

Induction of mucosal and systemic immune response by single-dose oral immunization with biodegradable microparticles containing DNA encoding HBsAg

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The purpose of this work was to assess the ability of plasmid DNA encoding hepatitis B virus (HBV) HBsAg encapsulated in poly(DL-lactide-co-glycolic acid) (PLGA) microparticles to induce local and systemic HBsAg-specific immunity following a single dose of oral immunization. RT-PCR analysis demonstrated prolonged transcription of plasmid DNA, consistent with the sustained expression and presentation of target antigen observed by confocal laser scanning microscopy, in gut-associated lymphocyte tissue (GALT) from mice immunized orally with plasmid DNA encapsulated into PLGA microparticles. Oral administration of PLGA-DNA microparticles induced a long-lasting and stable antigen-specific antibody response, both serum total antibody and intestinal IgA, in BALB/c mice. Mice immunized orally exhibited antigen-specific gamma interferon production and cytotoxic T lymphocyte responses in spleen and GALT after restimulation *in vitro* with HBsAg or tumour cells stably expressing HBsAg. In contrast, naked DNA vaccines given by intramuscular injection induced only systemic cellular and humoral responses to HBsAg, which were much lower than the responses elicited by oral DNA encapsulated in PLGA microparticles at equivalent doses. The results are encouraging with regard to obtaining good compliance and vaccination coverage with candidate plasmid DNA vaccines, especially in developing countries.

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INTRODUCTION

Development of a safe, effective and affordable vaccine represents the best hope for controlling the spread and consequences of the hepatitis B virus (HBV) epidemic worldwide. Recent data have indicated that immunotherapeutic strategies stimulating both cellular and humoral immune responses to HBV antigens are essential to cure chronic HBV infection (Trepo *et al.*, 2003). In this regard, DNA-based vaccination appears a particularly pertinent approach for chronic hepatitis B treatment, since it has been well-documented to elicit durable humoral and cell-mediated immunity including cytotoxic T lymphocytes (CTL) and cytokines in normal mice (Brazolot Millan *et al.*,

1998; Oka *et al.*, 2001), HBV-Tg mice (Oka *et al.*, 2001), ducks (Le Guerhier *et al.*, 2003) and chimpanzees (Davis *et al.*, 1996). It is a good candidate for immunization of non-responders to recombinant HBsAg vaccines and for therapeutic vaccination (Brazolot Millan *et al.*, 1998), providing a promising option for the development of subunit vaccines. In our previous study, intramuscular (i.m.) injection of plasmid DNA vectors encoding the middle envelope proteins could prevent experimental infection of tree shrews with HBV (Zhou *et al.*, 2003).

However, even at higher doses of DNA, human clinical trials of HBV DNA vaccine have only yielded much lower levels of immune responses than have been observed in small animals. Such a situation was also found with a human immunodeficiency virus (HIV) DNA vaccine (Tacket *et al.*, 1999; Boyer *et al.*, 2000). Therefore, the potency of DNA

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vaccines must be increased to enable this technology for successful human application.

Mucosal vaccination generates systemic and local immunity. The most attractive route of mucosal immunization is oral, because of its high patient compliance, ease of administration and applicability to mass vaccination. Recently, various attempts have been made to demonstrate the efficacy of biodegradable and biocompatible microspheres as particulate delivery systems in order to induce systemic and local immune responses following oral immunization (O'Hagan & Singh, 2003). A wide range of polymeric materials have been explored for their ability to enhance the immunogenicity of oral vaccines, such as poly(DL-lactide-co-glycolic acid) (PLGA) (Sharpe *et al.*, 2003; Herrmann *et al.*, 1999; Singh *et al.*, 2001), starch (Wikingson & Sjöholm, 2002), chitosan and other cationic polymers (Illum *et al.*, 2001; Twaites *et al.*, 2004), since microparticles of less than 10 µm are readily taken up by intestinal M cells, macrophages and other professional antigen-presenting cells (APCs), leading to antigen presentation in regional inductive immune sites (Kim *et al.*, 1999; Baras *et al.*, 1999; Okada & Toguchi, 1995). Of these, PLGA has already been approved as a component of a number of drug-delivery systems and has a long history of safe use in humans (Klencke *et al.*, 2002; Okada & Toguchi, 1995). More recently, it was shown that PLGA-encapsulated plasmid DNA elicited systemic and mucosal antibodies to the encoded antigen, as well as CTL responses after oral delivery in non-primate and primate models (Kaneko *et al.*, 2000; Sharpe *et al.*, 2003; Herrmann *et al.*, 1999; Singh *et al.*, 2001).

In this study, we evaluated the feasibility of using plasmid DNA encoding HBsAg encapsulated in PLGA microparticles to induce local and systemic HBsAg-specific immunity following single-dose oral immunization. The immunogenicity of encapsulated DNA administered by the oral route was compared with naked DNA vaccine injected i.m. The potential advantages of encapsulated DNA vaccine over naked DNA vaccines are discussed.

METHODS

Plasmid construction. The small envelope gene of HBV was amplified by PCR from the HBV genome (*adr* subtype) obtained from a patient's serum positive for HBV and was cloned into the *EcoRI/BamHI* sites of plasmid vector pVAX1 (Invitrogen Life Technologies). The resultant plasmid, pVAX(S) was amplified in *Escherichia coli*, isolated by alkaline lysis and purified by double banding in equilibrium gradients. Endotoxin was removed by multiple Triton X-114 extractions. All plasmid preparations were found to contain fewer than 50 endotoxin units (mg purified DNA)⁻¹ by the *Limulus* amoebocyte lysate assay.

