials

Hepatitis B core antigen and anti-HBc monoclonal antibody, Yc-3, were from The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan); Trizma base and Trizma hydrochloride from Sigma Chemical Co., St. Louis, MO; poly(lactide-co-glycolide) 50/50 (MW = 53,000) (PLGA) from Medisorb Technologies, Cincinnati, OH; polyvinyl alcohol (PVA, 98–99% hydrolyzed, Mw = 31,000–50,000) from Aldrich Chemical Company, Inc. Milwaukee, WI; analytical grade methylene chloride and gelatin from Nacalai Tesque Co., Kyoto, gelatin from Seiwa Kasei, Co., Tokyo, and Nitta Gelatin Co., Tokyo, Japan; affinity-purified peroxidase-labelled anti-mouse IgG, IgA goat serum from Chemical International Inc., Temewla, CA; affinity-purified antibody peroxidase labelled antimouse goat antibody from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD; and complete Freund's adjuvant (CFA) from Gibco, Grand Island, NY. All other reagents were of special reagent grade.

Stability Test of HBcAg and Effect of Additives on HBcAg Stability

Stability of HBcAg

To examine the stability of HBcAg itself, 1000 ng/ml of HBcAg solution was incubated for 3 days using various buffers (pH 3–9) at 4°C, room temperature, and 37°C and the immunoreactivity of this solution was measured after 0.5, 1, 2, 3, 4 and 5 min sonication with the strength employed being the same as that used for the preparation of PLGA microspheres containing HBcAg. The effect of freeze-thawing was evaluated by measuring the immunoreactivity of the HBcAg solution (1,000 ng/ml) after repetition of the freeze-thawing process once, twice, or three times.

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Effect of Various Additives on HBcAg Stability

Five milliliters of HBcAg solution (1–25 μ/ml) in the absence or presence of various additive solutions was emulsified with 5 ml of methylene chloride using 1 min sonication, then mixed by agitation for 0, 2, and 4 h. The buffer phase was used to determine the concentration by ELISA with microplates which had been subjected to coating overnight with 50 μl per well of 0.5% rabbit serum anti-HBc at 4°C. The microplates were then masked by incubation with 100 μl of pBS containing 2% BSA. The buffer phase sample (50 μl) containing HBcAg was added to the well and incubated for 30 min at 37°C, then washed three times in 100 μl of 0.05% of Tween20/PBS. After washing, 0.1 ng/ml of Yc-3 monoclonal antibody was added to the well as a 50-μl solution and incubated for 30 min at 37°C. The plates were washed three times then 50 μl of diluted HRP (horseradish peroxidase) labelled-goat anti-mouse IgG conjugates (1:30000) was added to the wells, followed by incubation at 37°C for 30 min. This was followed by washing five times in 100 μl of 0.05% Tween20/PBS. Finally, the reaction was stopped after 30 min with the addition of 50 μl of diluted 1 N H_2SO_4. ELISA plates were read with a plate reader and the results were expressed as the optical density at 450 nm.

Effect of Gelatin Type on HBCAg Stability to Methylene Chloride

The method was essentially the same as that described in the above paragraph. The gelatin concentration was fixed at 4% (w/w), and the pH of this solution was measured using a pH meter (Horiba F8-OD, Tokyo, Japan).

CD Spectrum Change Induced by Interaction Between HBcAg and Gelatin

The interaction between gelatin and HBcAg particles was evaluated by the change in the circular dichroism (CD) spectra (J-600, Nihon Bunko Co., Ltd., Tokyo, Japan). The band width was 1.0 nm, sensitivity was 20 mdeg/FS, and the time constant was 2.0 sec. Scanning speed was fixed at 20 nm/min for the range of 350–200 nm.

