Small-Scale Extraction of Transgenic Tobacco Cells

To measure both the levels of total soluble protein (TSP) and expression of antibody, 1 ml of suspension culture was transferred into a 2 ml microtube. One-tenth ml of 10× complete protease inhibitor mix (Roche Diagnostics K.K., Tokyo, Japan) in 0.5 M Tris pH 7.5 and 0.15 g each of 0.1 and 1 mm diameter glass beads (Sigma-Aldrich Japan K.K., Tokyo, Japan) were added. Tubes were shaken at 30 Hz for 5 min by Mixer Mill MM300 (Qiagen K.K., Tokyo, Japan) and centrifuged at 15,000*g* for 10 min to obtain the plant extract supernatants. TSP concentrations were determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Preparative Scale Extraction of Transgenic Tobacco Cells

For antibody purification, large-scale (100 ml × n) tobacco suspension cultures were sampled at 10 days after initiation of culture. The cells and media were separated by vacuum filtration through the filter paper (Advantec, ToyoRoshi, Tokyo, Japan). Media were centrifuged for 60 min at 18,000*g* and the supernatants were filtered through 0.45 μm filters for loading to the liquid chromatography. Cells were frozen overnight and thawed in 2 volumes of (v/v) extraction buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1× complete protease inhibitor cocktail). Cells were homogenized (Physcotron, Microtech Co., Tokyo, Japan) until over the 80% of cells were disrupted, as determined by microscopic observation. Cell debris was removed by centrifugation at 18,000*g* for 30 min. The protein in the supernatant was concentrated by 50% ammonium sulfate precipitation and the resulting pellet was suspended in phosphate-buffered saline (PBS). The suspension was filtered through 0.45 μm filters before liquid chromatography.

Liquid Chromatography

The filtered protein solutions were loaded onto a 10 ml Poros Protein A Plastic column (Applied Biosystems Japan Ltd., Tokyo, Japan) at a flow rate of 10 ml/min using a chromatography system (BIO CAD sprint, Applied Biosystems Japan Ltd.). After washing with 10 column volumes of PBS, bound protein was eluted with 5 column volumes of 0.1 M citrate pH 3.0/0.1 M NaCl and collected in 0.2 volumes of 1 M Tris for readjustment to a neutral pH. The buffer was changed to PBS, the eluate was concentrated using a 100 kDa molecular size cut-off filter (CentriPlus, Millipore Co., Billerica, MA), and filtered through 0.22 μm filters.

ELISA

ELISA plates (E.I.A/R.I.A; Costar, Corning, Inc., Acton, MA) were coated with 0.2 μg/well of purified adr subtype HBsAg (Meiji Dairies Corp., Tokyo, Japan) derived from huGK-14 hepatoma cells. Plates were blocked overnight with 3% skim milk (Wako, Tokyo, Japan) in PBS to inhibit non-specific binding. After washing with PBS containing 0.05% Tween-20 (PBST), 100 μl of either twofold serially diluted plant extracts or purified antibodies were added to the plates for 1 hour incubation at room temperature. Plates were rewashed with PBST, 100 μl of either horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated goat anti-human IgG specific antibody (Jackson Immuno-Research, West Grove, PA) at a dilution of 1:5,000 was added, and the plates were incubated for an additional 1 hr at room temperature. Unbound HRP-conjugate was washed away with PBST. Bound antibodies were detected by incubation for 30 min with tetramethylbenzidine base (TMB, Bio-Rad) and the optical density at 650 nm (OD_{650}) in each well was read. When AP conjugate was used, unbound conjugate was washed away with PBST, bound antibodies were detected by incubation for 1 hr at 37°C with *p*-nitrophenyl phosphate (*p*NPP) substrate (Bio-Rad), and the OD_{405} was read. The ELISA antibody titer was expressed as the highest dilution giving an OD_{405} of 0.1 U above that of the control wells without antigen.

ELISA Quantification of the Human IgG in Plant Extracts

ELISA plates were coated with anti-human IgG (0.5 μg/well; Jackson ImmunoResearch) then the test samples were added to the plates for 1 hr at 37°C following by blocking with 3% skim milk in PBS. Serially diluted human IgG (Jackson ImmunoReserch) was used as standard in all assays. After incubation, AP-conjugated anti-human IgG (Jackson ImmunoResearch) at a dilution of 1:5,000 was added and the plates were incubated for an additional 1 hr at room temperature. Plates were washed and incubated with *p*NPP substrate (Bio-Rad) for 1 hr at 37°C and the OD_{405} was read.

