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ORIGINAL ARTICLE

# Nanogram Doses of Alum-Adjuvanted HBs Antigen Induce Humoral Immune Response in Mice When Orally Administered

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**Abstract** Mucosal immunity elicited by plant-based and other orally administered vaccines can serve as the first line of defense against most pathogens infecting through mucosal surfaces, but it is also considered for systemic immunity against blood–borne diseases such as hepatitis B (HB). Previous oral immunization trials based on multiple administration of high doses of HBs antigen elicited an immune response; however, a reproducible and long-lasting immunization protocol was difficult to design. The objective of this study was to evaluate the effect of dose and timing of orally delivered alum-adsorbed antigen on the magnitude of the anti-HBs humoral response. Mice were immunized orally by gavage intubation or parenterally by intramuscular injection three times, once every 2 weeks, with doses of 5, 50, or 500 ng alum-adjuvanted HBsAg. A low dose (10 ng) of HBsAg was orally administered three times in different time intervals: 2, 4, 6, and 8 weeks. The three consecutive 5-ng oral doses of the antigen induced immune response at the protective level ($\geq$10 mIU/ml), significantly higher than the reaction elicited by three 50 or 500 ng doses. In contrast, intramuscular delivery of these doses did not differ significantly; however, they induced a five to six times higher immune response than oral immunization. The 8-week period between each of the three oral immunizations appeared to be favorable to the anti-HBs humoral responses compared with the shorter schedules. The results presented here clearly identify the importance of low doses of antigen administered orally in extended intervals for a significantly higher anti-HBs response. This finding provides some indications concerning the strategy of orally administered vaccines, including plant-based ones.

**Keywords** HBs antigen · HBsAg · Oral immunization · Anti-HBV oral vaccine

**Abbreviations**

| | |
|---|---|
| HBs, HBsAg | Hepatitis B surface antigen |
| HB | Hepatitis B |
| HBV | Hepatitis B virus |
| mIU/ml | Milli-international unit/ml–unit of anti-HBs antibody titer |
| S-IgA | Secretory IgA |
| GALT | Gut-associated lymphoid tissue |
| VLPs | Virus-like particles |
| APCs | Antigen-presenting cells |
| CT-B | Cholera toxin subunit B |
| LT-B | Heat-labile enterotoxin B |
| PBS | Phosphate-buffered saline |

## Introduction

It is currently a high priority worldwide to find an efficacious, cost-effective, and reliable method of mass immunization against hepatitis B (HB) and many other fatal diseases afflicting underdeveloped regions of the

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globe. The basic reason for this need stems from the fact that the number of chronic hepatitis B virus (HBV) carriers worldwide reached 350 million in 2000 (Kao and Chen 2002) and it is growing, mainly in developing countries. The disease has been spreading despite the fact that one of the most effective recombined vaccines has been available for 25 years and the unit price of HB vaccination has dropped to as little as $0.30 (Goldstein and Fiore 2001). Insufficient healthcare infrastructure in the poorest regions is, among other factors, a limitation which causes failure of mass HB vaccination attempts. Thus, economically feasible and easily implementable vaccination programs are desirable.

The mucosal immunization avenue meets current expectations for simplified procedures in regions with poor medical infrastructure (Azis et al. 2007; Mestecky et al. 2008). The first oral vaccine administered on a mass scale that proved to be very efficacious was attenuated polio virus against poliomyelitis (Koprowski et al. 1952), bringing preventive medicine closer to complete viral eradication (Kew et al. 2005). Despite increasing needs, only a few nasally or orally administered vaccines, for example against polio, cholera, and typhoid, based on purified and attenuated viruses have been approved for use (Brandtzaeg 2007). The development of alternative oral vaccines, including plant-based formulations, is a major research goal since they would not require advanced purification technologies and advanced medical equipment for their administration.

