Immunogenicity in humans of an edible vaccine for hepatitis B

Yasmin Thanavala*†, Martin Mahoney‡, Sribani Pal*§, Adrienne Scott*¶, Liz Richter‖**, Nachimuthu Natarajan‡, Patti Goodwin††, Charles J. Arntzen‖‡‡§§, and Hugh S. Mason‖‡‡

Departments of *Immunology, ‡Cancer Prevention, Epidemiology, and Biostatistics, and ††Institutional Protocols and Scientific Integrity, Roswell Park Cancer Institute, Buffalo, NY 14263; and ‖The Boyce Thompson Institute for Plant Research, Ithaca, NY 14853

Contributed by Charles J. Arntzen, January 7, 2005

A double-blind placebo-controlled clinical trial evaluated the immunogenicity of hepatitis B surface antigen (HBsAg) expressed in potatoes and delivered orally to previously vaccinated individuals. The potatoes accumulated HBsAg at ≈8.5 μg/g of potato tuber, and doses of 100 g of tuber were administered by ingestion. The correlate of protection for hepatitis B virus, a nonenteric pathogen, is blood serum antibody titers against HBsAg. After volunteers ate uncooked potatoes, serum anti-HBsAg titers increased in 10 of 16 volunteers (62.5%) who ate three doses of potatoes; in 9 of 17 volunteers (52.9%) who ate two doses of transgenic potatoes; and in none of the volunteers who ate nontransgenic potatoes. These results were achieved without the coadministration of a mucosal adjuvant or the need for buffering stomach pH. We conclude that a plant-derived orally delivered vaccine for prevention of hepatitis B virus should be considered as a viable component of a global immunization program.

mucosal immune response | oral vaccine | surface antigen | transgenic plant

Hepatitis B virus (HBV) is responsible for significant morbidity and mortality despite the availability of safe and efficacious injectable vaccines (1). In 1996 it was estimated that some 115 million people were infected with HBV even though a subunit vaccine isolated from yeast became available a decade earlier. Global mortality due to this disease is estimated to be 1 million cases per year. Data from two National Health and Nutrition Examination Surveys (NHANES II, 1976–1980; NHANES III, 1988–1994) were analyzed to examine trends in the prevalence of HBV infection in the U.S. No statistically significant declines in prevalence of HBV infection occurred between the two surveys, and >300,000 new cases occurred annually despite the availability of a hepatitis B vaccine (2, 3). Therefore, immunization rates in the U.S. remain below targeted objectives. The situation is even worse in countries that cannot afford the current licensed parenteral vaccines but in which morbidity and mortality is high because HBV is endemic. In some of these countries, the current vaccines are of limited availability and use because of the need for cold storage and significant cost of the vaccines.

The incredible success of vaccination in contributing to public health is attributable, in part, to the simplicity of vaccines. For polio, the simple incorporation of attenuated poliovirus on sugar cubes resulted in an oral vaccine that was universally acceptable. Impressive logistical advantages of orally administered vaccines were exemplified by two national vaccination days in 1996, when 121 million Indian children were vaccinated against polio at 650,000 immunization centers (4). Orally delivered vaccines made this enormous endeavor achievable at reasonable costs. The Indian effort was part of a global program to eradicate polio, for which success has been achieved in North and South America. Unfortunately, a simple oral formulation is not achieved easily for the new generation of subunit vaccines, which hold the greatest promise for disease prevention in the 21st century.

There is an urgent need to make the technically sophisticated field of subunit vaccines (which are almost exclusively biotechnology products) available in the poorer countries of the world in which infectious diseases are still the primary cause of death. We have been exploring a strategy of producing and delivering oral subunit vaccines as constituents of transgenic, edible plants such as potatoes, tomatoes, or bananas. Indigenous technology in developing countries could be harnessed for plant-derived vaccine production to overcome current constraints. Initial research on vaccine production in plants focused on expression of antigenic proteins to protect against pathogens that cause diarrhea, because oral immunization was likely to induce localized protection against enteric diseases (5–10). Parallel studies showed that hepatitis B surface antigen (HBsAg) could be expressed in plants, and it was immunogenic when partially purified and injected into mice (11). Moreover, when mice were fed transgenic potato tubers expressing HBsAg, both primary and booster serum antibody responses were elicited (12). These combined experiments demonstrated that plants can express, fold, assemble, and process foreign antigens and can provide both a simple vaccine-manufacturing process as well as a matrix suitable for oral immunization.

