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Supercritical fluid extraction provides an enhancement to the immune response for orally-delivered hepatitis B surface antigen

Celine A. Hayden[a], Emily M. Smith[a], Debra D. Turner[b], Todd K. Keener[a], Jeffrey C. Wong[c], John H. Walker[d], Ian R. Tizard[b], Rafael Jimenez-Flores[e], John A. Howard[a,*]

[a] Applied Biotechnology Institute, Cal Poly Tech Park, San Luis Obispo, CA 93407, USA
[b] Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843, USA
[c] Horticulture and Crop Science Department, California Polytechnic State University, San Luis Obispo, CA 93407, USA
[d] Department of Statistics, California Polytechnic State University, San Luis Obispo, CA 93407, USA
[e] Dairy Product Technology Center, California Polytechnic State University, San Luis Obispo, CA 93407, USA

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ABSTRACT

The hepatitis B virus continues to be a major pathogen worldwide despite the availability of an effective parenteral vaccine for over 20 years. Orally-delivered subunit vaccines produced in maize may help to alleviate the disease burden by providing a low-cost, heat-stable alternative to the parenteral vaccine. Oral subunit vaccination has been an elusive goal due to the large amounts of antigen required to induce an immunologic response when administered through the digestive tract. Here we show that high levels of HBsAg were obtained in maize grain, the grain was formed into edible wafers, and wafers were fed to mice at a concentration of approximately 300 μg/g. When these wafers were made with supercritical fluid extraction (SFE)-treated maize material, robust IgG and IgA responses in sera were observed that were comparable to the injected commercial vaccine (Recombivax®). In addition, all mice administered SFE wafers showed high secretory IgA titers in fecal material whereas Recombivax® treated mice showed no detectable titer. Increased salivary IgA titers were also detected in SFE-fed mice but not in Recombivax® treated mice. Wafers made from hexane-treated or full fat maize material induced immunologic responses, but fecal titers were attenuated relative to those produced by SFE-treated wafers. These responses demonstrate the feasibility of using a two-dose oral vaccine booster in the absence of an adjuvant to induce immunologic responses in both sera and at mucosal surfaces, and highlight the potential limitations of using an exclusively parenteral dosing regime.

1. Introduction

Despite the availability of an effective parenteral vaccine for over 20 years, hepatitis B virus (HBV) remains an important problem, with 240 million chronically infected patients worldwide [1]. The present recommendation for the vaccine consists of injecting a 10 or 20 μg dose of the HBV small surface antigen, HBsAg, as a primary dose followed by two boosting doses. Although seroconversion occurs in greater than 90% of the general population using commercialized vaccines [2], there are specific segments of the population that are poor responders or non-responders. Among them are the elderly, obese individuals, HIV-positive patients, and individuals with celiac disease, irritable bowel disease, Down syndrome or chronic kidney disease [3–13].

An oral vaccine may improve seroconversion in the general population by stimulating the immune system at mucosal sites, tissues that are traditionally primary sites of infection. In addition, an oral vaccine would be easier to administer and could increase compliance in populations that historically forego one or all of the HBsAg doses such as hemophiliacs [14], at-risk youth [15], transient populations [16], persons engaging in high-risk sexual activity [17,18], and healthcare workers [19]. On a global scale, a maize-produced oral alternative could provide a low-cost, heat-stable alternative to parenteral vaccines [2,20] and therefore improve coverage in remote areas or resource-poor areas that cannot afford the infrastructure for reliable cold storage, needle administration, and waste disposal.

Many attempts have been made to develop a viable oral vaccine system with some success. Encouragingly, when HBsAg was expressed in potato tissue and fed to human volunteers as a booster dose, an increase in antibody titer was observed in 63% of participants [21]. The authors speculated that an increased concentration

* Corresponding author. Tel.: +1 805 756 6458.
E-mail address: jhoward@appliedbiotech.org (J.A. Howard).

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of antigen would be needed to improve seroconversion rates but, unfortunately, highly concentrated HBsAg in potato tissue has not been forthcoming. Increased antigen concentrations have been recently achieved in maize and have shown improved responses in mice relative to the potato material [22].

A key advantage of cereal grains is their ability for long-term stable storage of recombinant proteins [23,24]. If the raw material is to be used for oral vaccine formulations, shelf life can be extended by removing lipids from the grain, leading to reduced rancidity, oil degradation and radical formation [25]. Several methods can be used for lipid extraction, including hexane extraction and supercritical fluid extraction (SFE) with carbon dioxide (CO_2). Hexane is a solvent that is routinely used for the extraction of oil from plant products, resulting in marketable vegetable and essential oils. Unfortunately hexane is a neurotoxin and therefore requires extensive safety precautions during extraction and disposal. Supercritical CO_2, formed at pressures above 74 bar and 31 °C, is emerging as an alternative commercial extraction solvent due to its relatively benign properties [26]. Using hexane extracted maize material as an oral vaccine can elicit a strong immunological response in mice [22], but SFE-treated material has not been tested for its immunogenicity.

In the present study, more highly concentrated maize material was assessed as a booster in mice relative to the commercial vaccine. Different maize lipid removal techniques were also implemented and assessed for their effect on immunologic responses in mice.

