ORIGINAL ARTICLE

Nanostructured SBA-15 silica as an adjuvant in immunizations with hepatitis B vaccine

Utilização da sílica nanoestruturada SBA-15 como adjuvante em imunizações com a vacina para hepatite B

Karina Scaramuzzi[1], Denise Cristina André Oliveira[2], Luciana Vieira Carvalho[1], Denise Vilarinho Tambourgi[1], Elisabeth Christina Nunes Tenório[2], Marisa Rizzi[3], Juliana Mussalem[3], Márcia Carvalho de Abreu Fantini[4], Viviane Fongaro Botosso[2], Osvaldo Augusto Sant´Anna[1]

ABSTRACT

Objective: To evaluate the applicability of SBA-15 silica as an adjuvant in immunizations with purified particles of the viral protein HBsAg, the main component of hepatitis B vaccine, Butang®, produced by Instituto Butantan. **Methods:** BALB/c mice orally or subcutaneously received 0.5 μg of HBsAg adsorbed/encapsulated to SBA-15 or adsorbed to $Al(OH)_3$. To assess the secondary immune response, a subcutaneous booster was administered 30 days after the first immunization. Individual serum and fecal samples of each group were periodically collected for specific antibody titration by ELISA. **Results:** Analysis of secretory IgA showed that mice orally primed with HBsAg on SBA-15 had increased levels of specific antibodies in primary and secondary immune responses. Specific serum IgA and IgG titers in HBsAg:SBA-15-orally immunized mice reached higher levels after the booster, demonstrating the effectiveness of oral vaccination with the use of silica. All immunized groups showed higher IgG1 levels. **Conclusion:** Our results clearly indicate the promising use of SBA-15 as an adjuvant, especially in oral immunizations.

Keywords: Hepatitis B; Oral vaccination; Adjuvants; Immunological memory

RESUMO

Objetivo: Demonstrar a aplicabilidade da sílica do tipo SBA-15 como adjuvante nas imunizações com a proteína recombinante HBsAg do vírus da hepatite B, principal componente da vacina Butang® produzida pelo Instituto Butantan. **Métodos:** Camundongos BALB/c receberam, pela via oral ou subcutânea, 0,5 μg do HbsAg adsorvido/encapsulado à SBA-15 ou adsorvido ao $Al(OH)_3$. Para avaliar a resposta imune secundária, uma dose de reforço foi administrada subcutaneamente 30 dias após a primeira imunização. Amostras individuais de soro e fezes foram coletadas periodicamente para titulação de anticorpos específicos por ELISA. **Resultados:** A análise de IgA secretada mostrou que camundongos imunizados pela via oral com HbsAg em SBA-15 apresentaram aumento nos níveis de anticorpos específicos nas respostas primária e secundária. Ainda, após o reforço, observaram-se maiores níveis de IgA e IgG séricas anti-HBsAg no grupo preparado com HBsAg:SBA-15 pela via oral. Todos os grupos imunizados apresentaram maior produção de IgG1. **Conclusão:** Os resultados indicam o usopromissor da sílica SBA-15 como adjuvante, especialmente nas imunizações pela via oral.

Descritores: Hepatite B; Vacinação oral; Adjuvantes; Memória imunológica

INTRODUCTION

Despite the availability of efficient prophylactic vaccines, human hepatitis-B virus (HBV) infection and its associated diseases still are a major public health problem. The World Health Organization (WHO) estimates that around 2 billion people worldwide show evidence of a former or current infection with HBV, and more than 350 million people are chronically HBV-infected. It is estimated that 1 to 1.5 million deaths per year occur due to HBV-related liver diseases, like cirrhosis and hepatic cancer[1-5].

Study carried out at the Immunochemistry Laboratory of Instituto Butantan – São Paulo (SP), Brazil.

[1] Immunochemistry Laboratory, Instituto Butantan, São Paulo (SP), Brazil.

[2] Hepatitis Division, Instituto Butantan – São Paulo (SP), Brazil.