Preparation of Microspheres and *In Vitro* Characterization of Microspheres

Preparation of Microspheres

The preparation method was essentially the same as that described previously (5). The external aqueous phase was 5% PVA solution containing 5% NaCl. HBcAg antigen was extracted as described in an earlier paper (11). The theoretical HBcAg loading was 0.25%. The HBcAg concentration in the extracted medium was determined by the ELISA described above. A Microtrac Particle Size Analyzer (Model 7995-30, Leeds and Northrup, North Wales, PA) was utilized to determine the microsphere average volume diameters and distributions. Scanning electron microscopy (SEM) was used to examine the surface and morphology of the microspheres. The method was essentially the same as that detailed in our previous paper (11).

Shape Confirmation of HBcAg Extracted from Microspheres by Sucrose Gradient Velocity Centrifugation

An extract of 1.5 ml from prepared microspheres was layered over 11 ml of 20–60% (w/w) linear sucrose gradient in PBS and centrifuged in an RPS 4-T rotor (Hitachi) at 30,000 rpm for 16 hr at 4°C. This centrifuged sample solution was fractionated into 17 samples of 0.7 ml each, and the HBcAg activity of each fraction was determined by ELISA.

In Vitro HBcAg Release Test

To determine the *in vitro* release of HBcAg from PLGA microspheres, the method described in our previous paper was used (11).

In Vivo Study

Animals

Five female Balb/C mice 8 weeks of age were used in each inoculation group. The mice, given laboratory chow and water *ad libitum*, were inoculated subcutaneously and orally using 100 μl. The required doses of microspheres were weighed and resuspended in 0.9% NaCl. The animals were bled and the separated serum samples were stored at −20°C until assayed.

Immunization Protocols

Groups of animals were inoculated with HBcAg solution (negative control), HBcAg formulated in CFA, and microspheres at 0 and 4 weeks. All animals were bled at 0, 4, and 8 weeks.

Measurement of Antibody

To measure the serum IgG antibody level, 96-well microtiter plates were coated overnight with 50 μg per well of 0.5% anti-HBc rabbit serum at 4°C, washed with PBS containing 0.05% Tween 20 (PBST) three times, then masked with 100 μl of PBS containing 2% BSA. After the washing, each well was filled with 50 μl of HBcAg solution (1 μg/ml), incubated for 30 min at 37°C, then washed with PBST three times. The immunized mice sera were subjected to fourfold serial dilutions starting from 1:100 dilution using PBS containing 2% BSA and added to each well. The plates were incubated for 30 min at 37°C, then washed with PBST three times. Color development of the immune complexes was performed with 50 μl of HRP labelled anti-mouse IgG goat antibody, H_2O_2 and a dye. After 30 min, the enzyme reaction was stopped by adding 50 μl of 1 N H_2SO_4. The ELISA plates were read with a plate reader and measured for optical density at 450 nm. The antibody titer was expressed as the reciprocal of the immune serum dilution that gave three times the optical density of the pre-immune serum at the same dilution.

To measure IgG or IgA levels in the fecal extract, the mice were placed in an empty cage for 4 hrs on the sampling day. The mice were allowed freedom of movement, and the excreted fresh feces (almost 30 blocks) were collected and dried. The feces were weighed and suspended in pH 7.4 PBS buffer (11%). The sample was centrifuged at 3,000 rpm for 10 min, and the supernatant IgG or IgA was determined by ELISA essentially

as described for the serum IgG measurement in the experimental section.

RESULTS AND DISCUSSION

Effects of Various Additives on HBcAg Stability

We examined the stability of HBcAg in various pH buffer solutions and found it to be comparatively stable at pH 6–9. For example, the antigenicity of HBcAg was maintained for almost 1 month in pH 7.4 (PBS buffer) at 4°C. However, HBcAg was unstable under acidic condition (pH 3–5); almost 50% was inactivated by incubation in pH 4.0 citrate buffer for 3 days. The sonication and freeze-thawing process did not affect the stability (detailed data not shown).

