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Immunology

Immunogenicity of transgenic plant-derived hepatitis B surface antigen

(plant-derived antigens/antibody production/T-cell proliferation)

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ABSTRACT **The focus of the Children's Vaccine Initiative is to encourage the discovery of technology that will make vaccines more readily available to developing countries. Our strategy has been to genetically engineer plants so that they can be used as inexpensive alternatives to fermentation systems for production of subunit antigens. In this paper we report on the immunological response elicited *in vivo* by using recombinant hepatitis B surface antigen (rHBsAg) purified from transgenic tobacco leaves. The anti-hepatitis B response to the tobacco-derived rHBsAg was qualitatively similar to that obtained by immunizing mice with yeast-derived rHBsAg (commercial vaccine). Additionally, T cells obtained from mice primed with the tobacco-derived rHBsAg could be stimulated *in vitro* by the tobacco-derived rHBsAg, yeast-derived rHBsAg, and by a synthetic peptide that represents part of the *a* determinant located in the S region (139–147) of HBsAg. Further support for the integrity of the T-cell epitope of the tobacco-derived rHBsAg was obtained by testing the ability of the primed T cells to proliferate *in vitro* after stimulation with a monoclonal anti-idiotype and an anti-idiotype-derived peptide, both of which mimic the group-specific *a* determinant of HBsAg. In total, we have conclusively demonstrated that both B- and T-cell epitopes of HBsAg are preserved when the antigen is expressed in a transgenic plant.**

On a global basis, hepatitis B virus (HBV) infection is probably the single most important cause of persistent viremia in humans. Current estimates establish that there are ≈300 million carriers of HBV in the world. Continued transmission of HBV is thus assured by the existence of this large reservoir of persistent human carriers (1). The worldwide problem of HBV infection and its association with chronic liver disease has necessitated the development of an effective vaccine. Because HBV cannot be propagated in tissue culture and the host range is limited to chimpanzees and humans, the first vaccine consisted of hepatitis B surface antigen (HBsAg) purified from the plasma of HBsAg carriers (2). However, concerns about the safety of serum-derived HBsAg resulted in the development of the first commercially available recombinant vaccine, produced by expression of HBsAg in yeast. Intramuscular injections of serum-derived HBsAg or yeast-derived recombinant HBsAg (rHBsAg) in healthy individuals result in effective immunization and protection from viral infection (2).

In many areas of the developing world the expense of immunization programs prohibits the use of the currently available vaccines for large segments of the population. This limitation led us to attempt the expression of HBsAg in plants with the hope of developing a product that is less expensive to produce and that would not require a cold chain during distribution.

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The first step in demonstrating concept feasibility for plant-based vaccine production came when we reported that the gene encoding HBsAg can be expressed in tobacco plants. The tobacco-derived rHBsAg self-assembles into subviral particles, which are virtually indistinguishable from serum-derived HBsAg and yeast-derived rHBsAg with respect to size, density sedimentation, and antibody binding (3). From these data we concluded that plants can produce an immunologically reactive antigen from an animal virus.

Here we report on the immunogenic properties of tobacco-derived rHBsAg and show that it can elicit HBsAg-specific antibodies in BALB/c mice and prime T cells *in vivo* that can be stimulated *in vitro* by the yeast-derived rHBsAg used in current vaccines.

MATERIALS AND METHODS

Preparation of rHBsAg from Tobacco. Transgenic plants expressing rHBsAg were grown from tobacco (*Nicotiana tabacum* cv. Samsun) seed harvested from plants that had been transformed by *Agrobacterium* infection as described (3). Leaves (200–300 g) were homogenized in a blender with liquid nitrogen, and the resulting powder was extracted overnight at 4°C in buffer (1 ml/g fresh wt) containing 0.1 M sodium phosphate (pH 7.4), 0.1 M sodium ascorbate, 0.1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride. The extract was centrifuged 30 min at 30,000 × *g* and additionally for 40 min at 40,000 × *g*. The resulting supernatant was concentrated by ultrafiltration with a YM30 membrane (Amicon) to 20–30 ml and frozen at −20°C.