[3] Farmacêutica Cristália, São Paulo (SP), Brazil.

[4] Department of Applied Physics, Universidade de São Paulo – USP, São Paulo (SP), Brazil.

Corresponding author: Osvaldo Augusto Sant´Anna – Immunochemistry Laboratory, Butantan Institute – São Paulo (SP), Brazil – Tel.: 11 3726-7222 – extension: 2001 – Email: gbrazil@usp.br

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Conflict of interest: none

HBV is a small enveloped DNA virus that belongs to the *Hepadnaviridae* family. The infectious particle contains an icosahedral nucleocapsid encompassing a circular DNA enclosed by a lipid bilayer envelope in which the proteins large (L), middle (M) and small (S) are inserted. In addition to the contagious virus particles, cells infected by HBV produce large quantities of noninfectious subviral particles of spherical or filamentous forms of 22 nm in diameter[6,7].

Prevention by vaccination is a unique and effective strategy to avoid disease. Several vaccine manufacturers have used recombinant DNA technology to express the S surface protein of HBV(HBsAg) in yeasts. In 1996, Butantan Institute initiated production of the recombinant vaccine for hepatitis B that also contains highly purified particles of the S recombinant protein adsorbed to aluminum hydroxide ($Al(OH)_3$). This vaccine, Butang®, has the same efficacy and safety as the imported vaccines and also employs genetic engineering techniques using *Hansenulla polymorpha* yeasts as biological vectors. This system enhanced protein expression four to ten times and strongly diminished the final costs of vaccine production[8].

Although considered efficient and harmless, the adjuvant in the Butang® preparation, $Al(OH)_3$ can lead to side effects like exacerbated local inflammation, granuloma, and even necrosis. Furthermore, alum predominantly elicits a T_H2 immune response polarization, which is less effective against viral infections[9,10].

Thus, there is a growing interest in investigating new immunization strategies and, especially, the development of adjuvants that do not interfere in the polarization of immune response, besides being secure, economically viable, chemically stable, and capable of positively modulating the immune response of poor responder individuals such as the elderly and the immunosuppressed[9,10].

Since the natural route of most infections is through the mucosa, and mucosal immunization is the most effective means of mimicking the induction of natural protection, it is important to highlight all the benefits of needle-free immunizations. Oral vaccines are easily administered and the side effects are minimal. Unfortunately, due to the harsh gastric environment, the development of local and systemic immunological response is impaired[11-16].

SBA-15 particles have a highly organized structure and, due to their physicochemical properties, they also have a great potential for application in different areas. These materials are able to interact with atoms, ions, and molecules, not only on the surface but also inside nanopores of approximately 10 nm in diameter[17-19]. This silica is synthesized in acidic medium and has a structure with remarkable thermal, hydrothermal, and mechanical stability (Figure 1). Recently, our group demonstrated the adjuvant potential of SBA-15 and currently several promising studies are underway at the Immunochemistry Laboratory of the Butantan Institute[20,21].

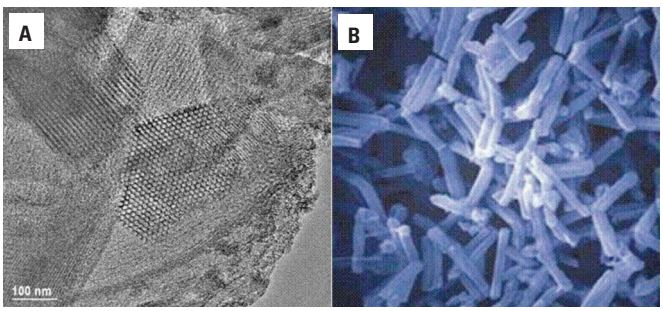

Figure 1. Mesoporous nanostructured SBA-15. (A) Transmission Electron Microscopy (TEM) of SBA-15 silica, highlighting its hexagonal ordered porous structure with mean pore diameter of around 10 nm. (B) Scanning Electron Microscopy (SEM) picture of SBA-15 particles of around 30 μm in diameter, showing their macroporous morphology

OBJECTIVE

To evaluate the applicability of SBA-15 silica as an adjuvant in immunizations with purified particles of the viral protein, HBsAg, the main component of the hepatitis B vaccine, Butang® produced by Butantan Institute.