2. Materials and methods

2.1. Maize material

All maize material used for the mouse study was derived from seed containing the HBG construct, as previously described [20]. All maize material used in the mouse studies was hybrid grain derived from heterozygous plants that contained genetic background from elite parent inbred lines 16038 and MBS5411. Control germ was G909 germ from the Grain Processing Corporation (Muscatine, IA).

2.2. Seed processing

HBG hybrid seed was soaked for 5 days in water (4 °C) to approximately 50% moisture, germ was extracted by hand, dried overnight at 37 °C to a final moisture of 6–15%, and ground to a fine cornmeal consistency. Ground germ was defatted by either hexane extraction or supercritical fluid extraction (SFE). Hexane extractions were conducted as previously described [20]. SFE treatment consisted of CO_2 extraction at 350 bar, 40–53 °C vessel temperature, using a 5L vessel in an SFT-250 (Supercritical Fluid Technologies, Newark, DE).

2.3. Wafer processing

Germ expressing HBsAg was treated with hexane or SFE to remove lipids. During this process, HBsAg concentration can either increase due to loss of lipids in the biomass or decrease due to loss of recombinant protein during the extraction method. To compensate for these changes in concentration, HBsAg germ materials were blended with control germ to obtain approximately equivalent concentrations of HBsAg in all germ flour used to make wafers. Control wafers were made with SFE-treated control germ. Each wafer contained 2.5 g germ flour and 0.65 g of ultrafine baker's sugar (C&H). Water was added to give a moldable consistency that was 15% of the germ weight for full fat material, and 25% for SFE- and hexane-defatted material. Wafers were hand-pressed into circular plastic molds (cat No.40116, Decagon/AquaLab, Pullman, WA) and dried in a VWR 1430 vacuum oven (VWR Scientific, Radnor, PA) at 58–62 °C, 21–22″ Hg until wafers lost >90% of the added water.

2.4. Antigen detection

To test HBsAg antigen levels in ground maize material or wafers, 100 mg samples were weighed out in duplicate and each sample was extracted in 1 mL PBS +1%TritonX-100. Four wafers were tested per treatment. HBsAg was assayed by sandwich ELISA, as described previously [22] with the following modifications: extracts were diluted 1:1000 and assayed using a monoclonal capture antibody (cat No. C01246M, Meridian Life Sciences, Memphis, TN), and a polyclonal HRP-conjugated detection antibody (cat No.B65811P, Meridian Life Sciences).

2.5. Mouse study

BALB/c inbred mice (Harlan) were randomly assigned to treatments 1 through 6 and housed individually. Eleven mice were assigned to treatments 1 through 3 and 10 mice were assigned to treatments 4 and 5. All treatments, except treatment 6, were injected with 0.5 μg of Recombivax® (Merck, Whitehouse Station, NJ) on day 0, and were boosted with full fat wafers, hexane-defatted wafers, SFE-defatted wafers, Recombivax®, or control wafers (treatments 1–5, respectively), with boosting initiated on day 112 and day 126 post-primary injection. For each boost, two wafers were offered per day for three consecutive days or a single intra-muscular Recombivax® injection was administered on the first day. Treatment 6 consisted of 5 mice which were injected with 0.9% sterile saline on day 0 and boosted with control wafers as above.

2.6. Anti-HBsAg antibody detection in mice

Blood samples were collected by submandibular venous puncture every 2–4 weeks, centrifuged to remove red blood cells, and stored in 50% glycerol at −20 °C. On boosting days, serum was collected a few hours prior to boosting. Fecal material was collected from cages that were cleaned 24 h prior to sampling, and samples were stored at −20 °C. Fecal samples were collected twice a week for the first 5 weeks, and again twice a week starting one day prior to the first boost and ending the week of the terminal bleed. Serum anti-HBsAg IgG and IgA were detected using a sandwich ELISA. Plates were coated with rHBsAg (cat No. R86872, Meridian Life Sciences), serum samples diluted 1:250, and HRP-conjugated anti-mouse IgG (cat No. ab6789, Abcam, Cambridge, MA) or AP-conjugated anti-mouse IgA (cat No. ab97232, Abcam) were used to detect IgG and IgA, respectively. For secretory IgA, 100 mg of fecal pellets were resuspended in 1 mL of 1%BSA in PBS containing a protease inhibitor (cat No. 11836153001, Roche Diagnostics GmbH, Mannheim, Germany) and diluted an additional 1:50 and used in the same assay as the serum samples. For saliva Ig, saliva was collected on day 141 post-primary injection, diluted between 1:5 and 1:25, and detected using the ETI-AB-AUK PLUS assay kit (DiaSorin, Saluggia, Italy) which calculates total anti-HBsAg Ig in mIU/mL based on the WHO 2nd International Standard. Serum Ig was also detected using the DiaSorin kit by diluting serum 1:50 or 1:500 so that titers fell on the linear part of the standard curve.

2.7. Immunoblot

One hundred milligrams of wafer material was first extracted three times in PBS +0.05% Tween 20 to remove native corn proteins that non-specifically bind to the detection antibody (it is