Obvious questions arise whether orally administered vaccines can be equally protective against diseases contracted by mucosal infection or non-mucosally transmitted diseases (Wang and Coppel 2008), such as HB (Kao and Chen 2002). However, numerous successful approaches to the construction of an oral vaccine against HB shows that the notion is not an unrealistic dream. Live attenuated prototype oral vaccines based on recombined vaccinia virus and adenovirus expressing the HBV surface antigen (HBs antigen, HBsAg) have proven to be effective in chimpanzee tests (Lubeck et al. 1989; Moss et al. 1984). An orally administered purified HBs antigen carried in stabilized vesicles (Borges et al. 2007; Shukla et al. 2008) as well as HBs-coding DNA immunization (McCluskie et al. 1999; Perrie et al. 2002) also stimulated an anti-HBs response. Many attempts have also been made in the last two decades to develop plant-based vaccines against HBV. Orally administered plant-associated HBsAg was shown to elicit a humoral response in mice (Joung et al. 2004; Kapusta et al. 1999; Kong et al. 2001; Richter et al. 2000) and humans (Kapusta et al. 1999; Kapusta et al. 2001; Mason et al. 2003; Thanavala et al. 2005). Studies on prototype oral vaccines were predominantly based on multiple administration of relatively high, i.e. microgram, doses of HBs antigen, usually supplemented with the cholera toxin subunit B (CT-B) or other adjuvants, as well as intestinal immunization combined with parenteral delivery of alum-adjuvanted HBsAg. However, despite some promising successes, reproducible and long-lasting oral immunization proved to be extremely difficult to establish.

We present here a novel approach to oral immunization showing, based on alum-adsorbed HBs antigen, that a very low, i.e. several nanogram, dose and extended prime/boost immunization timing are crucial for a significant and nominally protective serum and mucosal antibody response.

## Materials and Methods

### Mice Immunization

Mice immunization studies were conducted with approval No. 35/2004 of the Local Bioethical Commission at the Medical Academy, Warsaw. Female BALB/c mice aged 6–8 weeks were immunized with alum-adsorbed recombinant S antigen of HBV (HBsAg) from the commercial vaccine Engerix B® (GlaxoSmithKline, Belgium). In the first experiment, five mice per group were primed and boosted two times, once every 2 weeks, with 5, 50, or 500 ng of HBsAg per dose (Fig. 1a). The antigen was diluted in 50 µl of phosphate-buffered saline (PBS) and injected intramuscularly or suspended in 100 µl of PBS and administered orally by gavage intubation. In the second experiment, five mice per group were primed orally and boosted two times with 10 ng of HBsAg in 100 µl of PBS. The intervals between immunizations were 2, 4, 6, or 8 weeks. Control mice were administered PBS every 5 weeks (Fig. 2a).

### Sample Collection and ELISA Assays

Blood samples and feces were collected from the mice 5 days before immunization (pre-immune) and 10 days after each immunization (Figs. 1a, 2a). Blood samples were centrifuged at 3,000 rpm and 4°C for 10 min and sera were collected. Antibodies were extracted from the feces by suspending the fecal samples in 5 v (w/v) of PBS, incubating on ice for 15 min, grinding and incubating on ice for 10 min, shaking and incubating on ice for 15 min, and final shaking and centrifugation at 14,000 rpm and 4°C for 10 min. Total anti-HBs responses in the sera were assayed twice using a Hepanostika® kit (Organon Teknika, Boxtel, the Netherlands) and the antibody titers were calculated in mIU/ml using a standard serum provided in the kit. Secretory IgA (S-IgA) from feces and serum IgA were