METHODS

Animals

Female 8 to 12 week-old isogenic BALB/c mice, supplied by Butantan Institute, were maintained at the animal facilities of the Immunochemistry Laboratory, under ethical conditions according to the international rules of animal care by the International Animal Welfare Recommendations[22]. All animal experiments were approved by the Animal Use and Care Ethics Committees from the Institute of Biomedical Science of the *Universidade de São Paulo* and from the *Instituto Butantan.*

SBA-15 silica synthesis

SBA-15 samples were synthesized by using a poly-(ethylene oxide)-poly-(propylene oxide)-poly-(ethylene oxide) triblock copolymer (Pluronic P123, $EO_{20}PO_{70}EO_{20}$, M_{av} = 5800 - BASF Chemical Co., Mount Olive, NJ, USA) acting as a micellar template. The source of silica for polymerization was the tetraethyl orthosilicate (TEOS), acquired

from Fluka/Sigma Chemical Co. (Milwaukee, WI, USA). Hydrochloric acid was purchased from Fisher Scientific Co. (Pittsburgh, PA, USA). The synthesis and adsorption characterization of samples were performed as described by Matos et al.[23].

Mice immunization

BALB/c mice (n= 5/group) received, by subcutaneous (s.c) injection or gavage, 0.5 μg of the HBsAg protein adsorbed or not to SBA-15 in a final volume of 0.25 mL PBS. HBsAg were mixed at a ratio of 1:10 antigen: SBA-15, v/v (0.5 μg and 5 μg, respectively). The mixtures were kept at rest at 4°C for 24 hours before immunizations. As controls, we evaluated specific antibody levels in non-immunized animals. Mice were also immunized s.c. with 0.5 μg of the HBsAg adsorbed to 6.25 μg $Al(OH)_3$ in a final volume of 0.25 mL for further comparative analysis. To assess the secondary immune response, all experimental groups received by the s.c. route a second dose of antigen adsorbed or not to SBA-15 or $Al(OH)_3$, 30 days after the first dose.

Individual blood samples from the retro-orbital venous plexus and fecal samples were periodically collected for specific antibody titer detection and posterior quantification by Enzyme-Linked Immunosorbent Assay (ELISA).

Fecal pellet extract collection

Fresh fecal pellets from orally immunized BALB/c mice were collected and weighed. Five milliliters of an inhibitory solution (phenylmethylsulphonyl fluoride - PMSF) 1mM was added, BSA 1% in PBS per 1 g of fecal pellet. After 15 minutes, the material was vigorously vortexed and samples were centrifuged at 20,000 $\bar{x}$ g for 10 minutes. The supernatants were removed and stored at -80°C until the ELISA assay.

ELISA for anti-HBsAg antibodies

Microplates with high binding properties (Maxisorp Nunc International, Rochester, NY, USA) were coated with 1.5 μg/mL of HBsAg diluted in carbonate-bicarbonate buffer pH 9.6 and incubated for 1 hour at 37°C and then moved to 4°C for 18 hours. Next, the plates were washed with PBS containing 0.05% Tween (PBS-T) and then blocked with 0.5% gelatin in PBS for 2 hours at 37°C. The washing procedure was repeated. Samples were distributed, serially diluted in PBS-T/gelatin 0.5%, and incubated for 1 hour at 37°C to quantify IgG and its isotypes and for 18 hours to serum and secreted IgA. Microplates were submitted to another washing procedure and 100 μL of anti-IgG (diluted 1: 2000), anti-IgG1 and anti-IgG2a (diluted 1: 1000), or anti-IgA (diluted 1: 500) labeled with peroxidase (Promega Co., Madison, WI, USA) were added. The plates were washed and incubated in the dark at room temperature with a buffer substrate (20 mg OPD diluted in 40 mL citrate-phosphate buffer pH 5.0; H_2O_2 at 0.3%).The reaction was stopped with 50 μL/well of citric acid 0.2 M and the absorbance was measured at 450 nm using the ELISA reader (Multiskan - Labsystems, Helsinki, Finland). Titers were calculated neglecting 20% of saturation dilution absorbance and expressed as $\log_2$.