When poly(lactide-co-glycolide) (PLGA) microspheres containing HBcAg were prepared as described previously (5), loss of HBcAg antigenicity was observed. HBcAg was extracted from several batches of prepared microspheres, and ELISA measurements of the antigenicities of the extracted media showed that antigenicity had been lost during the process of microencapsulation into the PLGA matrix.

The major reason for the inactivation of HBcAg during the preparative process is thought to be its contact with methylene chloride. We conducted a simple stability test by emulsifying 5 ml of HBcAg solution (1–25 μg/ml) with 5 ml of methylene chloride by 1 min sonication. When the concentration in the buffer phase was measured by ELISA, no antigenicity of HBcAg was observed for any of the samples, showing that contact of HBcAg particles with methylene chloride led to the dramatic inactivation.

To select appropriate additives to protect the immunoreactivity of HBcAg, we examined the effects on HBcAg stability of various water-soluble additives such as polyethylene glycol, carboxy methylcellulose, cyclodextrins, sugars, and amino acids. The results are summarized in Fig. 1. Among various water-soluble additives, polyethylene glycol (PEG) 2000 showed a stabilizing effect to methylene chloride to some extent. The best stabilizing material was gelatin (Nacalai tesque; molecular weight distribution 10,000–150,000).

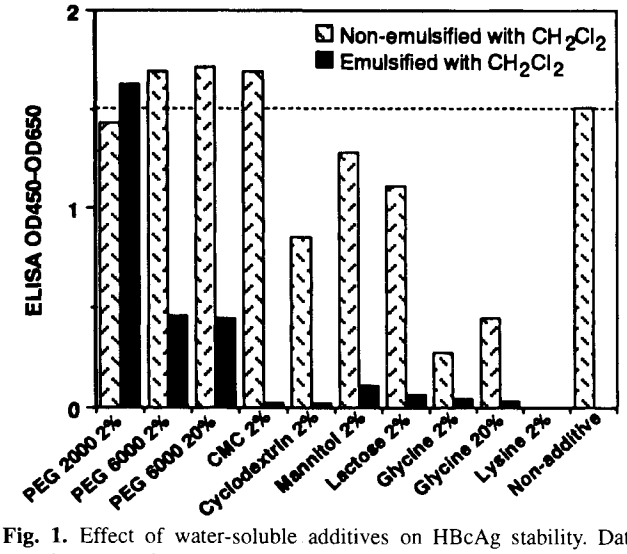

Fig. 1. Effect of water-soluble additives on HBcAg stability. Data was the mean of two experiments. The symbol "Non-emulsified with CH_2Cl_2" means that the ELISA activity of the mixture of HBsAg solution and additives was determined as the control. Initial HBcAg concentration was fixed at 25 ng/ml.

Figure 2 shows the effect of gelatin concentration on HBcAg stability to methylene chloride under room temperature or ice cooling. The stabilizing effect of gelatin increased as its concentration increased, with a plateau at above 4% (w/v); the effect of 6 or 8% (w/v) gelatin solution was almost the same as that of the 4% (w/v) solution (data for 6% and 8% gelatin addition were not shown in Fig. 2).

We also examined the effect of gelatin type on HBcAg stability to methylene chloride. Table 1 summarizes the immunoreactivity of HBcAg solution containing various types of gelatins (as 4% solution). Gelatin with a low molecular weight of 400 (type II in the table), for example, had a slight stabilizing effect. When the HBcAg solution containing the type II gelatin was emulsified with methylene chloride, its immunoreactivity decreased with time. As the molecular weight increased (between the range between 400 (type II) and 10000 (type IV)), the stabilizing effect seemed to rise in proportion to it. Different findings were observed for type III gelatin. With respect to pH, as shown in Table 1, that of type II gelatin (Mw = 700) was rather low (5.28). With gelatin type VII and VIII, having pH values of 4.46 and 5.52, respectively, the stabilizing effect was not sufficient. Thus, an acidic pH is not appropriate for improving the stability of HBcAg to methylene chloride even when the gelatin has a considerably high molecular weight (type VII; Mw = 80000–200000). Type IX gelatin with a pH value of 6.12 (as 4% solution) was also effective for improving HBcAg stability. The pH of the Nacalai gelatin was 6.20. These findings coincided well with the description of HBcAg stability in a different pH buffer and suggested that the pH of the gelatin is the key to stabilizing HBcAg. Another important factor is the molecular weight.