Preparation of PLGA. DNA was microencapsulated in PLGA as described previously (Tinsley-Bown *et al.*, 2000). Briefly, solutions of two PLGA polymers, RG502 and RG503 (Boehringer Ingelheim), were mixed 50:50 in ethyl acetate (4 ml) and emulsified with 0.4 ml pVAX(S) (10 mg ml⁻¹) in STE buffer (100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0). This emulsion was added

immediately to an 8% aqueous polyvinyl alcohol solution (66 ml) preheated to 30 °C and emulsified, resulting in the formation of a water-in-oil-in-water emulsion. Microparticles were harvested by centrifugation, washed several times to remove the polyvinyl alcohol and residual solvent and finally lyophilized. A control formulation with the empty vector pVAX1 encapsulated into PLGA microparticles was similarly manufactured. For the phagocytosis experiment, PLGA-pVAX(S) microparticles containing encapsulated rhodamine B were prepared individually with a target load of 0.25% (w/w) rhodamine in PLGA by the same process.

Microparticles were generated at least three times. DNA encapsulation ranged from 4.1 to 6.2 µg (mg microparticles)⁻¹ for lab-scale batches. The products showed regular and spherical microsphere morphology, with particle sizes in the range 2–9 µm with a median diameter of 5.7 µm, as examined with a particle size analyser and by scanning electron microscopy.

Immunization. BALB/c mice were bred and cared for in the animal facilities of the Second Military Medical University, Shanghai. Only female mice, 6–8 weeks old at the start of vaccination, were used. The microparticle formulation was suspended in 7.5% sodium bicarbonate solution in order to buffer gastric acid fluids. A single dose of oral administration was performed with pVAX(S) encapsulated into PLGA microparticles by using a 24-gauge feeding needle. The microparticle dose was 20 or 200 µg per mouse. In order to test the reproducibility of batches, these experiments were carried out three times with separately produced batches of microparticles. The control groups received 200 µg empty vector pVAX1 encapsulated into PLGA microparticles by the same route. Single-dose i.m. inoculation was performed with naked pVAX(S) (20 or 200 µg in 200 µl saline per mouse) in two other groups of mice, to which ketamine [100 mg (kg body weight)⁻¹] had been administered by i.m. injection 3 days before. A 100 µl formulation was injected in the tibialis anterior and another 100 µl was injected in the hamstring in the two hind legs of each animal. Sera were collected by retro-orbital bleeding 4 weeks after the immunization.

Fecal washes were prepared from fecal samples as described previously (Staats *et al.*, 1996). Briefly, fresh fecal samples (100 mg) were mixed with 1 ml PBS, incubated at room temperature for 15 min, vortexed and centrifuged in a microcentrifuge for 10 min. Supernatants were collected and stored at -20 °C until assayed for anti-HBsAg antibody.

Antibody responses induced by different DNA formulations.

HBsAg-specific total antibody and intestinal IgA were analysed by ELISA in serum or fecal washes from individual mice in each group with a commercial kit (SIIC KeHua Biotech Co. Ltd). Ninety-six-well plates were incubated with rHBsAg in PBS. The solid phase was incubated at 4 °C with HBsAg-specific antibody followed by incubation with horseradish peroxidase (HRP)-conjugated HBsAg or antibodies specific for mouse IgA. Serum from animals immunized with empty vector encapsulated into microparticles was used as the negative control (blanks). Binding of antibodies was measured as A₄₅₀ after reaction of immune complexes with substrate for HRP. Total antibody in serum was converted to mIU by comparison with a serially diluted standard antibody (Centers for Disease Control and Prevention China), while HBsAg-specific IgA titres were evaluated by A₄₅₀. Mean titres were calculated from at least three points of the linear portion of the titration curve.

Cell isolation. Mice were sacrificed 4 weeks post-immunization and spleens were removed aseptically. Splenocytes were purified on Ficoll-Paque cushions. Lymphocytes from gut-associated lymph tissue (GALT), including Peyer's patches and lamina propria, were dissociated into single cells by enzymic digestion as described by Polyanskaya *et al.* (2001). Briefly, the large and small intestines were

dissected from individual mice and the fecal material was flushed from the lumen. After the Peyer's patches were identified and removed from the intestinal wall, the intestines were opened longitudinally. Tissues were placed into 1 mM EDTA and incubated twice at 37 °C to remove the epithelial cell layer. To isolate lamina propria lymphocytes, tissues were cut into small pieces and incubated in medium containing 300 U collagenase type VIII ml⁻¹ (Sigma) for 50 min at 37 °C with stirring. Cells were carefully removed by discontinuous gradient centrifugation containing 75 and 40 % Percoll (Sigma).

RNA isolation and RT-PCR. Lymphocytes were isolated from Peyer's patches and lamina propria from six mice in each group at 24 h or 14 days after oral immunization with PLGA-encapsulated plasmid DNA at 200 µg per mouse. The muscle at the site of inoculation (tibialis anterior) and the draining lymph nodes (popliteal) were removed from six mice in each group at parallel time points after i.m. injection of naked pVAX(S) at the same dose. The muscle, lymph nodes or lymphocytes of six mice in each group were pooled for extraction of total cellular RNA. Total cellular RNA was isolated using TRIzol (Life Technologies) and further purified using RNase-free DNase (Qiagen). Single-stranded cDNA (1 µg) was primed with oligo(dT) and synthesized using the Promega reverse transcription system. Primers specific for the HBV HBsAg gene sequence were used to amplify a 675-bp product; mouse β -actin primers were used to amplify a 360-bp product which served as a positive control. RT-PCR products were analysed by electrophoresis on 1.5 % agarose gels containing ethidium bromide.

Confocal laser scanning microscopy. Lymphocytes were isolated and pooled from Peyer's patches of six mice in each group at 24 h or 14 days after oral immunization with PLGA-DNA at a dose of 200 µg per mouse. Lymphocytes were isolated and pooled from popliteal lymph nodes of six mice in each group at parallel time points after i.m. injection of naked DNA at the same dose. Surface staining was performed in freshly prepared FACS staining buffer (PBS, 0.5 % BSA) for 15 min on ice in the presence of saturating concentrations of PE-conjugated mAb against CD11c (Pharmingen) (2 µg in 100 µl). Subsequently, lymphocytes were washed and fixed for with 4 % formalin. For immunolabelling intracellular HBsAg, cells were stained with FITC-conjugated mAbs against HBsAg in freshly prepared permeabilization buffer (PBS, 0.2 % BSA, 0.2 % saponin) for 40 min. To block Fc γ receptor binding, samples were supplemented with 1 µg purified rat anti-mouse CD16/CD32 mAb (PharMingen).