Upon thawing, the sample was centrifuged for 10 min at 12,000 × *g*, and the supernatant was loaded onto 10–40% sucrose gradients, and these were centrifuged at 100,000 × *g* for 13–14 hr. Fractions were assayed for HBsAg by an Auszyme ELISA kit (Abbot). Briefly, samples were diluted in 200 ml of 0.02 M sodium phosphate/150 mM sodium chloride, pH 7.5 (PBS), and 50 μl of peroxidase-conjugated mouse anti-HBsAg and a mouse anti-HBsAg-coated bead were added to each sample. After incubation at room temperature for 16 hr the beads were washed four times with distilled water, and 300 μl of freshly prepared *o*-phenylenediamine·2 HCl solution was added. Samples were incubated for 1 hr at room temperature, the reaction was stopped with 1 ml of 1 M H_2SO_4, and the absorbance was measured at 492 nm. Positive fractions were pooled, dialyzed with PBS, and concentrated with a YM30 membrane to a final HBsAg concentration of 100 μg/ml or greater. The HBsAg concentration for samples purified in this manner was typically ≈3% of total protein.

Induction of Anti-HBsAg Antibodies. BALB/c mice (five per group) were immunized i.p. on days 0, 7, and 14 with the tobacco rHBsAg (0.5 μg per animal per injection) in complete Freund's adjuvant, incomplete Freund's adjuvant, and saline,

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; rHBsHg, recombinant HBsAg.

respectively. Control groups of mice were immunized with yeast-derived rHBsAg (Merck Sharpe & Dohme) as an alum-adsorbed precipitate at a dose of 0.5 μg per injection. Mice were bled before the start of immunization and thereafter at weekly intervals over a period of 16 weeks.

ELISA Assay for Measurement of Anti-HBsAg-Specific Antibodies. Mouse sera were evaluated for anti-HBsAg-specific antibodies using a commercially available AUSAB enzyme immunoassay (EIA) diagnostic kit (Abbott). Two hundred microliters of serum samples, appropriately diluted, were added to individual wells containing HBsAg-coated beads. After incubation at room temperature for 18 hr, the beads were washed with distilled water to remove unbound antibodies. The beads were then incubated with biotin-tagged HBsAg and peroxidase-conjugated rabbit anti-biotin for 2 hr at 40°C in a water bath. After being washed, the beads were transferred to individual assay tubes, and 300 μl of *o*-phenylenediamine·2HCl was added to each tube. After incubation at room temperature for 30 min the reaction was stopped by adding 1 ml of 1 M H_2SO_4, and absorbance was measured at a dual wavelength of 492:600 nm.

Isotype Distribution of the Anti-HBsAg Response. The isotype distribution of the anti-HBsAg response was determined by using the mouse typer sub-isotyping kit (Bio-Rad). Sera were diluted in PBS/0.05% Tween 20 (PBS-T) and incubated on HBsAg-coated beads for 2 hr. The beads were washed, and a 1:2 dilution of rabbit anti-mouse subclass-specific antiserum [Bio-Rad, enzyme immunoassay (EIA) grade Mouse Typer panel] was added for 1 hr. The beads were washed again, and a 1:3000 dilution of goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad) was added. After 1 hr the beads were washed and developed in a peroxidase substrate solution [2,2′-amino-di(3-ethyl-benzothiazoline sulfonate) and H_2O_2] for 30 min, and results were read on an automated EIA reader at 405 nm. The entire assay was done at room temperature, and each wash step consisted of five washes in PBS-T.

***In Vitro* Proliferation of Mouse Lymph-Node T Cells.** BALB/c mice were immunized in the hind footpads with tobacco-derived or yeast-derived HBsAg. The amount of antigen administered and the schedule of immunization were as described above. One week after the third injection the animals were sacrificed, the popliteal nodes were collected and teased apart, and the cells were purified as described (4). The enriched T cells were adjusted to a concentration of 2.5×10^5 cells per well. One hundred microliters of enriched T cells per well were plated in 96-well flat-bottomed plates along with 5×10^5 irradiated syngeneic spleen cells per well as a source of antigen-presenting cells. Stimuli diluted in complete medium were added to the cells in triplicate cultures. The stimuli were as follows: tobacco- and yeast-derived recombinant HBsAg (0.5, 0.1, 0.05, and 0.025 μg per well), monoclonal anti-idiotype 2F10 (5) and isotype control antibody 2C3 (50 and 20 μg of each antibody per well), 2F10 peptide (6), HBsAg peptide (4), and control peptide (0.5 and 0.25 μg of the peptides per well), ConA (1 μg per well), or medium alone. The cells were then cultured for 120 hr as described (4, 6). Proliferation, as measured by [^{3}H]thymidine incorporation, was determined by liquid scintillation spectroscopy. Results are expressed as the mean cpm of [^{3}H]thymidine incorporated in triplicate wells.