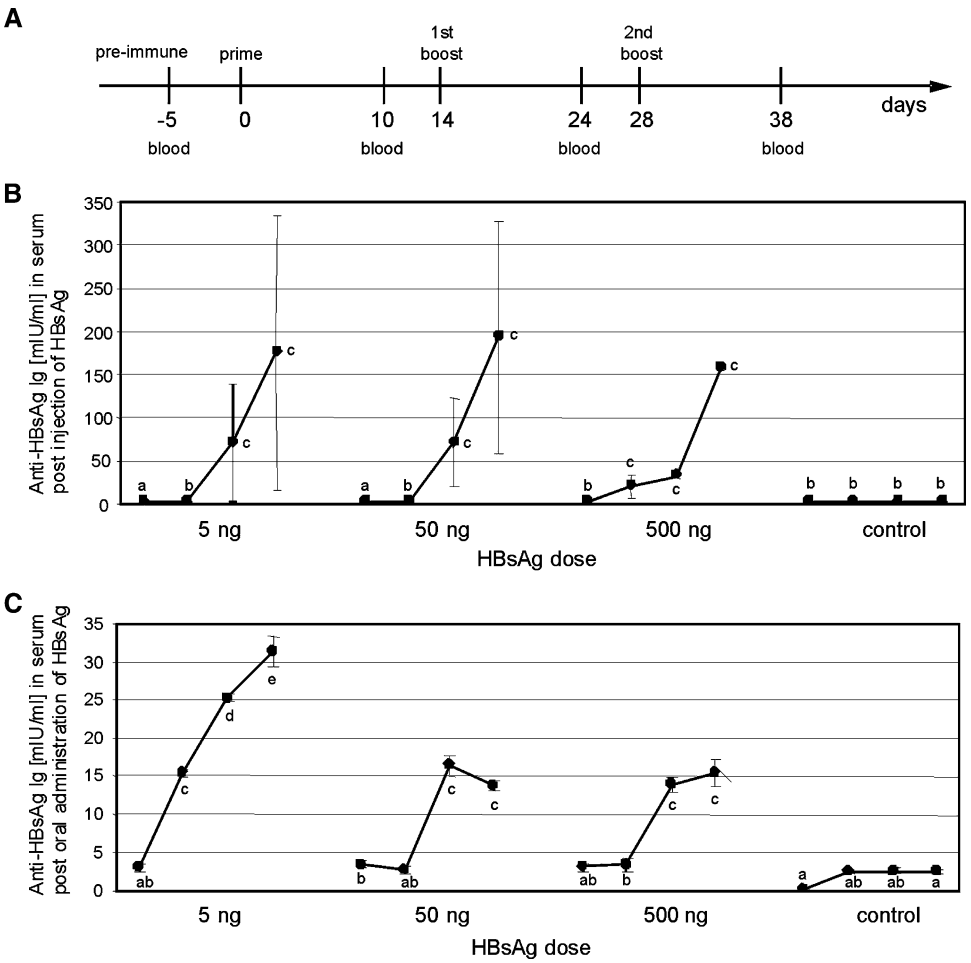


**Fig. 1** Anti-HBs response elicited in serum by immunization of BALB/c mice with 5, 50, or 500 ng of HBsAg. **a** Time course of HBsAg delivery every 2 weeks (prime on day 0, first boost day 14, second boost day 28) and blood sample collection at pre-immune date (day −5) and 10 days after each immunization (days 10, 24, and 38). **b** Immune response induced by parenterally administered HBsAg. **c** Immune response induced by orally administered HBsAg. Antibody titers are expressed in mIU/ml as arithmetic means and standard deviations (SD) of results obtained from five mice at the pre-immune date and 10 days after each immunization. One-way analysis of variance with repeated measurements was made and homogenous groups were obtained by multiple comparisons within and between the experimental groups using the Student–Newman–Keuls test for $p < 0.05$ (Statistica 6®). Statistically homogenous groups within an experiment (**b** or **c**) with (non)significant differences are marked by the *same* or *different letter* indexes. The scale of the *Y*-axis is different for the individual graphs to illustrate the course of antibody response in the particular immunization variants

monitored using a microplate ELISA. Microtiter PolySorp (NUNC, Denmark) plates were coated with 1 μg/ml of HBsAg (Biodesign, USA) in PBS (pH 7.4), incubated overnight at 4°C, then washed three times with PBS-Tween® 20 (0.05% v/v, PBST), blocked for 1 h at 37°C with 5% (w/v) fat-free milk in PBS, followed by washing with PBST. Samples of the feces extract or serum were serially diluted 1:10, 1:20, 1:40, and 1:80 with PBS and incubated for 1 h at room temperature. The plates were washed with PBST and incubated for 1 h at room temperature with 1:500 diluted polyclonal goat anti-mouse IgA conjugated with horseradish peroxidase (Sigma, USA), followed by washing with PBST. TMB liquid substrate (Sigma, USA) was added and the development of the reaction at room temperature and dark incubation was stopped after 30 min with 1 N sulfuric acid. The absorbance optical density at 450 nm ($OD_{450}$) was measured and the arithmetic means of the $OD_{450}$ values for the sample dilutions of 1:10, 1:20, and 1:40 (multiplied by the dilution factor) were calculated as the relative titer of the sample's IgA.

## Statistical Analysis

Titers of antibodies induced in the consecutive immunizations were calculated as arithmetic means with standard deviations (SD) of data obtained from the five mice of the same experimental group. One-way analysis of variance for repeated measurements was conducted. Homogenous groups were obtained by multiple comparisons within a