The question that we asked in the current study was: Will a plant-derived oral subunit vaccine comprised of an antigen from a nonenteric pathogen function at induction sites in the human gut and generate a systemic immune response that is predictive of disease prevention?

The present clinical trial evaluated the safety and immunogenicity of orally delivered HBsAg expressed in transgenic potatoes. In a randomized, placebo-controlled, double-blind trial, health care workers with a history of previous parenteral immunization with the licensed hepatitis B vaccine volunteered to consume multiple doses of transgenic or control potatoes. The subjects were evaluated for safety, reactions to the vaccine/vehicle, and immune response to HBsAg.

Materials and Methods

Generation of HBsAg Transgenic Tubers. To generate the HBsAg transgenic tubers, the HBsAg gene from a pMT-SA clone of a Chinese adr isolate of HBV (13) was inserted into a transformation plasmid vector pHB114, which was mobilized into

Freely available online through the PNAS open access option.

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; anti-HBs, anti-HBsAg; mIU, milli-international unit.

†To whom correspondence may be addressed. E-mail: yasmin.thanavala@roswellpark.org.

§Present address: Department of Molecular Genetics, Lerner Research Institute, Cleveland Clinic Foundation, NC/20, 9500 Euclid Avenue, Cleveland, OH 44195.

¶Present address: Wyeth, 401 North Middletown Road, Pearl River, NY 10965.

**Present address: Lexicon Genetics Incorporated, The Woodlands, TX 77381-4287.

‡‡Present address: Biodesign Institute at Arizona State University, Tempe, AZ 85287.

§§To whom correspondence may be addressed at the ‡‡ address. E-mail: charles.arntzen@asu.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0409899102

Agrobacterium tumefaciens (LBA4404), which then was used to transform *Solanum tuberosum* L. cv. Frito-Lay 1607 (FL1607). The transformed FL-1607 was cured of the *A. tumefaciens* and clonally propagated, and the FL-1607 HB114-16 line was selected for its high level of HBsAg expression (14). This line then was clonally propagated to multiply the number of plants, potted in soil, and grown in a greenhouse to produce the potato tubers that were used in this clinical trial. Lot number HB114-16-4 (transgenic) and F04 and F05 (placebo) were used for this trial.

Trial-Entry Criteria. Before entry into the study, all volunteers provided informed consent. All study participants had documented history of primary immunization series (three doses) between 1 and 15 years ago with the licensed hepatitis B vaccine and documented adequate serum IgG anti-HBsAg (anti-HBs) response (>10 milli-international units (mIU)/ml) to the primary vaccine series. All volunteers included in the study had current anti-HBs antibody titers ≤115 mIU/ml. All female subjects were tested by a urine pregnancy test no more than 2 days before the first dose of vaccine. The female subjects were instructed to use adequate birth control methods to prevent pregnancy for at least 3 months after the last vaccine dose. Criteria that resulted in noninclusion of potential volunteers in this trial included: known history of allergy to hepatitis B vaccine in any form; current anti-HBs levels >115 mIU/ml; known history of hepatitis B infection in the past; pregnancy or breast feeding; participation in any other investigational study within 30 days of enrollment in this study; currently active gastrointestinal disease including peptic ulcer, gastroesophageal reflux, inflammatory bowel disease, diverticulitis, or pancreatitis; use of prescription medication or over-the-counter histamine type 2 blockers for any of the above-mentioned diseases within 1 month of study enrollment; and any laboratory assay abnormality that suggested dysfunction of hematological, renal, or hepatic systems.

Diary-Card Responses. Subjects recorded their responses to specific questions concerning their tolerance of the vaccine. The diary card specifically elicited reactions in the categories of systemic reactions (malaise, myalgia), gastrointestinal reactions (nausea, vomiting, abdominal pain, change in bowel movements), and febrile responses and also allowed for other unsolicited comments to be captured for evaluation of reactions to oral immunization. Subjects recorded reactions on the evening of each vaccine dosing and for 3 days afterward. All study subjects were randomized into three groups by use of a centrally generated block randomization list. The list was provided only to the study pharmacist, who was unblinded to the group assignment of subjects. All other study personnel and study subjects remained blinded through the completion of the study. The medical investigator had the option to unblind the study for any individual subject in the event that the management of a subject (i.e., serious adverse event) required it.