RESULTS AND DISCUSSION

rHBsAg Derived from Transgenic Tobacco Can Induce Specific Antibodies. Fig. 1 shows that BALB/c mice immunized with the tobacco-derived rHBsAg can elicit a specific antibody response that compares favorably with that obtained after immunization with the yeast-derived rHBsAg (Merck Sharpe & Dohme). Specific antibodies were first detected in both groups of mice 1 week after the last antigen injection. In the group immunized with yeast-derived rHBsAg, the responses rose sharply, reached near-maximum values by week 3, and remained at a plateau thereafter. In comparison, the antibody levels in the group immunized with rHBsAg derived from transgenic tobacco increased steadily, reached maximum values at week 7, and remained at relatively high levels for the entire time course studied. This comparison is particularly relevant in view of the fact that the tobacco-derived rHBsAg was a crude protein preparation in which the HBsAg was <3% of the total protein in the immunization sample. The results suggest that elaborate purification procedures may not be necessary to ensure that plant-derived rHBsAg is sufficiently immunogenic.

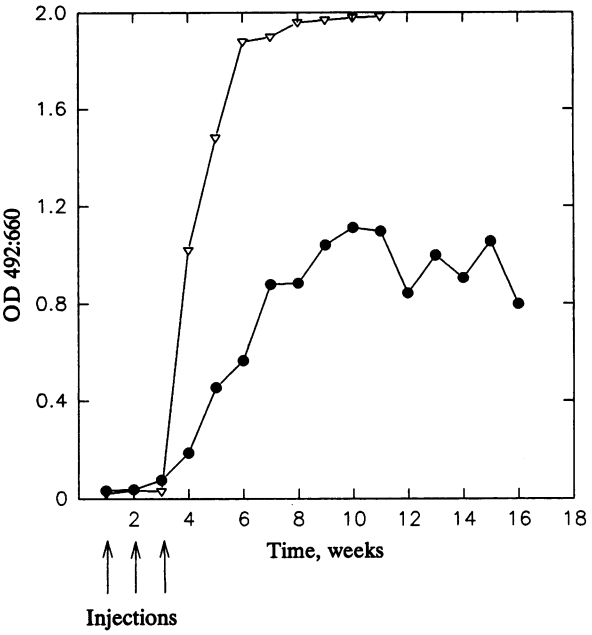

FIG. 1. Antibody response elicited by immunization with tobacco-derived rHBsAg and yeast-derived rHBsAg in BALB/c mice. Results are expressed as OD 492:660 nm at a 1:10 dilution for sera from the tobacco-derived rHBsAg experimental group (●) and 1:20 dilution for sera from the yeast-derived rHBsAg control group (▽).

Isotype Distribution of Anti-HBsAg Responses. An analysis of the isotype distribution of the anti-HBsAg response showed that mice immunized with the tobacco-derived rHBsAg produced HBsAg-specific antibody of all the IgG subclasses and also IgM antibodies (Table 1). In contrast, mice immunized with yeast-derived rHBsAg produced predominantly IgG1 and substantial amounts of IgG2b antibodies and lower levels of IgG2a and IgM antibodies. No response was elicited in the IgG3 subclass. In mice IgG3 antibodies along with IgG2a, IgG2b, and IgM are capable of activating complement, whereas IgG1 antibodies do not. In humans the IgG3 subclass