Statistical analysis

Results were expressed as mean values ± standard deviation. Statistical significance was determined by the unpaired *t*-test and set at $p < 0.05$ using GraphPad Prism 4.0 software (La Jolla, CA, USA).

RESULTS

Anti-HBsAg specific antibody response

Subcutaneous immunization

For the evaluation of specific IgG and its isotypes, blood samples were periodically collected for antibody titration. After the first dose, 7, 14, and 30 days, no serum antibody levels were detected in any of the experimental groups. IgG levels, after the booster, measured 7, 14, and 30 days post-second dose were higher in both SBA-15 (5.2 $\log_2$, 7.8 $\log_2$and 9 $\log_2$) and $Al(OH)_3$ groups (11 $\log_2$,11 $\log_2$ and 9.6 $\log_2$). A significant difference was detected in IgG titers between those groups at day 7 post-booster ($p < 0.05$), showing a better humoral response after s.c. immunization with $Al(OH)_3$ (Figure 2A). Therefore, it is patent that both adjuvants led to seroconversion.

Specific IgG subclasses responses were evaluated in both s.c. immunized groups after the second dose at days 7, 14, and 30 post-immunizations. Anti-HBsAg IgG1 titers were similarly high in both HBsAg:$Al(OH)_3$ (10.2 $\log_2$, 12.5 $\log_2$ and 12 $\log_2$) and HBsAg:SBA-15 groups (7.4 $\log_2$, 10.4 $\log_2$ and 9 $\log_2$) (Figure 2B). However, at day 7 post-immunization, a significant difference was observed between the groups ($p < 0.05$), clearly indicating that animals that received the formulation with $Al(OH)_3$ presented with higher levels of IgG1 (Figure 2B).

Specific IgG2a titers, detected 7, 14, and 30 days post-booster, were 8 $\log_2$, 8.5 $\log_2$ and 7.8 $\log_2$, respectively,in the $Al(OH)_3$ group and 5.8 $\log_2$, 6.6 $\log_2$, 6.8 $\log_2$ in the silica group (Figure 2C).