Lymphocytes were smeared onto carrier sheet glass covered with a glass slide. Cells were viewed with a Zeiss LSM 510 confocal microscope equipped with LSM 510 software version 2.02 and Ar/Kr (458 and 488 nm) and He/Ne (543 nm) lasers. The lens used was a Plan-Neofluar oil lens.

Gamma interferon (IFN- γ) production assays. Splenocytes and GALT lymphocytes from eight mice in each group were tested for cytokine responses to purified recombinant HBsAg protein (10 µg ml⁻¹). The IFN- γ assay was described previously (Meseda *et al.*, 2002). Supernatants were harvested after 72 h, when peak amounts of the cytokine could be measured. Supernatants from at least three separate wells were pooled and assayed for the presence of IFN- γ by ELISA, using a commercial kit (Jingmei Biotech). A range of dilutions of purified recombinant mouse IFN- γ was included as standards.

In vitro cytotoxicity assays.

Immunomagnetic enrichment of CD8⁺ lymphocytes. Splenocytes and GALT lymphocytes from eight mice in each group were pooled and tested for the ability to generate CTLs at 4 weeks post-immunization. Immunomagnetic enrichment of CD8⁺ lymphocytes was performed by a magnetic-activated cell sorter (MACS; Miltenyi Biotec), using microbeads conjugated with rat anti-mouse CD8 α

mAb (Miltenyi Biotec) according to the manufacturer's instructions (<http://www.miltenyibiotec.com/>). The preparation yielded enriched CD8⁺ lymphocyte populations of approximately 95 %, as confirmed by FACS analysis.

HBsAg-expressing CT26 cells. CT26 cells, a murine colon carcinoma cell line (H-2d, BALB/c mice), were permanently transfected with plasmid pcDNA3-(S), comprising the paternal plasmid pcDNA3 (Invitrogen Life Technologies) and an inserted sequence encoding HBsAg, under continuous selective pressure with 400 µg G418 ml⁻¹ (Gibco-BRL). G418-resistant and HBsAg-expressing clones designated CT26/S were selected and screened for by PCR amplification of the integrated gene and expression of HBsAg by ELISA in our previous work (Zhou *et al.*, 2003).

CTL response assays. Stable transfectant CT26/S cells were used to raise CTLs by *in vitro* stimulation of splenocytes from immunized mice of each group for 7 days. CTL effector cells (2×10^5) were co-cultured with the 2×10^5 CT26 cells (transfected with the vector DNA without insert) or stable CT26/S transfectants as targets in 200 µl wells for 4 h. Specific cytolytic activity of CTLs was tested by a non-radioactive and colorimetric assay, an alternative to the ⁵¹Cr-release assay for the quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase activity released from the cytosol of damaged cells into the supernatant with the Cytotoxicity detection kit (Roche). The procedure is described in the manufacturer's protocol (<http://www.roche-applied-science.com/pack-insert/1644793a.pdf>). The percentage of lysis was calculated as described by Sharpe *et al.* (2003).

Statistical analysis. The Kruskal–Wallis non-parametric rank sum test and Wilcoxon's matched pairs test were used to test the significance of differences in humoral and cellular immune responses. Analysis of variance (ANOVA) was also performed. Data were analysed using SAS 8.2 (SAS Institution Inc.). A *P* value <0.05 was considered significant.

RESULTS

PLGA-DNA microparticles phagocytosed into mouse monocyte macrophage cell line RAW 264.7 expressed HBsAg *in vitro*

The physical state of encapsulated DNA was examined following release into solution. Gel electrophoresis revealed a high proportion of the supercoiled form. To determine whether microparticles could be phagocytosed and to investigate expression of the encapsulated DNA within the cells, microparticles were added to monolayers of RAW 264.7 cells. As shown in Fig. 1, the amount of phagocytosed PLGA-DNA microparticles in RAW 264.7 cells increased with the co-incubation time up to 8 h.

Following incubation for 72 h, HBsAg was detectable in RAW 264.7 cells containing phagocytosed PLGA-DNA microparticles by Western blot analysis (Fig. 2) and by ELISA with a mAb against HBsAg (data not shown).

PLGA-DNA microspheres prolonged the expression and presentation of target antigen in GALT from immunized mice

Because antigen presentation is required to elicit an immune response, it was necessary to determine whether