Table 1. Isotype distribution of anti-HBsAg response

Immunogen	Week	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
Tobacco HBsAg*	1	0.34	0.30	0.62	0.91	0.70	0.03
	4	0.38	0.41	0.98	1.18	0.76	0.03
	13	0.34	0.35	0.97	1.02	0.87	0.02
Yeast HBsAg†	3–5	1.69	0.22	0.81	0.10	0.24	ND

Comparison of the isotype distribution of the anti-HBsAg response in mice immunized with either tobacco-derived rHBsAg or yeast-derived rHBsAg. All sera were tested at 1:50 dilution. Results are expressed as OD at 405 nm. ND, not determined.
*Values represent the mean from sera of two mice.
†Values represent the average of sera from two mice bled 3, 4, and 5 weeks after the third injection.

of antibodies is also the most efficient at activating the complement pathway.

Mouse T-Cell Stimulation. To examine the fidelity of the T-cell epitope expressed by the tobacco-derived rHBsAg, we examined the *in vitro* proliferative response of mouse lymph-node T cells. BALB/c mice were primed *in vivo* with the tobacco-derived rHBsAg, and the primed T cells were tested for their ability to proliferate *in vitro* to appropriate stimuli. Table 2 shows that T cells from mice primed *in vivo* with the tobacco-derived rHBsAg can be recalled *in vitro* to proliferate upon stimulation with not only the immunizing antigen (tobacco-derived rHBsAg) but also to yeast-derived rHBsAg and to a synthetic 9-aa peptide designated as the HBsAg peptide (CTKPTDGNC; ref. 4), which represents a partial analogue of the group-specific *a* determinant located in the S region (139–147) of HBsAg. This result is significant in that it indicates that the tobacco-derived rHBsAg had primed T cells *in vivo* that are crossreactive *in vitro* with the commercially used yeast-derived rHBsAg and that the reactivity is directed to the peptide sequence that is a part of the protective *a* determinant (S139–147) of HBsAg.

Proliferation of T cells was also elicited by a monoclonal anti-idiotype 2F10, and a linear 15-mer synthetic peptide was derived from the anti-idiotype sequence. We had previously shown (5) that monoclonal antibody 2F10 is an internal image anti-idiotype that mimics the group-specific *a* determinant of HBsAg. We had also demonstrated that immunization with this anti-idiotype can elicit HBsAg-specific antibodies and T cells in several mouse strains (4). Further, we had identified a 15-aa sequence (AVYYCTRGYHGSSLY) in the heavy-chain hypervariable region of this anti-idiotype that has partial residue homology with sequences of the *a* determinant epitopes of HBsAg (6). A 15-aa synthetic peptide corresponding to this area of homology can generate anti-HBsAg-specific antibodies when injected into mice and prime murine lymph node cells for an *in vitro* HBsAg-specific T-cell proliferative response (6). Thus, the ability of the anti-idiotype 2F10 and the 2F10 peptide to stimulate *in vitro* proliferation of T cells (Table 2) obtained from the lymph nodes of mice primed *in vivo* with the tobacco-derived rHBsAg further substantiates the observation that this antigen retains the appropriate epitopes expressed by both the yeast-derived rHBsAg and the anti-idiotype that mimics it.

Table 2. *In vitro* proliferation of T cells primed with transgenic plant-derived HBsAg

Stimulus	Concentration, μg/well	[^{3}H]Thymidine incorporation, cpm
Tobacco HBsAg	0.5	34,261
	0.1	20,091
	0.05	32,056
	0.025	15,175
Yeast HBsAg	0.5	41,247
	0.1	36,181
	0.05	25,576
	0.025	12,313
Anti-idiotype (2F10)	50	26,474
	20	9,337
	10	3,049
Isotype control (2C3)	50	676
	20	910
	10	2,165
2F10 peptide	0.5	29,612
	0.25	8,930
HBsAg peptide	0.5	39,357
	0.25	28,586
Control peptide	0.5	1,003
	0.25	1,023
None (medium)		3,171

The tobacco-derived rHBsAg can prime T cells *in vivo* that can be stimulated *in vitro* by yeast-derived rHBsAg, anti-idiotype 2F10, and synthetic peptides derived from HBsAg and the anti-idiotype. Proliferation was assessed as described. Results are expressed as the average cpm of [^{3}H]thymidine incorporation in two separate experiments.