Vaccine Preparation and Consumption. Study subjects fasted for 90 min before and after ingesting the potatoes. Study subjects were encouraged to consume their designated portion of potato over a maximum of 15 min. Vital signs were recorded before vaccine administration and 30 min after consumption of the vaccine dose. The potatoes were peeled immediately before ingestion to remove skin containing solanine, an alkaloid present in all green tissues of potatoes that can cause abdominal discomfort, nausea, or bitter taste. Each potato was cut into bite-size pieces and placed into an ice-cold water bath to prevent browning from oxidation. After completion of all peeling and dicing, 100- to 110-g doses of potato were weighed out by the study pharmacist for each subject according to randomized group assignment and subject identification number. A sample of processed potato from each group at each feeding was frozen for subsequent testing to verify antigen content.

Measurement of Anti-HBs Antibodies. Anti-HBs IgG was detected by using the AUSAB EIA (Abbott). Polystyrene beads coated with HBsAg were incubated with either the test human serum or set of standards or other controls for 18 ± 2 h at room

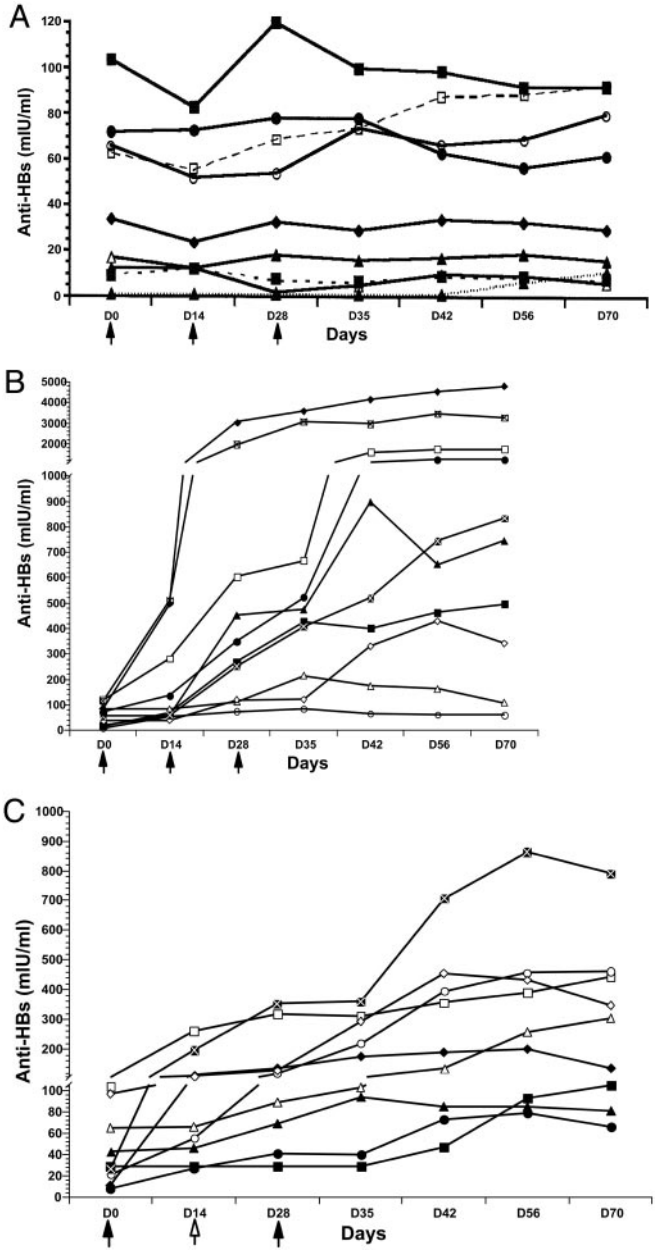

Fig. 1. Time courses for changes in serum anti-HBs IgG in individual subjects. (*A*) Nine volunteers in group one consumed placebo potatoes on days 0, 14, and 28 (open arrowheads). None of the volunteers had any significant changes in their anti-HBs-specific titers during the study. (*B*) Ten of 16 volunteers who ate three doses (group three) of HBsAg-containing transgenic potatoes on days 0, 14, and 28 (closed arrowheads) showed marked increases in anti-HBs titers. Each line represents changes in an individual volunteer's antibody titer throughout the study period. (*C*) Nine of 17 volunteers (group two) who received transgenic potatoes on days 0 and 28 (closed arrowhead) and nontransgenic potatoes (open arrowhead) on day 14 showed marked increases in anti-HBs titers. Note the discontinuities in the ordinate axes in *B* and *C* and consequent breaks in the curves of higher-titer subjects.