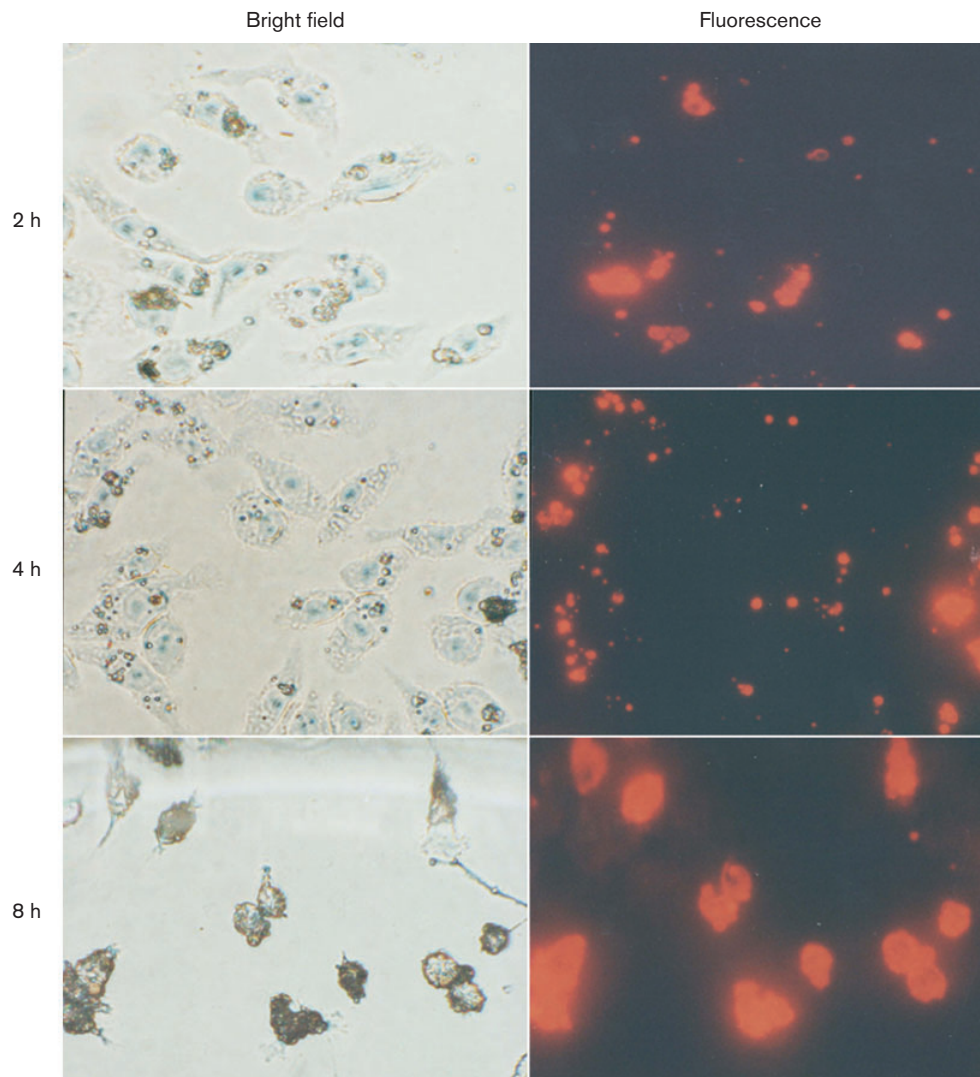


Fig. 1. RAW 264.7 cells phagocytose PLGA-encapsulated plasmid DNA microparticles. A 24-well plate was seeded with suspensions of the mouse monocyte macrophage cell line RAW 264.7 in DMEM containing 10% FCS and 0.1 mg kanamycin ml⁻¹ to give approximately 80% confluence after overnight incubation. Plasmid DNA-PLGA microparticles containing encapsulated rhodamine were prepared as described in Methods. Microparticles were suspended and added to monolayers with encapsulated DNA at a concentration of 20 µg per well. After different incubation times, wells were washed to remove excess microparticles and fresh growth medium was added. Phagocytosis was observed and photographed using a digital camera linked to a fluorescence microscope.

oral PLGA-encapsulated DNA microparticles direct DNA delivery to APCs *in vivo* by an RT-PCR detecting gene transcription in GALT (Peyer's patches and lamina propria). HBsAg-specific mRNA in the lymph nodes, or lymphocytes, as well as muscle at the site of inoculation, was analysed by RT-PCR in each group. As illustrated in Fig. 3, HBs mRNA expression was detected in both the Peyer's patches and lamina propria at 24 h and 14 days post-immunization with oral PLGA-DNA microparticles (200 µg per mouse). In contrast, mRNA expression was observed in the muscle and popliteal lymph node of mice immunized with naked DNA at 24 h, but not at day 14

post-immunization. The control group of mice inoculated with PLGA-pVAX1 microparticles, processed at the same time as PLGA-pVAX(S)-immunized animals, served as negative controls and exhibited no RT-PCR products, indicating that sample cross-contamination was unlikely. We also performed control PCR-only (no RT) reactions to confirm that HBsAg sequence detection depended on RT activity and was not the result of plasmid DNA contamination in the RNA preparation (data not shown).

We localized APCs involved in the expression and presentation of gene-encoding antigen in draining lymph nodes of

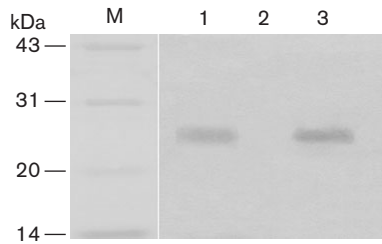


Fig. 2. Western blot analysis of phagocytosed microparticles expressing full-length HBsAg in RAW 264.7 cells. Suspended microparticles were added to monolayers of RAW 264.7 cells with encapsulated plasmid DNA pVAX(S) at a concentration of 20 μ g per well. After 6 h at 37 °C, cells were washed three times to remove excess microparticles and fresh growth medium was added. Following incubation for 72 h, cell monolayers were lysed and analysed by Western blotting using a mAb to HBsAg. Empty vector pVAX1 DNA encapsulated in microparticles served as a negative control. Lanes: 1, lysate of cells phagocytosing PLGA-pVAX(S) microparticles; 2, PLGA-pVAX1 microparticles; 3, purified recombinant HBsAg; M, markers.

six mice in each group with confocal laser scanning microscopy by double immunolabelling with PE-conjugated mAb against CD11c⁺ and FITC-conjugated mAb against HBsAg. Persistence of expressed and presented HBsAg was observed at superficial and intercellular sites of CD11c⁺ lymphocytes of Peyer's patches in mice immunized with PLGA-DNA microparticles at 24 h and 14 days post-immunization, while it was detected in CD11c⁺ lymphocytes from popliteal lymph nodes from the group of mice inoculated i.m. with naked DNA at 24 h, but not at day 14 post-inoculation (Fig. 4). There was also no detection in CD11c⁺ lymphocytes from Peyer's patches of mice at 24 h or 14 days after immunization with orally administered naked plasmid DNA at the same dose (data not shown). Our observations provided more direct evidence that sustained expression and presentation of target antigen took place in APCs from immunized animals, compared with other groups' efforts to localize APCs around the site where the encoded antigen protein was detectable after i.m. inoculation of DNA vaccine by immunohistochemical staining of CD11c⁺ dendritic cells and antigen protein (Oka *et al.*, 2001).