CONCLUSIONS

The field of vaccine development has advanced dramatically over the last 10 years as researchers have coupled advanced theories about the immune response with recombinant DNA technology to design novel subunit vaccines. At present one recombinant subunit vaccine (for HBV) is approved for commercial use in humans (7), and numerous others are being tested. In recognition of the potential international impact of new vaccine technology, a consortium of philanthropic organizations and the World Health Organization refocused their attention on vaccine research in 1992 by presenting a Children's Vaccine Initiative. The focus of the Children's Vaccine Initiative is to encourage the discovery of technology that will make vaccines available to developing countries where they are needed most (8). Priority areas identified were lower cost vaccines, vaccines that can be easily distributed in countries lacking refrigeration and health care infrastructure, and oral vaccines generally. Our strategy has been to determine whether genetically engineered plants can be used in lieu of traditional fermentation systems to produce candidate vaccines; if so, technology transfer to developing countries for vaccine production could be as simple as providing plants or plant seeds (9).

Because our previous studies had shown that tobacco leaf cells can express rHBsAg (3), we were interested in examining the immunological characteristics of this material and comparing it with yeast-derived rHBsAg, which is the current source of commercially available HBV vaccine. The results presented here conclusively show that rHBsAg from transgenic tobacco elicits HBsAg-specific B- and T-cell responses in mice; hence both the B- and T-cell epitopes are preserved in the plant-derived material. The development of an anti-HBsAg-specific antibody response to the tobacco-derived rHBsAg was qualitatively similar to that seen after immunization with the yeast-derived rHBsAg. Although the response to the tobacco-derived rHBsAg was of a slightly lower magnitude, the tobacco preparation used for immunization was relatively impure, with the rHBsAg representing <3% of the total protein in the sample in comparison with the highly purified yeast antigen. The ability of such an impure preparation to produce the antibody responses observed in this study is particularly significant because foreign antigens expressed in edible plant tissues for use as oral vaccines also will consist of impure mixtures with numerous other plant proteins.

The ability of the tobacco-derived rHBsAg to prime T cells for a subsequent HBsAg-specific T-cell proliferative response to *in vitro* stimulation (Table 2) with yeast-derived rHBsAg indicated that the T-cell epitopes of both antigens are similar. More importantly, the primed T cells were also stimulated by a 9-aa synthetic HBsAg peptide that represents a partial sequence of the group-specific *a* determinant located in the S region (139–147) of HBsAg. The integrity of the T-cell epitope was also tested by using another set of reagents—namely, an internal image anti-idiotype (2F10) that mimics the group-specific *a* determinant of HBsAg and a 15-aa synthetic peptide (2F10 peptide; ref. 6) that represents the area of homology between the anti-idiotype 2F10 and the *a* determinant of HBsAg. T cells from mice immunized with the tobacco-derived rHBsAg proliferated specifically to anti-idiotype 2F10 and to the 2F10 peptide but did not proliferate to an isotype-matched control antibody or to a control peptide. Collectively these results show that the transgenic tobacco-derived rHBsAg

retains the immunological properties needed to elicit HBsAg-specific B- and T-cell responses.

In our studies of antigen production in plants thus far, we have used tobacco as the initial test system. This approach has allowed analysis of gene expression in comparison with an extensive literature that has developed around this facile tool for molecular biologists. Unfortunately, tobacco tissues are rich in toxic alkaloids that prevent direct animal feeding studies. Now that it is obvious that there are no inherent limitations to the expression or aggregation of rHBsAg in tobacco cells and that the protein expressed is immunologically active, we have developed transformation systems for other plant species. For example, by using a patatin promoter that drives tuber-specific protein expression (10), we have achieved expression levels of rHBsAg in potato tubers comparable with those in tobacco leaves. Because we anticipate that accumulation levels of any foreign protein in plant cells may be limited when the polypeptide simply accumulates in the cell cytoplasm, studies must be directed to the construction of transformation vectors that contain DNA sequences that target polypeptides to specific cellular compartments. Furthermore, expression levels in edible plant tissues must be maximized so that oral immunogenicity of HBsAg in untreated edible plant tissues can be evaluated.

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