Inhibition ELISAs

Affinity measurement was carried out by inhibition ELISA [Burton et al., 1991]. ELISA plates were coated overnight at 4°C with 0.2 μg/well of purified HBsAg. Wells were blocked with 3% skim milk in PBS. The amount of each MAb that gave half-maximum binding to HBsAg-coated plates was determined by ELISA. HBsAg was diluted by twofold steps from 2 μg/ml (the average MW of the proteins in the HBsAg manufactured by Meiji Dairies Corp. is 25.5 kDa) to 62.5 ng/ml in PBS with 3% skim milk and mixed with the same volume of 2× antibody solution. A 50 μl/well aliquot of the HBsAg-antibody mixture was added to the plates. The plates were incubated for 90–120 min at room temperature and washed three times with PBST before addition of a 1:5,000 dilution of HRP-conjugated goat anti-human IgG specific antibody (Jackson ImmunoResearch). After 1 hr of incubation, followed by washes, TMB (Bio-Rad) was added. The OD_{650} was read after 30 min. The HBsAg concentration that gave 50% inhibition of maximum binding reflected the mAb affinity, which is

approximately the same as the binding constant [Burton et al., 1991].

Flow Cytometric Analysis

The hepatocellular carcinoma cell line PLC/PRF/5 (Alexander cell line), which expresses HBs antigens (adw) on the cell surface, was cultured in 3 cm plastic dishes (Asahi Technoglass Co., Chiba, Japan). When cells reached confluence, medium was discarded, 1 ml of each antibody was added to $2–5 \times 10^5$ cells/dish, and cultures were held on ice for 1.5 hr. After washing twice with PBS, a 1:10 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG specific antibody (ICN Pharmaceuticals, Inc., Aurora, OH) was added and plates were incubated for 30 min at 37°C. At the end of the incubation, cells were trypsinized, filtered through nylon mesh, and sorted by flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ).

Microcytotoxicity Assay

Complement-dependent cytotoxicity activity was assayed to test the reactivity of the MAb Fc region with complement. The Alexander cells were cultured to confluency in a 96-well plate, incubated on ice for 1.5 hr with 50 µl of purified test antibody, followed by incubation with rabbit complement (Veritas, Tokyo, Japan). Ten microliters of Cell Counting Kit8 (DOJIN, Tokyo, Japan) was added and cell viability was estimated by measurement of OD_{450}, as specified by the manufacturer's protocol.

RESULTS

Generation of Transgenic Tobacco Cells

The constructs, designated p29, p30, and p31, are shown in Figure 1. To obtain the active full length IgG antibodies, protein fragments of heavy and light chain must be delivered to the secretary pathway through the ER. Three series of transgenic tobacco BY-2 suspension cells (B29, B30, and B31) were obtained by *Agrobacterium*-mediated transformation with the numerically corresponding binary plasmids. Small-scale protein extracts of 7 day cultures of several kanamycin resistant clones from liquid medium culture were used for ELISA. The B29-4, B30-3, and B31-7 cell lines, with the specific cell lines designated after the hyphen, were selected for further analysis because they gave OD values in ELISA and cell growth in the liquid medium that were higher than the other cell lines that were tested (data not shown).

The time course of the anti-HBs synthesis was determined by quantifying amounts of human IgG proteins in the total extracts of the culture that consisted of both the medium and cell extracts, and TSPs were quantified (Fig. 2). The anti-HBs titers and human IgG proteins showed the almost same time course of synthesis, with peak titers observed at 10 days after inoculation, but the time courses were different from that of TSP. The amounts of both IgG and TSP in the