Taken together, the results suggested that plasmid DNA encapsulated into PLGA microparticles can be substantially distributed to the lymph nodes after oral administration, partly explaining the systemic and local immunogenicity of orally administered plasmid DNA vaccines. Our observation that sustained expression and presentation of target antigen correlated with the prolonged transcription of plasmid DNA in GALT 14 days post-immunization demonstrated that PLGA-DNA microparticles prolonged the expression and presentation of HBsAg in APCs with CD11⁺ phenotype in draining lymph nodes of immunized mice.

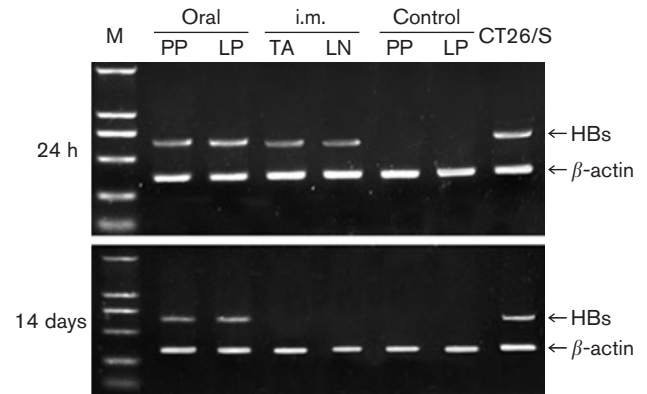


Fig. 3. RT-PCR detection of HBs mRNA in muscle and draining lymph nodes of mice immunized with PLGA-DNA microparticles. Lymphocytes were isolated from Peyer's patches (PP) and lamina propria (LP) from six mice in each group at 24 h or 14 days after oral immunization with PLGA-encapsulated plasmid DNA at 200 μ g per mouse. The muscle at the site of immunization, tibialis anterior (TA), and draining lymph nodes (LN) were removed from six mice in each group at parallel time points after i.m. injection of naked pVAX(S) at the same dose. Control mice received PLGA-pVAX1 microparticles orally. Muscle, lymph nodes or lymphocytes of mice from each group were pooled for extraction of total cellular RNA. HBsAg-specific mRNA in tissues or lymphocytes of each group was detected by RT-PCR using HBsAg-specific primers (top band). Total RNA extracted from CT26/S cells stably expressing HBsAg served as positive controls. Amplification with β -actin-specific primers was included as a quality control for cellular RNA (bottom band). Each lane is representative of results obtained from pooled tissues and cells of mice in each group. M, Markers.

Enhanced HBsAg-specific local and systemic humoral responses following oral immunization with PLGA-DNA

Because DNA vaccines have most frequently been delivered either by i.m. injection of naked DNA plasmid or by particle bombardment of skin tissue, we first compared levels of HBsAg-specific immune responses in mice immunized i.m. or orally with naked or PLGA-encapsulated plasmid DNA encoding HBsAg at 4, 8, 12, 16 and 20 weeks post-immunization. HBsAg-specific total antibody responses were detectable at different times after single oral immunization of PLGA-encapsulated DNA (20 or 200 μ g per mouse), with a peak response at week 12 (Fig. 5a). The formulation of oral PLGA-DNA (20 μ g per mouse) increased potency to levels almost equivalent to that induced by i.m. injection of naked DNA (200 μ g per mouse) ($P > 0.05$). A significantly higher level of specific antibody response was induced when the oral dose of PLGA-encapsulated DNA was increased to 200 μ g per mouse. The peak titre was approximately twice as high as that induced by i.m. injection of naked DNA at the same dosage ($P < 0.001$). Therefore, by two separate criteria,