Figure 3 summarizes the CD spectra for HBcAg in the presence or absence of gelatin (Nacalai Tesque). The CD spectrum of the HBcAg solution showed that it changed dramatically on contact with methylene chloride. The absorbance of about 230 nm disappeared completely, coinciding with the loss of HBcAg immunoreactivity immediately after contact with meth-

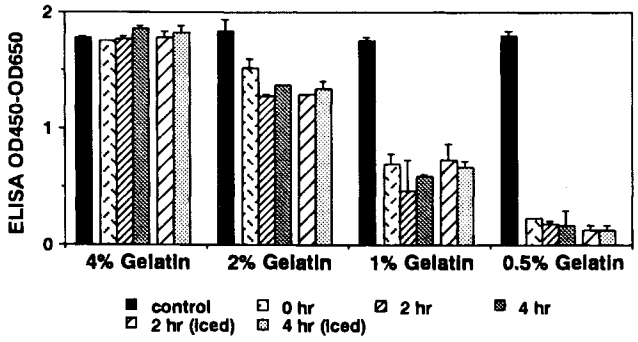

Fig. 2. Effect of gelatin concentration on HBcAg stability to methylene chloride. Each horizontal bar represents the mean and S.D. of three experiments. Initial HBcAg concentration was fixed at 25 μg/ml. For example, the symbol for 4 hr (iced) means that the emulsification of the HBcAg solution with methylene chloride was continued for 4 hr under cooling, after the water-phase HBcAg activity had been determined by ELISA.

Table 1. Effect of Gelatin Type on HBcAg Stability Against Dichloromethane

Gelatin type	M.W.	pH (4% conc.)	Immunoactivity of HBcAg after emulsified with CH_2Cl_2 (%)[a]		
			0 hr	2 hr	4 hr
I	10000–150000	6.20	99 ± 5	101 ± 4	102 ± 3
II	400	6.61	94 ± 4	88 ± 9	54 ± 8
III	700	5.28	84 ± 4	65 ± 4	46 ± 7
IV	1000	6.57	95 ± 5	92 ± 12	75 ± 6
V	2000	6.15	96 ± 7	93 ± 2	80 ± 5
VI	10000	6.05	97 ± 4	95 ± 5	90 ± 3
VII	80000–200000	4.46	90 ± 8	76 ± 5	58 ± 9
VIII	50000–180000	5.52	94 ± 2	80 ± 7	68 ± 6
IX	50000–200000	6.12	98 ± 7	94 ± 4	95 ± 4

Note: I: Nacalai Tesque Inc. II ~ V: Seiwa Kasei Co., Ltd. IV~IX: Nitta Gelatin Inc.
[a] The activity was represented as the percentage compared to the control HBcAg activity.

ylene chloride. The CD spectrum of gelatin and HBcAg mixture solution did not change on contact of the mixture solution with methylene chloride, with the CD spectrum being almost the same as that of the gelatin itself. These CD findings suggest that the gelatin molecular network enwraps HBcAg particles and thereby offers protection from the attack of methylene chloride molecules.

We next performed the microencapsulation of HBcAg in the presence of 4 or 8% (w/v) gelatin in the internal aqueous phase and 5% PVA solution containing 5% NaCl in the external aqueous phase under room temperature or ice cooling. An increase of gelatin % in the internal aqueous phase and cooling process led to an increase in loading efficiency. When 8% (w/v) of gelatin (Mw = 10,000–150,000) was added to the internal aqueous phase under cooling, the loading efficiency reached 61%, indicating a beneficial effect. The one of the reasons for this considerably high loading is the stabilizing effect of the gelatin (Nacalai Tesque) itself, but another reason may be the increased viscosity of the internal aqueous phase containing gelatin due to the cooling. At over 8% (w/v) gelatin concentration, the viscosity of the first emulsification liquid becomes too large to treat, thereby producing larger microspheres. Thus, the most effective gelatin concentration was concluded to be 8% (w/v). Microspheres were fairly monodispersed and spherical according to morphology study, and the mean volume diameter of the prepared microspheres was 4.27 μm ± 1.23 μm. The microspheres contained 0.15% of HBcAg.