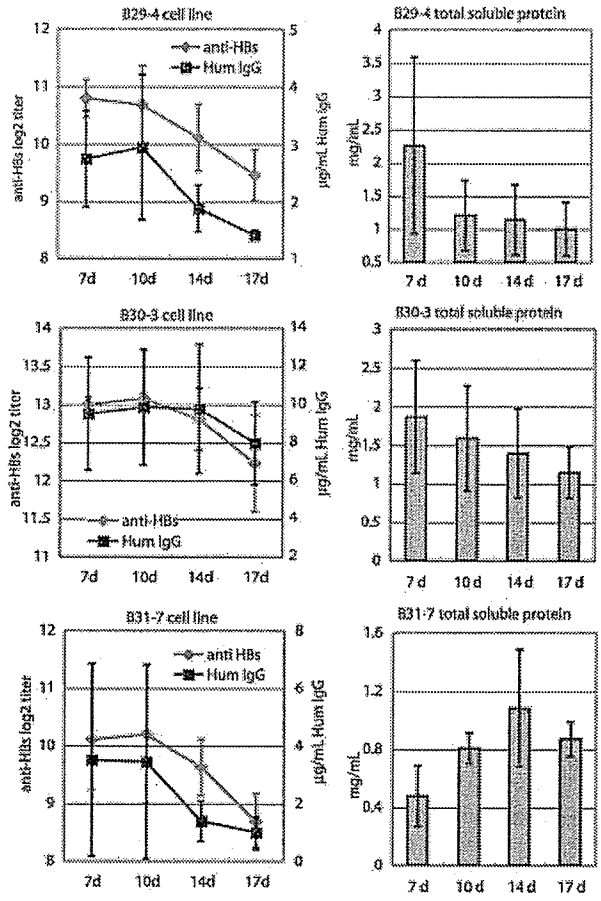

Fig. 2. Time course of antibody production in transgenic cell lines. B29-4, B30-3, and B31-7 cultures were sampled from 7 to 17 days after inoculation. Both anti-HBs titers and human IgG in samples were measured by ELISA. The amounts of total soluble proteins (TSP) were measured by Bradford assay. The results are presented as the mean ± SD (n = 3).

extracts of B30-3 cells were relatively stable from 7 to 17 days. In contrast, the B29-4 cells had rapid reductions in the amount of TSP after 10 days. The B31-7 cells also had reduced levels of IgG after 10 days, despite the peak of TSP detected at 10 days. The percentages of human IgG in the TSP were approximately 0.2% for B29-4, 0.6% for B30-3, and 0.25% for B31-7 cells. Despite the differences in the SSs for the three cell lines, the ratios of antibodies in the TSP were of same order, but the time points at which saturation density was reached were different. B30-3 cells grew well and rapidly reached saturation density. However, B31-7 cells grew slowly and did not reach saturation density until 10–14 days.

Purification of the Antibodies From Transgenic Tobacco Cells

Every 10 days the cells and medium from the cultured suspension cells were separated by paper filtration for purification of the recombinant MAb. Figure 3 shows the maximum yields of the preparative scale IgG purifica-