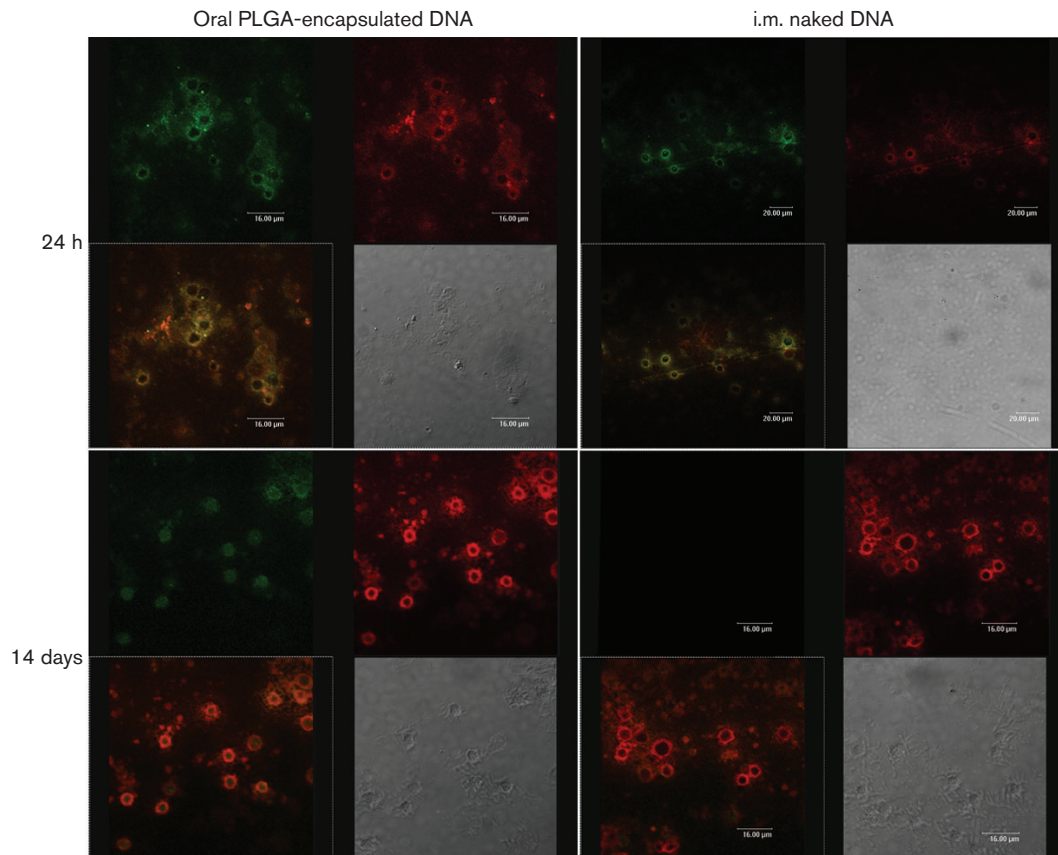


Fig. 4. Persistence of expressed and presented HBsAg in APCs with CD11⁺ phenotype from draining lymph nodes of immunized mice observed by confocal laser scanning microscopy. Lymphocytes were isolated and pooled from Peyer's patches of six mice in each group at 24 h or 14 days after oral immunization with PLGA-encapsulated plasmid DNA at 200 µg per mouse. Lymphocytes were isolated and pooled from popliteal lymph nodes of six mice in each group at parallel time points after i.m. injection of naked DNA at the same dose. Surface staining was performed in the presence of saturating concentrations of PE-conjugated mAb against CD11c (red). Subsequently, lymphocytes were fixed with 4% formalin and stained with FITC-conjugated mAb against HBsAg in permeabilization buffer to immunolabel membranous or intracellular HBsAg presented or expressed (green). Lymphocytes were smeared onto carrier sheet glass and viewed by confocal laser scanning microscopy.

increase in titre and decrease in DNA dose, PLGA formulation of DNA greatly increased HBV HBsAg DNA vaccine potency.

In subsequent studies, we analysed induction of intestinal HBsAg-specific IgA responses by immunization with naked or PLGA-encapsulated DNA. As shown in Fig. 5(b), significant production of fecal IgA was detected in mice vaccinated by PLGA-pVAX(S) at 4, 8, 12 and 16 weeks, consistent with the antigen expressed in GALT described above, suggesting that a mucosal antibody response had been induced. In contrast, mucosal IgA responses were not detected in stools of mice injected i.m. with the naked DNA. Finally, no total antibody or IgA response was observed when PLGA-pVAX1 microparticles were used. Thus, oral immunizations with PLGA-DNA expressing HBsAg induced specific local and systemic humoral responses.

Enhanced HBsAg-specific local and systemic IFN- γ production following oral immunization with PLGA-DNA

It is accepted that strong antibody responses are important for clearing HBV and protection against HBV infection. There is also increasing evidence to show that the Th1 cell, CTL response to HBV and the associated antiviral cytokines (IFN- γ , TNF- α , IL2) developed may play a key role in virus resolution during natural infection (Trepo *et al.*, 2003; Rico *et al.*, 2001). The ability of PLGA microspheres containing HBsAg-encoding plasmid DNA to facilitate cellular uptake by the intestinal epithelium and APCs prompted us to analyse whether this delivery system induces cellular immunity in local and systemic lymphoid tissues. The production of IFN- γ released from splenocytes and GALT in immunized mice after restimulation with purified rHBsAg *in vitro* was measured. Results representative of

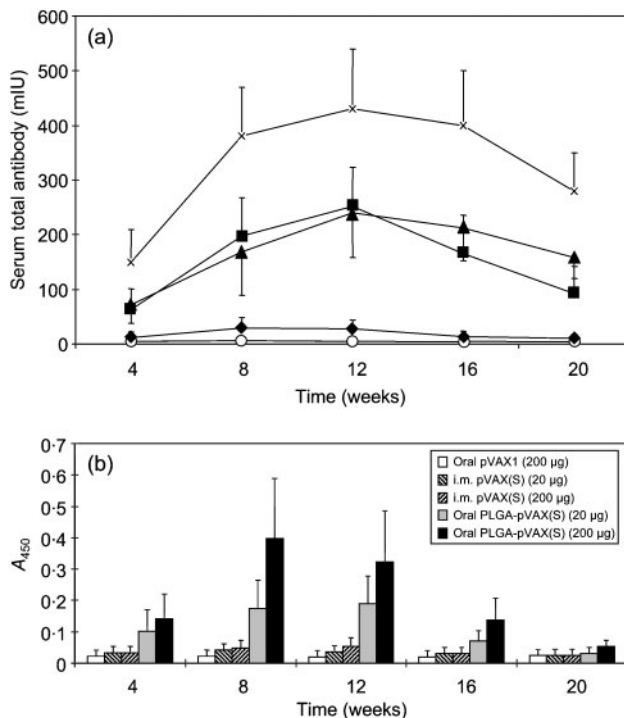


Fig. 5. Induction of HBsAg-specific total antibody (a) and IgA (b) in serum and fecal washes of BALB/c mice immunized i.m. with naked DNA or by oral administration of PLGA-encapsulated plasmid DNA encoding HBsAg. Single-dose oral administration was performed with plasmid DNA formulated on PLGA-encapsulated microparticles at 20 (a, $\blacktriangle$) or 200 (a, $\times$) µg per mouse. Naked plasmid DNA [20 (a, $\blacklozenge$) or 200 (a, $\blacksquare$) µg per mouse] was inoculated i.m. via the tibialis anterior. Control mice received pVAX1 formulated on PLGA-encapsulated microparticles (a, $\circ$). At the indicated times post-immunization, HBsAg-specific total antibody and IgA elicited were analysed in serum and fecal washes from individual mice in each group. Titres were determined as outlined in Methods. Data represent mean $\pm$ SD concentrations per group and per time point from one representative experiment of three performed, where $n=18$.

three separate experiments are shown in Fig. 6. A significant trend towards enhanced IFN- γ responses was observed in groups of mice immunized with pVAX(S) encapsulated in microspheres (20 or 200 µg per mouse), compared with the response elicited by i.m. naked pVAX(S) injection at the same dose (Fig. 6). The amount of IFN- γ produced by splenocytes from mice immunized with encapsulated pVAX(S) (200 µg per mouse) [PLGA-pVAX(S), 1980 pg per 10^6 splenocytes] was approximately twice that produced by cells from mice immunized with the same amount of naked pVAX(S) ($P<0.001$).