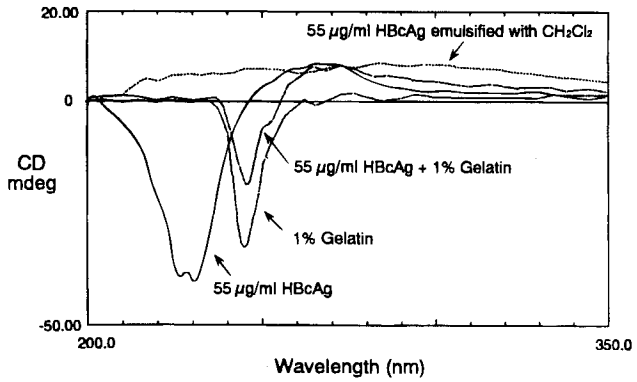

Fig. 3. Change of CD chromatograms of HBcAg by addition of gelatin.

Shape Confirmation of HBcAg

The ELISA employed in the present study was designed to recognize HBcAg as a particle antigen. HBcAg antigen was extracted from 0.15% HBcAg-loaded PLGA microspheres and its particle characteristics were established by sucrose gradient velocity centrifugal technique.

As shown in Fig. 4A (sample extracted from microspheres) and Fig. 4B (control HBcAg solution), the extracted sample showed a peak with a sucrose concentration of 42.3%, a value almost the same as the control HBcAg sedimentation profile. Thus, the HBc particles extracted from the prepared microspheres had almost the same sedimentation coefficient as the control HBcAg.

Release Test

The *in vitro* release profile (pH 7.4 Tris buffer) of prepared PLGA microspheres containing 0.15% HBcAg is shown in Fig. 5. Within 2 days, 60 to 70% of the incorporated HBcAg was released, with no further release being observed up to 7 days. Even after 4 weeks, the released fraction was about 60%, with about 30% of the HBcAg remaining unreleased (data not shown).

Previous research has demonstrated long sustained release of proteins or peptides from PLGA microspheres *in vitro* (12–14). Lai *et al.* (15) examined the release of an antitumor agent from PLGA microspheres several microns in diameter and reported that about 60% of the drug was released within a day. We found essentially the same release profile. Ogawa *et al.* (16) has also reported a 30% burst release with no additional release of peptide from PLA microspheres 20 μm in diameter. The absence of additional release was frequently observed for the PLGA microsphere system, and a discrepancy was noted between the *in vitro* and *in vivo* release rates for this system. One reason for it was that the *in vitro* release medium employed did not reflect the *in vivo* erosion rate of the PLGA polymer. We therefore evaluated the burst-released fraction for HBcAg-loaded PLGA microspheres with a small diameter. We had previously found that the extent of the *in vitro* burst release did not affect the immunological response *in vivo* in a study using ovalbumin as a model antigen (5). Even though we did not perform a precise *in vitro* release–*in vivo* immunological