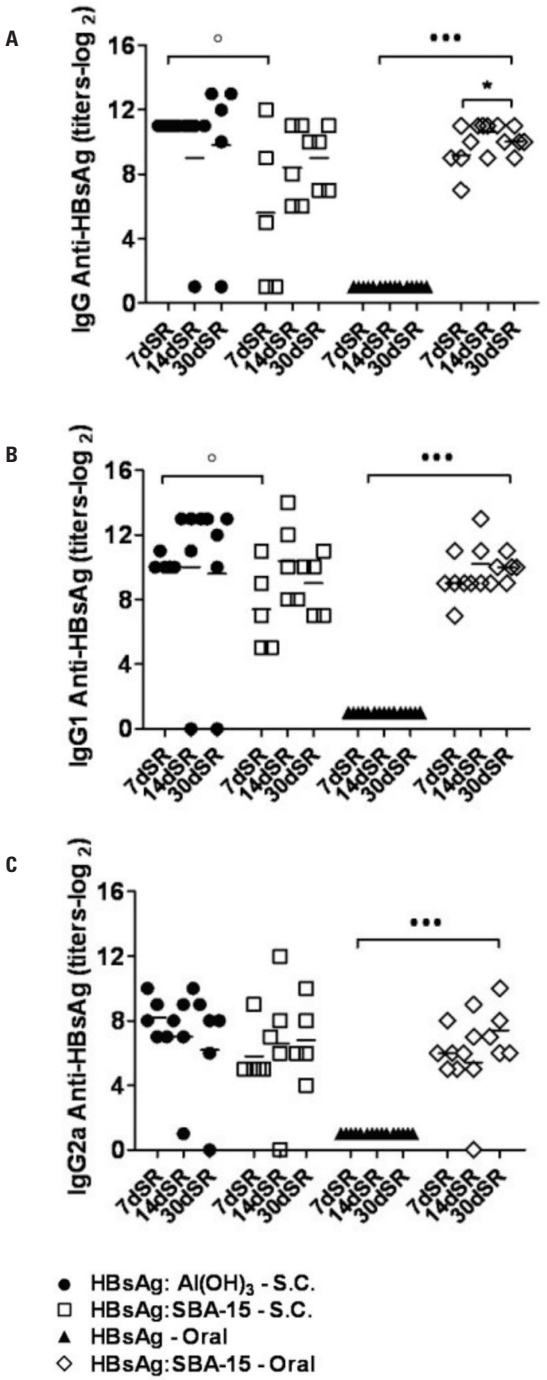

Figure 2. Antibody production by orally or subcutaneously immunized BALB/c mice. (A) Serum IgG, (B) IgG1, (C) IgG2a anti-HBsAg levels (n = 5 animals/group). Antibody titers measured by ELISA 7, 14 and 30 days after booster, administrated subcutaneously 30 days after first immunization. Group of animals immunized with rHBsAg was used as reference for the unpaired Student *t* test analysis, *p < 0.05; ••• p < 0.001. Antibodies titers at days 7, 14 and 30 after booster were also used as reference for the unpaired Student *t* test analysis, ° p < 0.05

Animals immunized with $Al(OH)_3$ showed higher titers of anti-HBsAg IgG1 in comparison to IgG2a, with statistical difference ($p < 0.05$), demonstrating a slightly predominance of a T_H2 response (Figure 3).

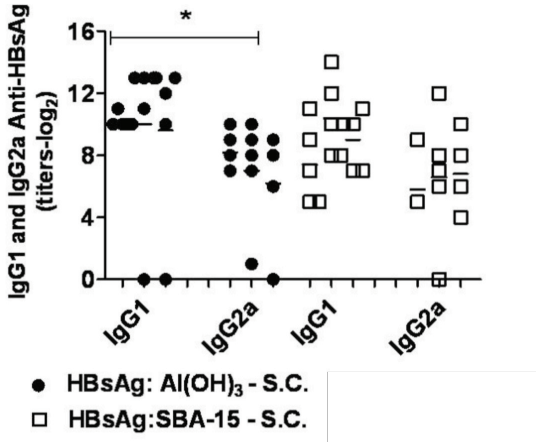

Figure 3. IgG1 and IgG2a antibody production by subcutaneously immunized BALB/c mice. Serum IgG1and IgG2a anti-HBsAg antibody levels (n = 5 animals/group). Antibody titers measured by ELISA 7, 14 and 30 days after booster, administrated subcutaneously 30 days after first immunization. IgG1 and IgG2a responses between the groups were used as reference for the unpaired Student *t* test analysis, *p < 0.05

Oral immunization

To determine the use of SBA-15 as an oral adjuvant, BALB/c mice were immunized with HBsAg in PBS or HBsAg adsorbed to silica. After the first administration, no specific serum antibodies titers (IgG, IgG1, IgG2a and IgA) were detected in either group.

The sera collected 7, 14, and 30 days after second s.c. immunization were evaluated for specific IgG and its isotype responses. Mice immunized only with HBsAg did not show seroconversion (Figure 2A).