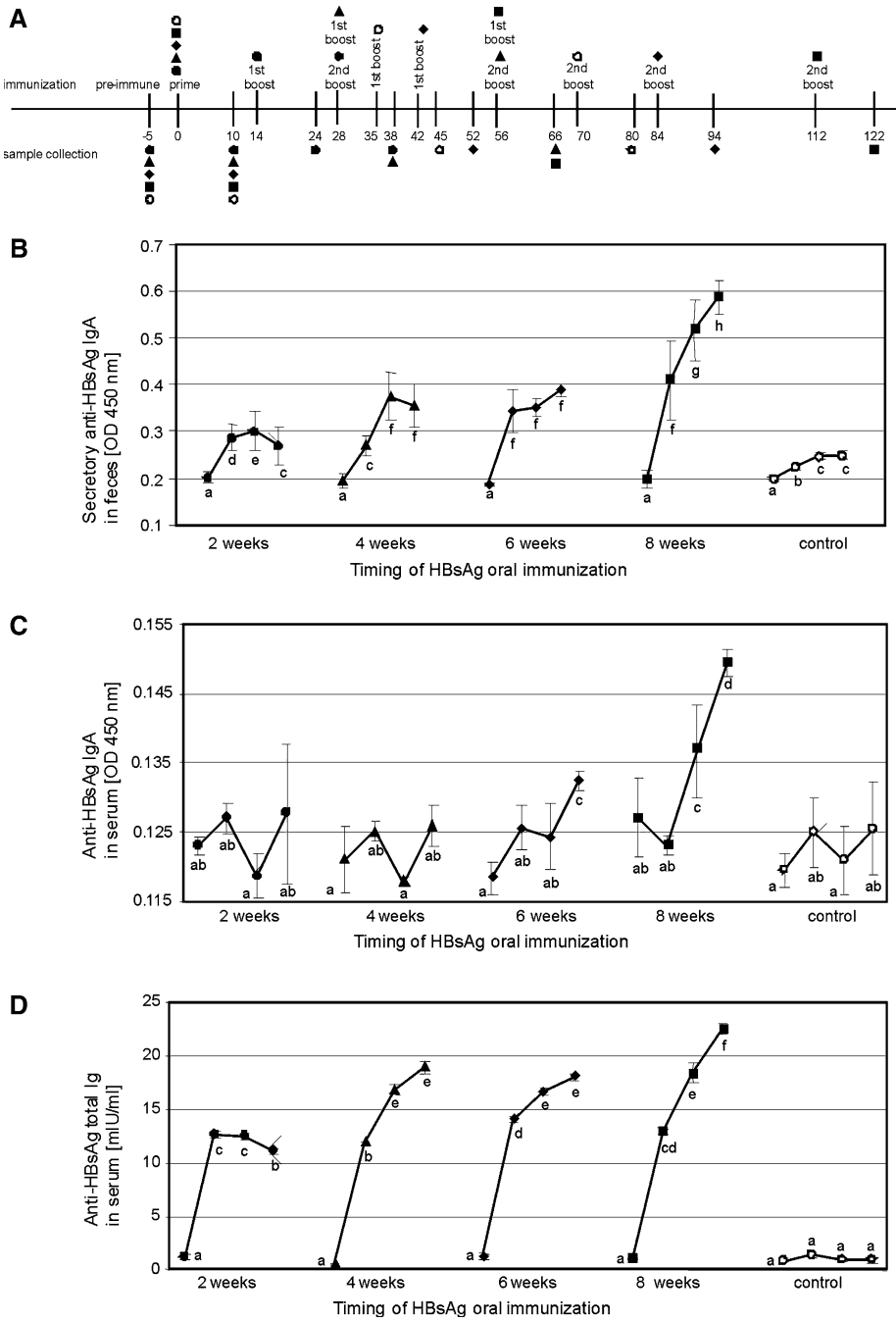


**Fig. 2** Anti-HBs immune response elicited by oral immunization of BALB/c mice with 10 ng of HBsAg administered with diverse timing. **a** Time course of HBsAg delivery (prime on day 0 for all schedules, *filled circle* 2 weeks: days 14 and 28, *filled triangle* 4 weeks: days 28 and 56, *filled diamond* 6 weeks: days 42 and 84, *filled square* 8 weeks: days 56 and 112, *open circle* control 5 weeks: days 35 and 70) and blood and feces sample collection at pre-immune date (day −5) and 10 days after each immunization (day 10 for the prime of all schedules, days 24 and 38 for 2-week boosts, days 38 and 66 for 4-week boosts, days 52 and 94 for 6-week boosts, days 66 and 122 for 8-week boosts, days 45 and 80 for control); **b** response of secretory IgAs in intestine; **c** response of serum IgAs; **d** response of total serum antibodies. Antibody titers are expressed as the OD at 450 nm (**b**, **c**) or in mIU/ml (**d**) as arithmetic means and standard deviations (SD) of results obtained from five mice at pre-immune and 10 days after each immunization. The titer of S-IgAs or serum IgAs for an individual mouse was calculated as the arithmetic mean of the $OD_{450}$ values for sample dilutions 1:10, 1:20, and 1:40 (multiplied by the dilution factor). One-way analysis of variance with repeated measurements was made and homogenous groups were obtained by multiple comparisons within and between the experimental groups using the Student–Newman–Keuls test for $p < 0.05$ (Statistica 6®). Statistically homogenous groups for the Ig class (**b**, **c**, or **d**) with (non)significant differences are marked by the *same* or *different letter* indexes. The scale of the *Y*-axis is different for the individual graphs to illustrate the course of antibody response in the particular immunization variants