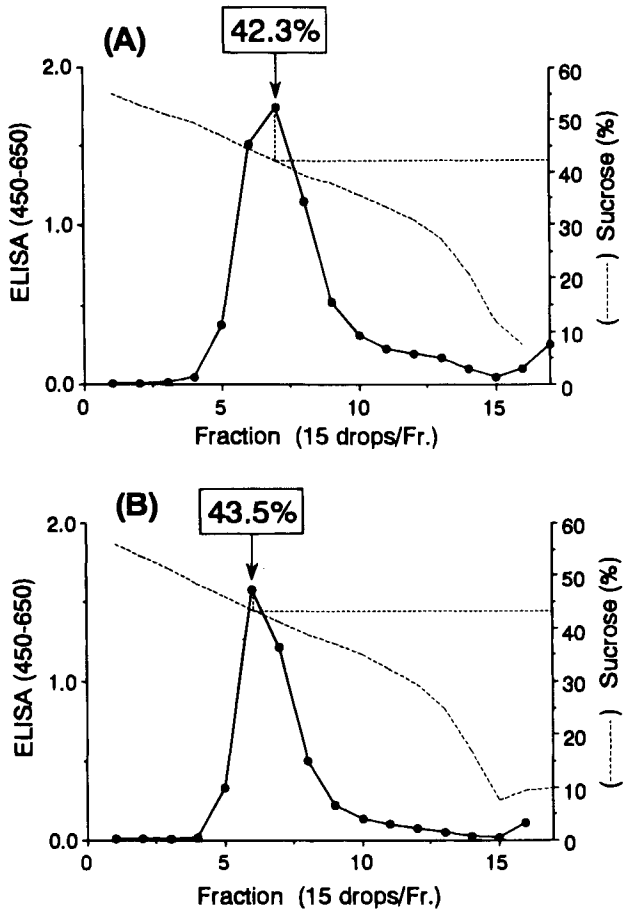

Fig. 4. Shape confirmation of HBcAg extracted from 0.15% HBcAg loaded-microspheres by sucrose gradient velocity centrifugal technique. The top figure (A) shows the elution profile for HBcAg extracted from prepared microspheres, whereas the bottom one (B) shows the profile of control HBcAg.

response correlation study here, HBcAg was incorporated into microspheres as a stable form and was released gradually as degradation proceeded with polymer erosion *in vivo*. Therefore, the unreleased fraction of the HBsAg in the *in vitro* release test seems to play a key role in maintaining the immunological response.

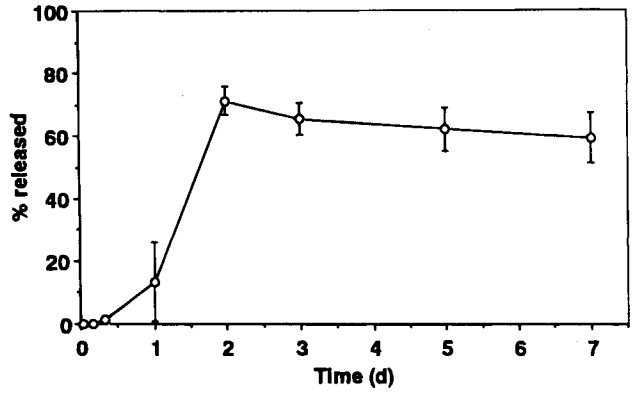

Fig. 5. Release profile of HBcAg from PLGA microspheres containing 0.15% HBcAg in ph 7.4 Tris buffer. Each point represents the mean ± S.D. of three experiments.

Table 2. Anti-HBc IgG Antibody Titer in the Serum of Mice Inoculated with Microspheres Containing HBcAg

	MS (S. C)	MS (oral)	HBc plain (S. C)	HBc + CFA (S. C)	HBc plain (oral)
4 weeks after	120000	90000	3500	15000	1000
1st Inoculation	40000	1000	7200	190000	1000
	15000	1000	3100	190000	1000
	170000	1000	3500	68000	1000
	64000	1000	7600	17000	< 1000
4 weeks after	310000	130000	175000	360000	1000
2nd	460000	1000	130000	—	1000
Inoculation	125000	1000	54000	780000	1000
	390000	1000	52000	150000	1000
	160000	1000	185000	250000	28000

Note: Each inoculation was done at 0 week and 4 weeks. HBcAg dose was fixed a 3 μg/dose.
— in the table means the mice died during the experiments.