Table 1. Increase in anti-HBs antibody titer in 9 of 17 volunteers who ate three doses of HBsAg-transgenic potatoes

Volunteer no.	Day 0, mIU/ml	Fold increase							
		Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56	Day 70
1	17	1.2	4.1	8.2	15.8	25.2	23.6	27.2	29.2
2	9	1.0	5.9	8.2	8.2	9.4	7.2	6.8	6.7
3	20	2.1	2.9	4.2	22.6	23.8	44.9	32.6	37.3
4	85	0.9	5.8	14.3	36.0	42.0	48.9	53.3	56.3
5	120	1.2	2.4	3.3	5.0	5.6	13.2	14.3	14.3
6	72	1.1	1.9	3.8	4.9	7.3	15.3	17.0	17.0
7	85	0.9	1.0	0.9	1.3	2.5	2.1	1.9	1.3
8	40	0.9	1.0	1.8	3.0	3.1	8.3	10.8	8.6
9	50	0.9	1.1	1.5	4.5	7.3	9.3	13.3	14.9
10	115	1.9	4.4	9.2	17.1	26.7	25.8	30.0	28.4

Volunteers 1–5 and 10 had at least a 2-fold increase in their individual antibody titers after one dose of trangenic potato eaten on day 0.

temperature. At the end of the incubation period, beads were washed and incubated in a solution containing biotin-tagged HBsAg and rabbit antibiotin conjugated with horseradish peroxidase at 40 ± 1°C for 2 h. Unbound biotin–antibiotin complex was removed, and the beads were washed. Next, *O*-phenylenediamine solution containing hydrogen peroxide was added to the bead, and the reaction was allowed to proceed for 30 min at room temperature. The reaction was stopped by the addition of 0.5 M sulfuric acid. The intensity of yellow color that develops is proportional to the amount of anti-HBs bound to the beads. The absorbance is measured at 492 nm in a Quantum II spectrophotometer. The AUSAB quantitative standard panel was used to convert OD values to mIU/ml.

Results

The drug substance used in this clinical trial was HBsAg expressed in tubers of the potato variety FL-1607. The line of potato selected for this study is designated FL-1607 HB114-16, and the antigen content was 8.5 ± 2.1 μg of HBsAg per g of transgenic potato, as detected by AUSZYME monoclonal ELISA (Abbott). The physical form of the potato-derived antigen was characterized (15). The HBsAg extracted from transgenic tubers cosedimented with yeast-derived HBsAg in rate-zonal sucrose gradients; however, electron microscopy showed accumulation of long tubules in endoplasmic reticulum-derived vesicles. The electrophoretic mobility on Western blots indicated that the potato-expressed HBsAg was similar to yeast-derived antigen but differed in the occurrence of a doublet monomer, suggesting partial glycosylation (15). The tissue from the untransformed potato tubers did not express any proteins that were reactive with antibodies to HBsAg.

Before entry into the trial, 168 previously vaccinated healthy adult health care workers were screened by a quantitative AUSAB assay (Abbott) for their current anti-HBs titers. Only those volunteers who had antibody titers of ≤115 mIU/ml qualified for inclusion in the study. Forty-two adult health care workers 25–58 years of age were enrolled in the study. There were 30 females and 12 males; 37 were Caucasian, 3 were African American, and two were other race. All volunteers were in excellent physical health, verified by medical history and a battery of blood tests (renal, hepatic, and hematologic) before entry into the study at day 0 and at three additional time points (days 14, 28, and 42) during the study. Study subjects were randomized into three groups and completed self-assessment diaries.

The volunteers in the three groups received either 100–110 g of placebo potatoes on days 0, 14, and 28 (group one), HBsAg-transgenic potatoes on days 0 and 28 and placebo potatoes on day 14 (group two), or HBsAg-transgenic potatoes on days 0, 14, and 28 (group three). The entries of the diary cards from the study subjects were analyzed on each of three occasions in categories of abdominal pain, bowel movement related, nausea, muscle pain, fatigue, headache, and other symptoms. Comparison of the adverse effects between the group of volunteers that ingested the placebo vaccine and each of the experimental groups did not show any statistical significance when using the Kruskal–Wallis test (16).