particular group and between different treatment groups using the Student–Newman–Keuls test. Differences were considered significant with $p < 0.05$. The statistical analysis was conducted using Statistica 6® software (StatSoft, USA).

## Results

The immunological responses of the BALB/c mice to the different doses of alum-adjuvanted HBsAg delivered with the same time intervals (Fig. 1a) showed profiles that were specific to the administration pathway, i.e. oral or parenteral. The levels of anti-HBs antibodies induced by injected HBsAg exceeded 150 mIU/ml and were at least five to six times higher than those observed after oral administration of the antigen. However, differences in individual reactions, expressed as the *SD* of the means, were observed (Fig. 1b). Mice responded equally to the highest dose of HBsAg (500 ng), while individual responses stimulated by the lower antigen doses of 50 ng and, particularly, 5 ng were distinctly diverse; for instance, some mice reacted prominently to the third antigen dose (287 mIU/ml), while others responded weakly (10–12 mIU/ml). However, the mean antibody levels were similar regardless of the total administered dose (15, 150, or 1,500 ng). Orally administered HBsAg induced a markedly lower immune response than the injected antigen; however, insignificant differences in the individual immune responses of the mice were observed as well as an opposite trend in the immune level in the aftermath of the antigen dosage (Fig. 1c). The lowest antigen dose (5 ng) stimulated the most intensive and rapid reaction. The concentration of anti-HBs antibodies increased gradually and reached the maximum (31.3 mIU/ml) after the second boost. Responses to the 50- and 500-ng doses of HBsAg were significant in comparison with those of the control or pre-immunized mice, however they were significantly lower (maxima: 16.5 and 15.4 mIU/ml) than to the 5-ng dose at both prime and boosts. The negative effect of higher HBsAg doses was particularly marked after the second boost, as an insignificant increase or even a decrease in antibody level was observed.

The timing (Fig. 2a) of the oral immunizations significantly affected antibody production, both in the intestinal mucosa and in the serum (Fig. 2b–d). The levels of S-IgA and serum IgA antibodies are expressed as $OD_{450}$ since appropriate tests calibrated in IU are not available for these particular antibody isotypes. However, a comparison of the antibody level curves following immunization facilitated an evaluation of local and systemic responses (Fig. 2b, d). For all the investigated timings, differences in anti-HBs antibody titer were insignificant among the particular experimental groups. The concentrations of anti-HBs S-IgA antibodies increased significantly compared with the control and pre-immune levels for the 4-, 6-, or 8-week and transiently for the 2-week intervals between immunizations (Fig. 2b), but the final levels of S-IgA varied with the timing schedule of antigen administration. The highest increase in S-IgA titer was observed for the 8-week interval between immunizations compared with the shorter timings. The levels of S-IgA production for the 4- or 6-week immunization intervals were comparable and significantly less efficient than the 8-week immunization, but more efficient than immunization every 2 weeks (Fig. 2b). A trend of increasing induction of serum IgA following extended immunization intervals was observed, although not as evidently as for S-IgA, and the induction of IgAs in the serum was most probably considerably lower than in the mucosa (Fig. 2c). No significant anti-HBs serum IgA response was elicited after immunization performed at 2- or 4-week intervals. The levels of IgA antibodies in the serum increased significantly with longer breaks between immunizations, after the second boost for the 6-week interval and after the first and second boosts for the 8-week interval (Fig. 2c). The trends in total systemic response were similar to those observed for the mucosal response of S-IgA (Fig. 2d). The titers of serum antibodies induced by 10 ng of HBsAg (12.6–22.4 mIU/ml) significantly increased compared with the control and pre-immune levels for all the tested immunization intervals (Fig. 2d); however, it was lower than the response induced by 5 ng of the antigen (31.3 mIU/ml). Shorter breaks elicited anti-HBs serum antibodies at a lower level, and for the 2-week timing some decrease in antibody level was even observed after the second boost. Significantly, the most efficient induction of immune response was observed for the longest, 8-week, interval, compared with both the pre-immune and the prime value as well as with other timings of immunization. Generally, longer intervals between immunizations progressively elicited more efficient anti-HBsAg immune responses, as shown by the levels of mucosal S-IgA and serum antibodies. The observed maximum titers of serum antibodies (31.3 and 22.4 mIU/ml, Figs. 1c, 2d) elicited by orally administered alum-adsorbed HBsAg were several times lower than those induced by the injected antigen (Fig. 1b). However, these concentrations were above 10 mIU/ml, the accepted cut-off value for predicting protection in humans.