On the other hand, IgG response in the group HBsAg:SBA-15 was higher: 9.2 $\log_2$,10.6 $\log_2$ and10 $\log_2$ at days 7, 14, and 30 after booster, showing a statistical difference 14 days after thesecond dose, compared to the first 7 days ($p < 0.001$) (Figure 2A). Anti-HBsAg IgG1 titers were 9 $\log_2$,10.2 $\log_2$,10$\log_2$; while IgG2a titers were 6 $\log_2$, 6.2 $\log_2$, 7.4 $\log_2$ (Figure 4) in those animals immunized with silica. Mice immunized without SBA-15 did not respond. After the first oral immunization with HBsAg:SBA-15, there was apredominance of a T_H2 response, with higher production of IgG1 (9.7 $\log_2$) instead of IgG2a (6.4 $\log_2$) ($p < 0.005$) (Figure 4).

Specific serum IgA titers were only detected in the group HBsAg:SBA-15, 10 days after the s.c. booster (4.5 $\log_2$) (Figure 5A).

Analysis of secretory IgA (s-IgA) showed that antibody titers of the group orally immunized with HBsAg:SBA-15 were higher than the s-IgA levels of the group immunized without silica. Ten days after the first immunization, s-IgA titers were 3 $\log_2$ in HBsAg:SBA-15 group and remained stable after the s.c. booster (Figure 5B).

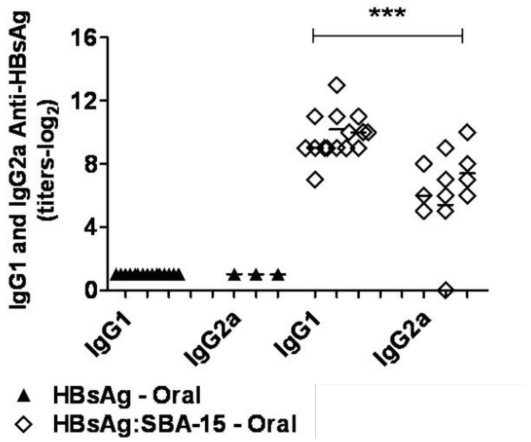

Figure 4. IgG1 and IgG2a antibody production by orally immunized BALB/c mice. Serum IgG1and IgG2a anti-HBsAg antibody levels (n = 5 animals/group). Antibody titers measured by ELISA 7, 14 and 30 days after booster, administrated subcutaneously 30 days after first immunization. IgG1 and IgG2a response between the groups were used as reference for the unpaired Student *t* test analysis, *** $p < 0.05$