Blood was collected from each study volunteer on days 0, 7, 14, 21, 28, 35, 42, 56, and 70 for measurement of serum anti-HBs using the AUSAB kit with a set of standards (AUSAB quantitation panel) that enables the OD values to be expressed as mIU/ml of serum. None of the nine volunteers who had ingested three doses of nontransgenic potatoes had any significant changes in their anti-HBs titers during the study (Fig. 1*A*). In contrast, 10 of 16 volunteers (63%) who ate three doses of HBsAg-containing transgenic potatoes (on days 0, 14, and 28) showed marked increases in antibody titers compared to their own day-0 titers ($P < 0.01$) (Fig. 1*B*). Among these individuals, six showed at least a doubling of their anti-HBs titers ($P < 0.01$) after consuming one dose of transgenic potato, and four of the six showed a ≥4-fold increase ($P < 0.01$) after one dose (Table 1). The statistical significance was tested by using Fisher's exact test (17). All responders showed gradual increases in titers, with apparent boosting effects of the second and third doses. Four volunteers in the three-dose group achieved final titers of >1,000 mIU/ml (Fig. 1*B*), with the highest at 4,785 mIU/ml. Nine of the 17 volunteers who received transgenic potatoes only twice (days 0 and 28) also showed increases in anti-HBs titers ($P < 0.01$) (Fig. 1*C*). Of these volunteers, five of the nine showed at least a doubling ($P < 0.05$) after one dose, and two showed a ≥4-fold response after the first dose (Table 2). The highest titer obtained in the two-dose group was 863 mIU/ml (Fig. 1*C*).

Statistical methods were used to contrast the antibody responses of the three groups, involving all volunteers who completed the trial. Responses of controls (group one) were contrasted versus those who received two doses of transgenic potatoes (group two) versus those who received three doses of transgenic potatoes (group three) to test the hypothesis that the effect of group one is less than or equal to group two, which is less than or equal to group three. The differences between the three groups were statistically significant when using the Jonckheere–Terpstra test (16) ($P < 0.05$). The comparisons between groups two (two doses) and three (three doses) revealed no statistically significant differences.

Discussion

We found that ingestion of transgenic potatoes expressing HBsAg by previously vaccinated volunteers provoked increases

Table 2. Increase in anti-HBs antibody titer in 10 of 16 volunteers who ate two doses of HBsAg-transgenic potatoes

Volunteer no.	Day 0, mIU/ml	Fold increase							
		Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56	Day 70
1	29	1.0	1.0	1.0	1.0	1.0	1.6	3.2	3.6
2	8	1.9	3.4	6.1	5.1	5.0	9.1	9.9	8.3
3	43	0.9	1.1	1.8	1.6	2.2	2.0	2.0	1.9
4	11	0.6	10.4	10.4	12.4	16.0	17.4	18.2	12.4
5	104	1.2	2.5	2.6	3.1	3.0	3.4	3.8	4.3
6	21	1.1	2.6	5.3	5.7	10.0	18.8	21.8	22.0
7	65	1.1	1.0	1.0	1.4	1.6	2.1	3.4	4.7
8	97	1.0	1.2	1.1	1.3	3.0	4.7	4.5	3.6
9	26	1.3	7.6	12.7	13.6	13.9	27.2	33.2	30.4

Volunteers 2, 4–6, and 9 showed a doubling in antibody titer after the first dose of trangenic potato eaten on day 0.

in the titers of serum antibody specific for HBsAg in 19 of 33 subjects, which is remarkable in view of the facts that (*i*) the vaccine was delivered without any adjuvant, (*ii*) the HBsAg, an antigen derived from a nonenteric pathogen, was delivered orally, and (*iii*) the recombinant subunit HBsAg was a nonreplicating vaccine. The anti-HBs titers were boosted up to 56-fold after three doses (Table 1) or up to 33-fold after only two doses (Table 2) of transgenic potato. There was no significant correlation between the individual absolute titers at the start of the study and the maximal titers at the end.