## Discussion

The development of vaccines acting through mucosal surfaces, mainly oral, is considered to be an approach for the mass prevention of HB and other diseases in the growing human population. Oral vaccines, preferably plant-derived

ones, although generally less effective than parenteral ones and still under development, are postulated as supplements to injected vaccines since they would not require complex medical infrastructure and equipment. Moreover, in contrast to vaccines based on attenuated pathogens, for example polio vaccine (Koprowski et al. 1952), oral subunit vaccines such as HB and some others containing only particular antigen(s) are supposed to be safer and thus possible to control and administer with a partially reduced medical staff. However, vaccines targeted to gut-associated lymphoid tissue (GALT) encounter two main problems, i.e. antigen stability through the alimentary tract and oral tolerance, which should be taken into account in the development of appropriate antigen formulations and immunization protocols with plant-based (Kirk et al. 2005; Koprowski 2002) and other orally delivered vaccines (Mestecky et al. 2007; Poonam 2007).

The aim of this study was to investigate the dose of HBs antigen applied orally, and, as a reference, intramuscularly, for a significant induction of immunity by prime/boost immunization and a subsequent nominally protective level of anti-HBs antibodies. The second aim of the study was to determine the timing of oral prime and boost immunizations. Studies of these parameters affecting humoral response were initiated before further detailed research on the antigen formulation and oral immunization protocol using plant-derived material. We decided to examine the anti-HBs antibody levels elicited by the recombined HBs antigen (Engerix B®, GlaxoSmithKline) because humoral response plays an essential role in the protection against HBV and the induction of a high level of anti-HBs antibodies is the general strategy in the design of anti-HBV vaccines (Brocke et al. 2005; Hollinger 1996).

The presented results demonstrate that low dosage and extended timing are essential for efficacious oral vaccination with HBsAg and these parameters affect parenteral and oral immunization differently (Figs. 1, 2). As may have been expected, injected HBsAg induced a several times higher immune response than orally delivered antigen; however, injected low antigen doses (5 ng) elicited distinct individual reactions (Fig. 1b). The presented data are consistent with the common knowledge that the antigen dose positively correlates with the humoral immune response in the case of HBs parenteral immunization (Brocke et al. 2005; Hollinger 1996) as $CD4^+$ lymphocytes are stimulated by almost permanent interaction with the antigen-presenting cells (APCs) bearing antigen (Obst et al. 2005). A possible explanation for the high variability in the individual responses is connected with the observation that low doses of an antigen delivered intramuscularly might be randomly distributed throughout the muscular tissue, with a varying degree of accessibility to APCs and B and T cells (Zinkernagel 2000). Therefore the low antigen dose appeared to be ultimately sufficient, on average, to elicit an immune response, but the reactions might be case dependent (Fig. 1b), while the low dosage of orally delivered antigen induced uniform responses (Fig. 1c). Thus the significant variability in the individual reactions to injected low antigen doses would undermine a possible theoretical comparison of equivalence between oral and parenteral low-dose antigen delivery, apart from the fact that the immunization mechanisms are different for parenteral and mucosal antigen administration. Although the mean level of response following antigen injection was considerably superior to that of oral delivery, it was shown that HBsAg administered orally at a low dose and extended timing elicited a significant response and finally reached a titer of >10 mIU/ml of anti-HBs serum antibodies (Figs. 1c, 2d) considered to be the minimal protective response (International Group 1988). The trend of increasing immune response following a reduction of antigen dose observed in the first experiment was verified in the second, in which 10 ng of HBsAg elicited a response of 12.6–22.4 mIU/ml compared with 31.3 mIU/ml induced by 5 ng of HBsAg. The induction of relatively equal immune responses by nanogram HBsAg doses probably resulted from the fact that GALT creates a tuned immune milieu to which even traces of foreign antigen have access (Mestecky et al. 2007), which makes it possible to mount a strong humoral mucosal immune response (Brandtzaeg 2003) and immunity may be efficiently spread from the local mucosa to the periphery (Mowat 2003).