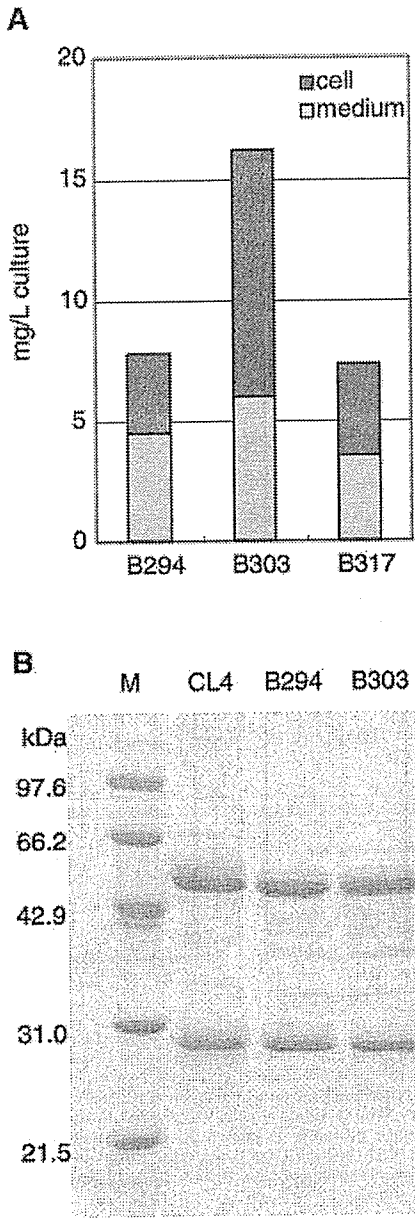

Fig. 3. Antibody purification from transgenic cell lines. A: IgG protein yields from B29-4 and B30-3 cultures. IgGs were purified from both cells and media of 10 day cultures with protein A columns. The B294 and B303 MAbs were quantified by ELISA. The maximum yield of the six times preparative scale purification procedures were indicated. B: SDS–PAGE analysis of purified antibodies. Purified CL4 (from culture medium), B294, and B303 MAbs (from medium and cell extracts) were analyzed using 12.5% SDS–PAGE. M, molecular weight marker.

tion from each of the tobacco cell lines cultured in n × 100 ml scale cultures for 10 days. The quantities of purified antibodies from medium were similar among the B29-4, B30-3, and B31-7 cell lines. However, the quantities of the antibodies recovered from the cells were varied. The maximum value after purification was 16.2 mg of human IgG from 1 L of a 10 day culture of B30-3 cells. The Coomassie-stained SDS–PAGE of purified MAbs (10 μg each), parental CL4MAb, MAb B294, and MAb B303, from both medium and cell extracts is shown in Figure 3B. The total amount of antibody purified from B31-7 culture was insufficient for analysis. The major bands of heavy (H) and light (L) chains of the MAbs were almost all the same size. A difference among the three MAbs was the presence of weakly staining bands between the positions of the H and L chains. The positions of the extra bands were different for the original CL4 and the MAbs produced in plants. MAbs B294 and B303 showed similar patterns of extra bands. It was not clear whether the extra bands were the result of nonspecific binding of the plant proteins to Protein A column or degradation products of the IgGs.

Quantitative Analysis of Plant Produced Antibodies

The antigen binding activities were compared among the purified MAbs, parental CL4, B294, and B303 MAb. The MAbs were quantified by ELISA and then serially diluted over the same range as for quantification for comparison by ELISA of the HBs binding activities by ELISA (Fig. 4.). No obvious differences in antigen-binding were observed among the antibodies when tested from 0.3 μg/ml (=2.0 nM) to 73 pg/ml (=49 pM).

For a more detailed comparison among these MAbs, inhibition ELISA was done to determine the relative affinity constants (Fig. 5). In five independent assays, (data not shown) that used the HBsAg concentrations giving 50% inhibition, the relative affinity constants, which ranged from 1 to 1.26×10^{-8} M, were almost the same among MAbs B294, B303, and CL4.

Antibody Reaction With Cell Surface HBs

The Alexander cells were incubated with HBIg or HBs MAbs and the binding of antibodies was confirmed by flow cytometric analysis (FACS). Human cytomegalovirus (CMV) neutralizing MAb, TI23 (IgG1/kappa), was

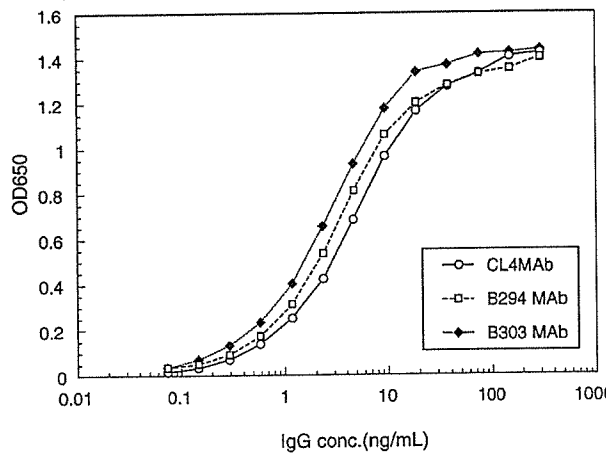

Fig. 4. Quantitative analysis of purified antibodies. Serially diluted solutions of the CL4, B294, and B303 MAbs were analyzed using the anti-HBsAg ELISA. The typical result of five experiments are shown.