In Vivo Immune Response in Mice

Antibody titers in mice given HBcAg intragastrically or orally are summarized in Table 2. The IgG titer levels of mice

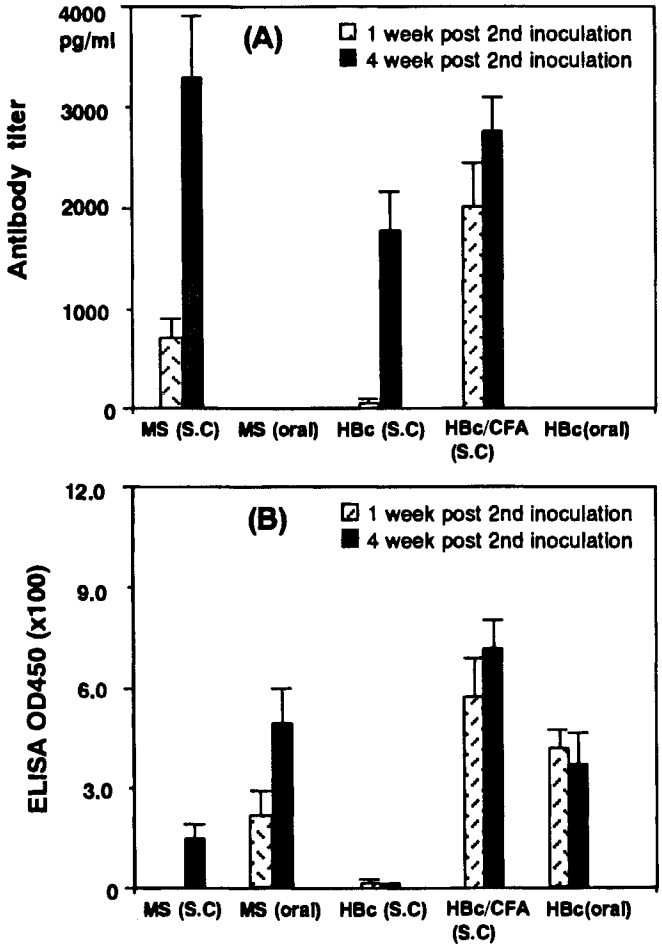

Fig. 6. Anti-HBc IgG (top fig, A) or IgA (bottom fig, B) antibody titer in the fecal extract of mice inoculated subcutaneously with microspheres containing 0.15% HBcAg. Each horizontal bar represents the mean and S.D. of 4–5 experiments.

inoculated with microspheres containing 0.15% of HBcAg subcutaneously at 4 weeks, were compared with those of mice given saline/HBcAg (negative control) and CFA/HBcAg (positive control). As shown in Fig. 6A, a considerably high IgG level was also detected in the fecal extract when mice were inoculated subcutaneously with microspheres. Nellore *et al.* studied the microencapsulation of HBsAg by the solvent/extraction method and its *in vivo* sucbutaneous inoculation (8). However, they did not characterize the prepared microspheres such as by determining HBsAg loading, and in addition, the observed adjuvant effect was low with subcutaneous inoculation. In the present study, we confirmed HBcAg to be incorporated in microspheres as an active form and also found that this resulted in a significant immuno-adjuvant effect.

Oral inoculation with PLGA microspheres containing 0.15% HBcAg was useless for vaccination. The anti-HBcAg IgG antibody level in serum was low except for one mouse (titer: 90,000). For the fecal IgA level, oral administration of HBcAg solution, HBcAg-loaded microspheres and subcutaneous administration of CFA slightly raised the anti-HBcAg antibody level which was considerably low, as shown in Fig. 6B. One reason for this is the low antigenecity of the HBcAg particle. In fact, HBcAg, which displays its immunoreactivity only as a particle which has two epitopes, seems to be fragile. Another reason for the low antigenicity seems to be the poor uptake of microspheres into Peyer's patch as described in the previous study (17). To explain these phenonmena, further experiments are needed.