Normally regulated GALT discriminates pathogens from harmless food and commensal antigens, sustaining, respectively, protective immunity and suppressive oral tolerance (Pamer 2007). In general, soluble monomeric antigens are labile and induce tolerance (Richman et al. 1978). In reverse, HBsAg and other antigens self-assembled into relatively durable virus-like particles (VLPs), constitute effective immunogens, also when delivered to intestinal mucosa (Agnello et al. 2006; Borges et al. 2007; Li et al. 2001; Shukla et al. 2008). Moreover, some VLPs administered through mucosa induce a T cell independent B cell response (Yao et al. 2004), similarly to VLPs in periphery (Bachmann and Zinkernagel 1996). Another, synergistic, mechanism which might be activated by the oral delivery of HBsAg VLPs is called the “danger signal”, which alerts the immune system (Matzinger 1994) through pathways common to the majority of viral infections. Thus, HBsAg assembled into highly immunogenic but safe VLPs (McAleer et al. 1984), is a suitable candidate for an anti-HBV oral vaccine and it is also proposed as a carrier for heterologous epitopes (Boisgerault et al. 2002). Depending on the dosage of a particulated antigen, as HBsAg, and the duration of its exposure to APCs, a local mucosal response may develop into an adaptive systemic immune response

and induce memory cells or may lead to unresponsiveness, including oral tolerance to an antigen (Garside and Mowat 2001; Mowat 2003; Strobel 2001). The mechanisms of the mucosal immune response are still under study, but it is known that high doses of antigen and/or its repeated frequent exposure may induce oral tolerance by anergy, clonal deletion, Treg-dependent suppression, or other mechanisms (Chen et al. 1995; Friedman and Weiner 1994; Heath and Carbone 2001; Taams et al. 1998).

Based on the above-mentioned facts, it could be hypothesized that alum-adsorbed HBsAg was taken up in the intestine (Mestecky et al. 2007) and then activated mucosal immune response and then systemic response. The results also allow us to assume, very carefully, that the oral immunization schedule based on antigen delivery at a low dosage and extended timing is sufficient, or might even be advisable, for the induction of an immune response at the protection level and that at the same time it may probably reduce the potential risk of acquired tolerance.

Nanogram HBsAg doses administered with an extended timing appeared to be comparable or equivalent in eliciting an immune response to multiple delivery of tens or hundreds of micrograms of HBsAg (Borges et al. 2007; Joung et al. 2004; Kong et al. 2001; Mason et al. 2003; Richter et al. 2000; Shukla et al. 2008; Thanavala et al. 2005) or other antigens, for example heat-labile enterotoxin B (LT-B) of *Escherichia coli* (Mason et al. 1998; Tacket et al. 2004), capsid protein of the Norwalk virus (Ball et al. 1999; Huang et al. 2005), CT-B of *Vibrio cholerae* (Jiang et al. 2007), the rabies virus (Yusibov et al. 2002), and rotavirus (Choi et al. 2002). High doses of antigens absorbed by GALT probably initiate an initial response, although it is possible that repeatedly exposed antigens could be tolerated (Swarbrick et al. 1979). Indeed, frequent and/or abundant antigen oral delivery is explored for this very reason as a method of inducing suppression of humoral immune response and consequently hyporesponsiveness in the therapy of chronic HB and hepatocellular carcinoma (Gotsman et al. 2000; Gotsman et al. 2002), or desensitization in allergic gastrointestinal diseases (Huibregtse et al. 2007; Wu et al. 2007).

The immune response observed after oral immunization with low doses (5–10 ng) of alum-adjuvanted HBs antigen was several times lower, although still at the nominally protective level, than those obtained in studies applying tens of micrograms of the antigen. The high dose of orally applied HBsAg elicited humoral responses, albeit only when the plant-associated antigen was administered along with the mucosal adjuvant CT-B and/or one of the immunizations, prime or boost, was parenteral using alum-adsorbed HBsAg (Joung et al. 2004; Kong et al. 2001; Mason et al. 2003; Richter et al. 2000; Thanavala et al. 2005). However, the delivery presented here of a low HBsAg dose induced an equally efficient or slightly higher immune response, both mucosal and systemic, than that elicited by orally/parenterally delivered micrograms of alum-adsorbed HBsAg, but unadjuvanted with CT-B (Kong et al. 2001) or intragastrically gavaged micrograms of the encapsulated antigen adjuvanted with synthetic CpG oligonucleotides (Borges et al. 2007; Shukla et al. 2008).