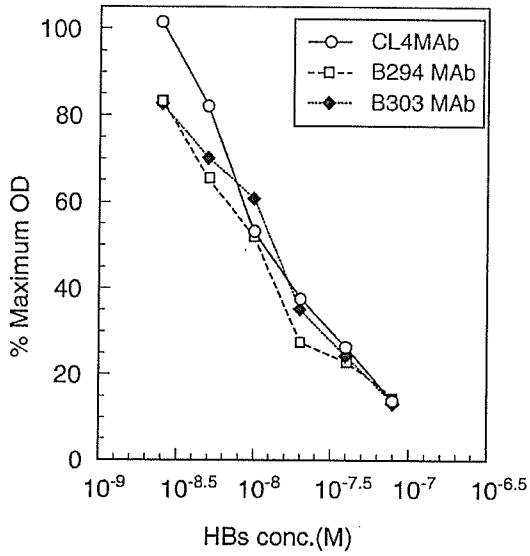

Fig. 5. Inhibition ELISA assays of purified antibodies. Affinities of the CL4, B294, and B303 MAbs were estimated by inhibition ELISA. The HBsAg was used as solid-phase antigen and serially diluted soluble HBsAg was used as inhibitor. The concentration of HBsAg, which gave 50% inhibition of maximum binding reflected MAb affinity, which is approximately the same as the binding constant. The typical result of five experiments is shown.

used as negative control [Masuho et al., 1987]. These cells were clearly labeled by HBIg, CL4, and B303 MAbs (Fig. 6). When the concentration of antibodies was increased to 40 μg/ml for CL4Mab or 20 μg/ml for MAb B303, the FACS profile was altered to a greater extent than when the HBIg concentration was increased to 20 IU/ml.

Complement-Dependent Cytotoxicity

It has been reported that anti-HBs antibodies have complement-dependent cytotoxicity in cells expressing HBs antigens on their surface [Shouval et al., 1982]. To confirm that the Fc domains of plant produced MAbs are functionally equivalent to those of HBIg, complement-dependent cytotoxicity test were done. When Alexander cells were reacted with the parental CL4Mab, MAb B303, positive control HBIg, or negative control TI23 MAb in the presence of rabbit complement, a dose-dependent increase in cytolysis was observed with all anti-HBs antibodies tested, but not with the T123 negative control (Fig. 7). There were no significant differences between the human antibodies and the plant-produced antibody for the complement binding function of the Fc region.

DISCUSSION

This is the report of the production in plant cells of a full-length human IgG that was synthesized using the gene encoding an HBV neutralizing MAb. As such, the plant derived MAb would be expected to be fully active in neutralizing HBV in vivo. The quantities of antibody

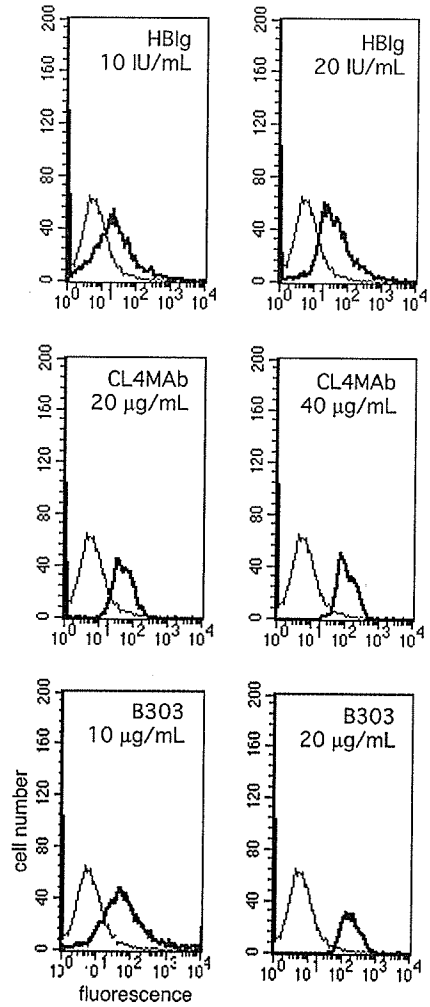

Fig. 6. Binding assay of the antibodies to cell surface HBsAg. The Alexander cells with surface HBsAgs were treated with HBIg, CL4, B303, and TI23 MAbs. Cell-antibody complexes were detected with FITC-conjugated goat anti-human IgG specific antibody and analyzed by flow cytometry. The number of cells are shown on the vertical and immunofluorescence intensity is shown on the horizontal axis. The result of the TI23 MAb (20 μg/ml) treatment is overlaid on each graph as negative control and is shown with a thin line. The typical result of five experiments are shown.