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REFERENCES

1. J. Freund. The effect of paraffin oil and mycobacteria on antibody formation and sensitization. *Am. J. Clin. Pathol.* **21**:645–656 (1951).
2. G. W. Beebe, A. H. Simon, and S. Vivona. Long-term mortality follow up of army recruits who received adjuvant influenza virus vaccine. *Am. J. Epidemiol.* **95**:337–344 (1972)
3. D. T. O'Hagan, K. Palin, S. S. Davis, P. Artursson, and I. Sjoholm. Microparticles as potentially orally active immunological adjuvants. *Vaccine* 7:421–424 (1989).
4. J. H. Eldridge, C. J. Hammond, J. A. Meulbroek, J. K. Staas, R. M. Gilley, and T. R. Tice. Controlled vaccine release in the gut-associated lymphoid tissues 1. Orally administered biodegradable microspheres target in the Peyer's patches. *J. Contr. Rel.* **11**:205–214 (1990).
5. T. Uchida, S. Martin, T. P. Foster, R. C. Wardley, and R. C. Grimm. Dose and load studies for subcutaneous and oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin. *Pharm. Res.* **11**:1009–1015 (1994).
6. T. Uchida and S. Goto. Oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin as vaccine formulation: particle size study. *Biol. Pharm. Bull.* **17**:1272–1276 (1994).
7. T. Uchida, S. Goto, and T. P. Foster. Particle size studies for subcutaneous delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin as vaccine formulation. *J. Pharm. Pharmacol.* **46**:1214–1221 (1995).
8. 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. & Tech.* **46**:176–180 (1992).
9. N. Nath, K. Hickman, S. Nowlan, D. Shah, J. Philips, and S. Babler. Stability of the recombinant hepatitis B core antigen. *J. Clin. Microbio.* **30**:1617–1619 (1992).
10. A. Budkowska, A. Kalinowska, and A. Nowoslawski. Identification of two HBeAg subspecificities revealed by chemical treatment and enzymatic digestion of liver-derived HBcAg. *J. Immunol.* **123**:1415–1416 (1979).
11. T. Uchida, A. Yagi, Y. Oda, and S. Goto. Microencapsulation of ovalbumin in poly(lactide-co-glycolide) by oil-in-oil (o/o) solvent evaporation method. *J. Microencapsulation* **13**:509–518 (1996).
12. S. Benita, J. P. Benoit, F. Puisieux, and C. Thies. Characterization of drug loaded poly (dl-lactide) microspheres. *J. Contr. Rel.* **73**:1721–1724 (1984).
13. P. Edman, E. Bjork, and L. Ryden. Microspheres as a nasal delivery system for peptide drugs. *J. Contr. Rel.* **21**:165–172 (1993).
14. T. Heya, H. Okada, Y. Tanigawara, Y. Ogawa, and H. Toguchi. Effects of counteranion of TRH and loading amount on control of TRH release from copoly (dl-lactic/glycolic acid) microspheres prepared by an in-water drying method. *Int. J. Pharm.* **69**:69–75 (1991).
15. Y.-L. Lai, R. C. Mehata, A. A. Thacker, S.-D. Yoo, P. J. McNamara, and P. P. DeLuca. Sustained bronchodilation with isoproterenol poly(glycolide-co-lactide) microspheres. *Pharm. Res.* **10**:119–125 (1993).
16. Y. Ogawa, Y. M. Yamamoto, S. Takada, H. Okada, and T. Shimamoto. Controlled-release of leuprolide acetate from polylactic acid or copoly(lactic/glycolic) acid microcapsules: influence of molecular weight and copolymer ratio of polymer. *Chem. Pharm. Bull.* **36**:1095–1103 (1988).
17. P. G. Jani, W. Halbert, J. Langridge, and A. T. Florence. The uptake and translocation of latex nonospheres and microspheres after oral administration to rats. *J. Pharm. Pharmacol.* **41**:809–812 (1989).