Approximately 40% of the volunteers were nonresponders to the HBsAg oral vaccine in transgenic potatoes, as measured by serum antibodies. Design of this study did not include assays to determine mucosal immune responses involving secretory antibody induction or other sensitive indicators of induction of antibody-secreting cells; it is therefore not possible to rule out any immune response in those individuals whose titers were not increased by eating the transgenic potatoes. It is relevant that use of the commercial HBsAg parenteral vaccine, which does include an adjuvant (alum), does not always lead to successful immunization. Nonresponsiveness remains a problem for a group of vaccinees (18–21), and booster vaccination to maintain protective titer levels has been discussed (22, 23). Moreover, oral delivery in human subjects of a recombinant adenovirus containing the HBsAg gene failed to stimulate serum anti-HBs (24), although that construct may have been compromised by deletion of an important adenovirus gene.

The speed and amplitude of serum anti-HBs titer increases in this study were variable among volunteers, as is expected for human subjects. The rather gradual increase in titers, on average, raises the possibility that oral presentation of HBsAg in potatoes represents a priming response rather than a boosting response achieved via memory cells that were established when the volunteers were immunized previously by injection. However, several subjects showed marked increases in anti-HBs titers at day 14 after only one dose, which supports the interpretation of a boosting mechanism. Additional studies with naive (not previously immunized volunteers) will be needed to clarify the nature of the immunization with potato-delivered antigen.

Introduction of an oral vaccine for HBV could greatly impact global immunization acceptance. We are greatly encouraged that this prototype study of human immunization against HBsAg gave a strong and sustained systemic antibody response in ≈60% of the volunteers who ate transgenic potatoes. It is worth noting that in this trial no buffering of stomach pH or any mucosal adjuvant was used, which is in contrast to our preclinical studies in which the use of a mucosal adjuvant was critical. We believe that in future trials the incorporation of a potent mucosal adjuvant might confer a substantial advantage in both magnitude and rate of response. Additionally, the dosage of the HBsAg can be increased, because this study has established a "ranging" value. This dosage increase can be accomplished by improvement in the HBsAg-expression constructs used to create transgenic plants and by processing the plant material to yield a more highly concentrated form of the protein.

In addition to preventing the primary disease episode, vaccination can lessen long-term effects of HBV pathology. Liver cancer is an important public health problem, ranking fifth in frequency of cancer worldwide. There are several risk factors for liver cancer, including exposure to hepatitis B, with 76% of all cases of liver cancer being found in Asia. A joint Food and Agriculture Organization of the United Nations/World Health Organization expert committee concluded that, given a limited public health budget, a more substantial reduction in liver cancer would be achieved by vaccination against HBV (no vaccine for hepatitis C virus is yet available) and reduction in the prevalence of carriers (25). A significant decline in the incidence of liver cancer has occurred among cohorts of HBsAg-vaccinated newborns and children in Taiwan (26). Thus, it is a sensible use of scientific knowledge as well as an ethical approach to use scarce resources where and how they will do the most good; in other words, give new vaccination strategies for HBV a higher priority, especially those that could be implemented very cost-effectively throughout the developing world.

The demonstrated success in this prototype study of oral immunization for HBV with an orally administered subunit vaccine provides a strategy to solve a global problem. We believe that the data presented here provide compelling evidence that orally delivered subunit vaccines can provide a useful component in a program of prophylaxis against nonenteric and enteric diseases.

We acknowledge the excellent assistance provided by M. Bluff, B. Cotter, G. Elkin, L. Gugino, D. Kirk, A. M. McNamara, J. Parsons, T. Smietana, J. Solomon, and J. Whalen. All potatoes used in the study were grown at The Boyce Thompson Institute, and the able assistance of its greenhouse staff is gratefully acknowledged. We are grateful to the volunteers who participated in this trial for their full, enthusiastic, and conscientious cooperation. Dr. Frank J. Malinoski initiated the preparation of the Investigation for New Drug proposal for this study. We thank Mrs. Cheryl Zuber for excellent help in manuscript preparation. Funding to support research for the use of HBsAg in transgenic plants for oral vaccination is provided by National Institutes of Health Grant AI42836 (to Y.T., C.J.A., and H.S.M.) and a World Health Organization grant (to Y.T., C.J.A., and H.S.M.). The clinical trial was funded by Roswell Park Cancer Institute.