Consistent with the mucosal IgA response, induction of IFN- γ responses to HBsAg was detected only in GALT derived from mice immunized orally with PLGA-DNA encoding HBsAg. In contrast, the production of IFN- γ in

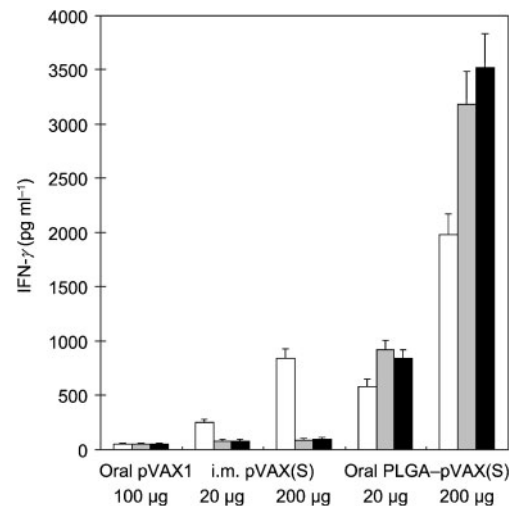


Fig. 6. HBsAg-specific IFN- γ production in splenocytes and lymphocytes from Peyer's patches and lamina propria. Splenocytes (open bars) and lymphocytes isolated from Peyer's patches (filled bars) and lamina propria (shaded bars) were obtained at week 4 after oral immunization with PLGA-encapsulated plasmid DNA encoding HBsAg at two different doses (20 or 200 µg per mouse) or after i.m. inoculation of naked plasmid DNA (20 or 200 µg per mouse). Pooled splenocytes or lymphocytes of each group were stimulated with purified rHBsAg (10 µg ml $^{-1}$). Supernatants were harvested after 72 h and assayed for production of IFN- γ by ELISA. Values are from a representative experiment of three performed and are presented as mean $\pm$ SD concentrations of triplicate wells, where $n=8$.

HBsAg-stimulated cultures established from lamina propria and Peyer's patches of mice immunized i.m. with naked plasmid DNA was at a background level (Fig. 6). Cultures tested positive for IFN- γ only when the mice had been immunized and the cultured cells were restimulated with HBsAg. Non-stimulated cells did not secrete IFN- γ (data not shown).

PLGA-DNA microspheres facilitated HBsAg-specific local and systemic CTL responses

Since previous studies demonstrated that intragastric administration of plasmid encapsulated in PLGA microparticles could prime specific CTLs (Kaneko *et al.*, 2000; Sharpe *et al.*, 2003; Herrmann *et al.*, 1999), we analysed HBsAg-specific CTL responses of CD8 $^{+}$ lymphocytes isolated from spleen, lamina propria and Peyer's patches of mice in each group by immunomagnetic enrichment after oral and i.m. immunizations. Stable transfectant CT26/S cells expressing HBsAg were used as target cells to raise CTLs by *in vitro* stimulation of splenocytes or lymphocytes from immunized mice of each group for 7 days. No HBsAg-specific CTL activities of splenocytes were detectable at an effector:target cell ratio (E:T) of 100:1 in mice after i.m. immunization of naked DNA at a dose of 20 µg per mouse. A CTL response of splenocytes

was detectable with a low specific lysis of 24 % at the same ratio in mice after a single i.m. inoculation of naked DNA at 200 µg per mouse, almost equivalent to that elicited in mice immunized by single-dose oral administration of PLGA-DNA (20 µg per mouse). Increased-dose oral administration of PLGA-DNA (200 µg per mouse) markedly enhanced the CTL response of splenocytes, with 43 % specific lysis at the same E:T ratio, which was approximately twice that elicited by immunization with naked DNA at the same dose ($P<0.05$) (Fig. 7).

Mucosal CTL responses induced by orally delivered PLGA-DNA microparticles (200 µg per mouse) were significantly stronger than those measured in cultures established from lamina propria and Peyer's patches after i.m. immunization with naked DNA at the same dose, which was at a background level ($P<0.001$) (Fig. 7).

The CTL activity was HBsAg specific, since mice immunized with PLGA-pVAX1 did not induce any detectable

HBsAg-specific lysis and no CTL activity was observed in any of the groups of mice when parental CT26 cells were used as target cells (data not shown). Results obtained from three similar experiments were comparable.

DISCUSSION

The mucosa of the gastrointestinal tract, some 400 m² of surface area in adulthood (Miller *et al.*, 1993), is rich in lymphocytes. It has been regarded as an ideal route of immunization. However, orally administered naked DNA has little immunogenicity because of its easy decomposition during passage through the acidic environment of the stomach, so additional encapsulation would be necessary for oral administration. Several commercially available oral vaccines are based on live-attenuated organisms, such as vaccines against poliovirus, *Vibrio cholerae* and *Salmonella typhi*. Recently, mice immunized with attenuated *Salmonella typhimurium* delivering plasmid DNA encoding HBsAg by the oral route showed significantly stronger CTL responses than those receiving recombinant HBsAg vaccination, while comparable to i.m. immunization with naked DNA (Woo *et al.*, 2001). Nonetheless, the advantages of avoiding an antivector immune response may outweigh the increased efficiency of vector delivery.