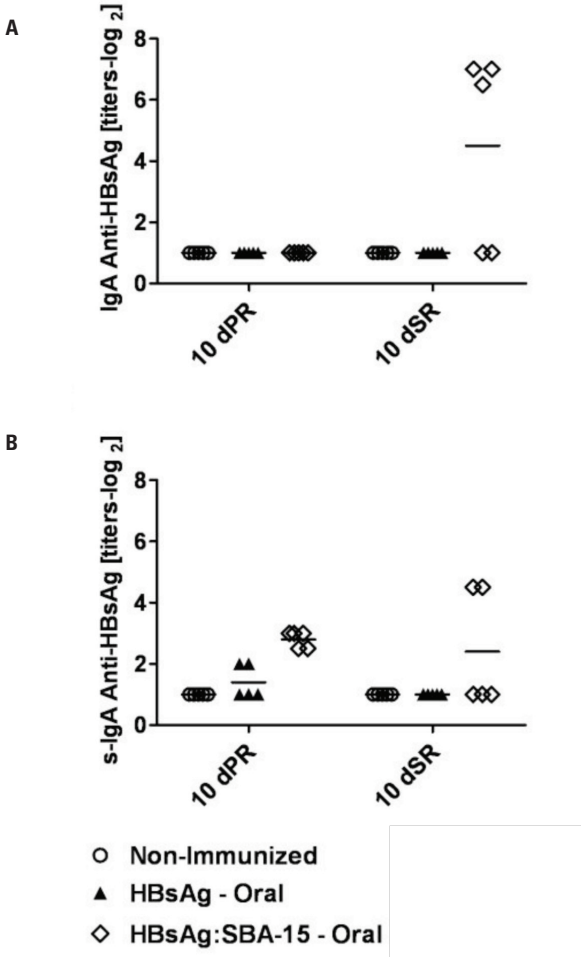

Figure 5. Antibody production by orally or subcutaneously immunized BALB/c mice. (A) Secretory IgA (s-IgA) and (B) serum IgA anti-HBsAg levels (n = 5 animals/group). Antibody titers measured by ELISA during primary and secondary immune responses. Subcutaneous booster was administrated 30 days after first oral immunization. Groups of animals non-immunized and immunized with rHBsAg were used as reference for the unpaired Student *t* test analysis

DISCUSSION

The immune system is a complex network that involves activation, regulation, and suppression, resultant characteristics of interactions involving cells and molecules. This essentially pleiotropic network is controlled by gene polymorphism of high adaptive values of traits (MHC, Complement System, genes that regulate the expressions of variable regions of immunoglobulin), and the main immunobiological functions (antibody production, inflammatory reactivity, tolerance) are submitted to polygenic controls that are, at least partially, independent.

The influence of environmental factors in the expansion of infectious diseases, such as the introduction of new antibiotics and vaccines that exert selective pressure on a given pathogen, changes the resistance rates of a microorganism. There are other environmental factors that act on both the capacity and the intensity of immuneresponsiveness to a given immunogen, modulating the genetically determined traits. This is the case of the adjuvants, either artificial or natural.

That is why it is so important to establish efficient vaccination schedules, with a proper planning of doses of antigens and suitable gaps between immunizations[24].

Besides the efficiency of all commercially available vaccines against Hepatitis B, it is always important to consider alternatives to decrease the boosters and the costs per dose. Moreover, the possibility of developing orally administered vaccines is of great importance. Needle-free immunizations have a wide range of advantages compared to other routes of administration. However, there are limitations for the success of an oral vaccine. Protein antigens usually fail in generating detectable systemic and local immune responses due to the hash gastric environment[15].

Recent results obtained from oral immunizations with Hepatitis A vaccines and human gamma globulin (HGG) adsorbed to SBA-15 have shown the applicability of SBA-15 as a support for antigens, protecting the epitopes from degradation and allowing the development of an efficient and specific immune response[25].

Comparing the formulation that uses $Al(OH)_3$ as adjuvant to its recombinant protein HBsAg adsorbed to SBA-15, it was clear that, after s.c. immunizations, a strong antibody response against HBsAg was obtained. Nevertheless, when we first administered orally a dose with the recombinant protein on silica, it was clear that the immune system was primed leading to an equal humoral response compared to the parenteral immunization (Figure 2). It is known that higher doses of antigens or the repetition of the vaccination did not essentially result in protection. Consequently, this is a significant result when we think about combining

oral and parenteral administration. Perhaps it will be possible to diminish the number of vaccine doses, since antibody production was detected after a second booster shot, and not only after a third dose as usual. Moreover, it will be possible to introduce oral immunizations on the vaccine schedule.

Another relevant fact is that, after the first oral immunization, good local antibody production (s-IgA) was identified, proving its efficacy and strengthening our plans to study the use of this silica in oral vaccines (Figures 4 and 5). After oral priming, it is possible that specific immune cells were activated, leading to the successful development of immunological memory.

As is already known, HBsAg tends to induce a T_H2 immune response with a major production of IgG1. This T_H2 pattern may be due to the subcutaneous administration and the presence of $Al(OH)_3$ in the vaccine formulation[26].

CONCLUSION

Our results corroborate the idea of the promising use of SBA-15 silica as an adjuvant/vehicle, even for oral immunizations. Compared to the HBV vaccines currently used, SBA-15 could anticipate and enhance the humoral response after oral and subcutaneous immunizations with a slightly predominance of a T_H2 type immune response. In oral immunizations, it is believed that these particles act in the physical protection of antigens or immunodominant epitopes, helping in their slow release and efficient activation of the immune system, and in the competent induction of immunological memory.

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