1. Kane, M. (1995) *Vaccine* **13,** S47–S49.
2. Coleman, P. J., McQuillan, G. J., Moyer, L. A., Lambert, S. B. & Margois, H. S. (1998) *J. Infect. Dis.* **178,** 954–959.
3. McQuillan, G. M., Coleman, P. J., Kruszon-Moran, D., Moyer, L. A., Lambert, S. B. & Margolis, H. S. (1999) *Am. J. Public Health* **89,** 14–18.
4. Bloom, B. R. & Widdus, R. (1998) *Nat. Med.* **4,** Suppl., 480–484.
5. Haq, T. A., Mason, H. S., Clements, J. D. & Arntzen, C. J. (1995) *Science* **268,** 714–716.
6. Mason, H. S., Ball, J. M., Shi, J. J., Jiang, X., Estes, M. K. & Arntzen, C. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5335–5340.
7. Mason, H. S., Haq, T. A., Clements, J. D. & Arntzen, C. J. (1998) *Vaccine* **16,** 1336–1343.
8. Tacket, C. O., Mason, H. S., Losonsky, G., Clements, J. D., Levine, M. M. & Arntzen, C. J. (1998) *Nat. Med.* **4,** 607–609.
9. Tacket, C., Mason, H. S., Losonsky, G., Estes, M. K., Levine, M. M. & Arntzen, C. J. (2000) *J. Infect. Dis.* **182,** 302–305.
10. Tacket, C. O., Pasetti, M. F., Edelman, R., Howard, J. A. & Streatfield, S. (2004) *Vaccine* **22,** 4385–4389.
11. Thanavala, Y., Yang, Y.-F., Lyons, P., Mason, H. S. & Arntzen, C. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3358–3361.
12. Kong, Q., Richter, L., Yang, Y. F., Arntzen, C. J., Mason, H. S. & Thanavala, Y. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 11539–11544.
13. Mason, H. S., Lam, D. M. K. & Arntzen, C. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11745–11749.
14. Richter, L. J., Thanavala, Y., Arntzen, C. J. & Mason, H. S. (2000) *Nat. Biotechnol.* **18,** 1167–1171.
15. Smith, M., Richter, L., Arntzen, C., Shuler, M. & Mason, H. (2003) *Vaccine* **21,** 4011–4021.
16. Hollander, M. & Wolfe, D. (1973) in *Nonparametric Statistical Methods*, eds. Bradley, R. A., Hunter, J. S., Kendall, D. G. & Watson, G. S. (Wiley, New York), pp. 114–137.
17. Argesti, A. (1996) in *An Introduction to Categorical Data Analysis*, eds. Barnett, V., Bradley, R. A., Fisher, N., Hunter, J. S., Kadane, J. B., Kendall, D. G., Scott, D. W., Smith, A. F. M., Teugels, J. L. & Watson, G. (Wiley, New York), pp. 39–44.
18. Stevens, C. E., Alter, H. J., Taylor, P. E., Zang, E. A., Harley, E. J. & Szmuness, W. (1984) *N. Engl. J. Med.* **311,** 496–501.
19. Weber, D. J., Rutala, W. A., Samsa, G. P., Santimaw, J. E. & Lemon, S. M. (1985) *J. Am. Med. Assoc.* **254,** 3187–3189.
20. Goldwater, P. N. (1997) *Vaccine* **15,** 353–356.
21. Jilg, W. (1998) *Vaccine* **16,** Suppl., S65–S68.
22. Barash, C., Conn, M .I., DiMarino, A. J., Jr., Marzano, J. & Allen, M. L. (1999) *Arch. Intern. Med.* **159,** 1481–1483.
23. Jilg, W., Schmidt, M., Deinhardt, F. & Zachova, R. (1984) *Lancet* **2** (8400), 458.
24. Tacket, C. O., Losonsky, G., Lubeck, M. D., Davis, A. R., Mizutani, S., Horwith, G., Hung, P., Edelman, R. & Levine, M. M. (1992) *Vaccine* **10,** 673–676.
25. Henry, S. H., Bosch, F. X., Troxell., T. C. & Bolger, P. M. (1999) *Science* **286,** 2453–2454.
26. Chang, M.-H., Chen, C.-J., Lai, M.-S., Hsu, H.-S., Wu, T.-C., Kong, M.-S., Liang, D.-C., Shau, W.-Y. & Chen, D.-S. (1997) *N. Engl. J. Med.* **336,** 1855–1859.