In the current studies, we demonstrated that plasmid DNA encoding HBsAg encapsulated into PLGA microparticles induced local and systemic HBsAg-specific immunity following a single dose of oral immunization, combining the advantages of DNA vaccination with the ease of administration by the oral route and concomitant induction of mucosal immune responses. It was shown that the PLGA-DNA microparticle formulation not only augmented the immune responses, but also reduced the dose required for comparable responses to plasmid DNA in saline.

Our observations at day 14 post-administration of encapsulated DNA clearly showed that the sustained expression and presentation of target antigen correlated with our demonstration by RT-PCR that HBsAg mRNA was detectable in the draining lymph node under conditions where injection of naked DNA does not give detectable signal, providing direct evidence of immunopotentiality. Because extracellular DNA is rapidly degraded and cleared within hours after immunization (Singh *et al.*, 2001; Lew *et al.*, 1995), the antigen-specific immune response to naked DNA may be dependent on the APCs present at the site of immunization. It seems reasonable to hypothesize that PLGA-DNA microparticles may promote immunity by acting as a means of prolonging the release of the plasmid DNA encoding the target antigen and preventing its degradation through encapsulation into a complex structure of polymeric material (Singh *et al.*, 2004; Hedley, 2003). The next investigation was to analyse quantitatively the proportion of APCs involved in expression and presentation of target antigen among total APCs in draining lymph nodes from immunized animals by flow-cytometry

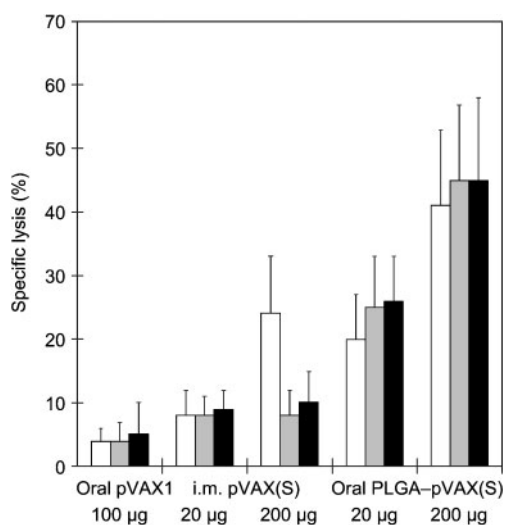


Fig. 7. HBsAg-specific CTL response in splenocytes and lymphocytes from Peyer's patches and lamina propria. Splenocytes (open bars) and lymphocytes isolated from Peyer's patches (filled bars) and lamina propria (shaded bars) were obtained at week 4 after immunization with oral PLGA-encapsulated plasmid DNA encoding HBsAg at two different doses (20 or 200 µg per mouse) or by i.m. inoculation of naked plasmid DNA (20 or 200 µg per mouse). Immunomagnetic enrichment of CD8⁺ lymphocytes from pooled splenocytes and lymphocytes of each group was performed with microbeads conjugated with rat anti-mouse CD8 mAb. Responder cells (2.0×10^7) were restimulated *in vitro* with mitomycin C-treated (25 mg ml^{-1}) CT26/S cells (1.0×10^6) stably expressing HBsAg at 37 °C. After a 1-week *in vitro* culture, the specific cytolytic activity was tested in a non-radioactive and colorimetric assay against target cells. The E:T ratio was 100:1 in all of these assays. Values for specific lysis are from a representative experiment of three performed and are presented as mean $\pm$ SD specific lysis of triplicate cultures, where $n=8$.

detection using double immunolabelling with Abs against an APC marker and target antigen, providing direct evidence that PLGA-DNA microparticles directed delivery to APCs and facilitated uptake into APCs, reflecting the two advantages of the microparticle formulation: controlled release and target delivery (Singh *et al.*, 2004; Hedley, 2003).

Vaccination at mucosal surfaces may result in humoral and cellular responses, both systemic and local, the latter not only at the site of vaccination, but also at distant mucosal epithelia. This is a particularly important observation in relation to local and systemic HBsAg-specific immune responses including antibody and CTL responses, since it has previously been reported that oral immunization with a human papillomavirus 16 virus-like-particle vaccine (Nardelli-Haeffliger *et al.*, 2003) or live-attenuated typhoid vaccine Ty 21a (Kutteh *et al.*, 2001) have been shown to be well tolerated, immunogenic and protective in women, inducing potent immune responses in the genital tract and other mucosa. Importantly, it has been reported that sexual transmission is one of the main routes of HBV infection, and a quarter of sexually transmitted HIV patients were infected simultaneously with HBV (Trepo *et al.*, 2003; Kura *et al.*, 1998). Antibody and CTL responses in the genital mucosa play major roles in protection against sexual transmission of HIV-1 in humans (Garulli *et al.*, 2004). Subsequent studies using PLGA microparticles with encapsulated DNA will directly assess the ability of mucosal immunization to induce CTL responses in the genital mucosa. In this respect, the current observations of CTL responses in GALT following oral immunization with PLGA-DNA are very encouraging.

It was notable that the HBsAg-specific CTL activities elicited in our present study by a single immunization of PLGA-DNA microparticles were relatively low. It is unlikely that the current formulation is optimal. Therefore, further studies will be necessary to determine the effect of multiple doses and the kinetics of DNA delivery for an optimal vaccination protocol. New approaches, in particular promising prime-boost strategies of consecutive DNA priming followed by boosting with purified proteins, could be useful to improve the immunogenicity of these PLGA-DNA-based vaccines, as reported by Sharpe *et al.* (2003).

In summary, this study describes the potential of oral DNA vaccination for induction of mucosal and systemic HBV HBsAg-specific cellular and humoral responses. The results may facilitate the development of a new type of protective HBV vaccine in humans.

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