A positive activity of alum oxide particles as an adjuvant or carrier for oral immunization with HBs antigen is likely since alum-adsorbed HBsAg might accumulate more effectively in GALT than the encapsulated antigen (Borges et al. 2007; Shukla et al. 2008). However, some data indicate that this adjuvant is rather one of several factors affecting oral immunization efficiency. Data on the immune response induced by plant tissue expressing HBsAg suggest that exogenous adjuvants are not indispensable for immunization (Kapusta et al. 1999). Endogenous DNA particles containing immunostimulatory unmethylated CpGs motifs and other plant components might possibly act as some adjuvants (Wang et al. 2002). The precise impact of alum oxide on HBsAg oral immunization efficacy remains unknown and requires further analyses, including comparisons with unadjuvanted HBsAg. Exploitation of alum oxide as a mucosal adjuvant for human oral vaccination would be as difficult to approve as applying CT-B or LT-B due to their certain, possibly unsafe activities. The data presented here indicate, however, that for oral vaccines, mainly plant-derived ones, other mucosal immunostimulators may be essential and need to be studied, although the immunization schedule affects immune response at least to a comparable extent.

Some earlier reports on oral immunization confirm the positive effect of a lowered antigen dosage and extended timing on the adaptive immune response. The impact of a low dose (5 ng/mouse; Fig. 1c) and longer intervals between antigen administration on immunogenicity presented here (Fig. 2) is consistent with the previous observation that the humoral response was higher and reached the $\geq$10 mIU/ml protective level in human adult volunteers fed on days 1 and 60 (Kapusta et al. 1999) than in those fed on days 1, 7, and 30 (Kapusta et al. 2001) with 1–2 μg/person plant-associated HBsAg. No humoral response was observed when mice were fed on seven consecutive days with plant-based HBsAg in a total dose of 1 μg (data not shown), which also suggests that multiple and high-dose antigen oral administration is conducive to tolerance rather than immunity. The importance of low dosage and extended timing for oral immunization could also be confirmed for CT-B or LT-B which, despite being soluble proteins and untypical mucosal antigens, are absorbed by M cells thanks to their ganglioside-binding properties and then elicit an immune response in the intestinal mucosa (Holmgren et al. 1994; Rask et al. 2000). Some boost effect was observed when CT-B was delivered

as late as 40 days post priming (Arakawa et al. 1998). In the case of LT-B, increasing the dose, i.e. from 3.3 to 33 μg, did not significantly affect mucosal and systemic responses in mice (Lamphear et al. 2002). In another study, orally administered LT-B turned out to be immunogenic at low doses, i.e. 20 or 200 ng (Beyer et al. 2007). An impact of dosage and timing on the direction of the mucosal response was also found for ovalbumin, for which IgG response in mice was enhanced by low-dose feeding, while suppression was induced by high-dose early oral boosting (Franco et al. 1998; Peng et al. 1989).

Some results of nasal immunization also corroborate the finding of low-dose-dependent oral immunization efficiency, as the mechanisms of the immune response are analogous for different mucosal surfaces. For instance, low and high antigen doses administered to the nasal mucosa triggered comparable immune reactions to those observed for recombined *Salmonella typhimurium* expressing HBV nucleocapsid (Nardelli-Haefliger et al. 2001) or inactivated rabies virus (Yoneda et al. 2008). Recent findings confirm that low antigen doses of influenza, i.e. 40 ng, supplemented with the adjuvant ISCOMATRIX™ and delivered to the pulmonary mucosa induced serum antibody levels equivalent to a dozen or so micrograms of the antigen delivered parenterally (Wee et al. 2008).

Parameters for oral vaccination such as dosage, timing, adjuvant, antigen formulation, and immunization route will have to be accurately calibrated for any oral vaccine. Oral immunization with HBsAg requires further detailed studies on humoral and cellular response, possible tolerance induction, and the like. However, the presented results of oral immunization with alum-adjuvanted HBsAg, being consistent with some other data, support the roles of dosage and timing in the immune response and show increased antigen immunogenicity, facilitating the induction of an anti-HBs response at the protective level. This study of low-dose oral immunization provides some insight for research on oral immunization using plant-based or other recombined vaccines.

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