produced were consistent with levels that would be expected from a plant production system [Conrad and Fiedler, 1998]. The average yield of the recombinant protein in our system using plant SS (B303) was usually 0.1–2% of TSP. The MAbs were expressed using three types of signals; specifically, human derived LS, dicotyledonous calreticulin derived SS, and monocotyledonous hordothionin derived SS. The use of hordothionin derived SS transgenic tobacco cells did not consistently result in expression levels of antibody that were sufficient for preparative scale purification. Overexpression of some proteins, when the monocotyledonous signal peptide was used or the processing site of the SS was not correct, were burden for the protein secretion pathway and

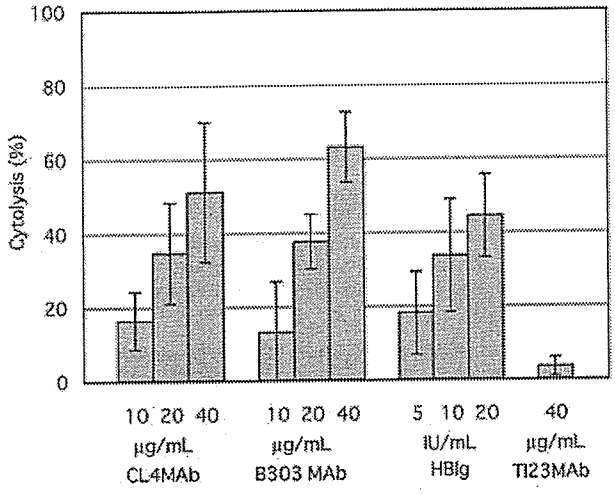

Fig. 7. Antibody induced complement-dependent cytotoxicity. The Alexander cells were treated with CL4 and B303 MAb. Hepatitis B immunoglobulin (HBIg) was used as positive control, and TI23 MAb was used as negative control. Columns and vertical bars represent the mean cytotoxicity values ± SD (n = 3).

resulted in less-than-optimum cell culture conditions. The transgenic tobacco cells containing dicotyledonous SS grew more vigorously and expressed the antibodies more consistently than cells made transgenic with human LS. The human derived signals that originate from the immunoglobulin leader sequence have usually been used for production of antibodies in transgenic plants because it is thought that the correct antibody processing is dependent upon the signal peptide. However, plant derived signal peptide was more suitable for both overexpression of the protein and stable growth of the host culture. Similar results have been reported for the stable expression of the HBs antigen in tobacco cells by plant signal peptide [Sojikul et al., 2003]. When culture conditions and the purification protocols are optimized, yields of full-length recombinant antibodies in tobacco suspension culture were 25 mg/L culture [Fischer et al., 2003]. In addition, when transgenic plants are used as bioreactors as an alternative to suspension cultures, it is easy to scale-up production simply by enlargement of the field area for plant growth.

Even though the transgenic expression systems provide an attractive vision for a source of plant derived MAbs for clinical use, there are problems that need to be solved before implementation of this strategy. Since HBIgs require intravenous injection to prevent the transmission of HBV from mothers to infants, recombinant antibodies used as substitutes for HBIg cannot be antigenic in humans. There are some differences in the protein glycosylation patterns between human and plants. However, studies of mice injected with a recombinant mouse IgG isolated from plants showed that, despite some differences in the glycan groups present on the recombinant antibody, neither the antibody nor the glycans were immunogenic [Chargelegue et al., 2000]. If necessary, the human type of glycosylated antibodies can be expressed in plants. The key technology for transgenic plant production of humanized glycosylated protein has been reported. The introduction of human galactosyltransferase (GalT) into plant cells results in the production of recombinant antibodies with human type β1,4-galactose residues [Palacpac et al., 1999; Bakker, 2001]. The next step for humanization of the glycosylation pattern will be to reduce the plant specific glycan residues, α1,3-fucose and β1,2-xylose. This may be possible by depression of glycosylation enzymes by inhibitory technologies such as RNA interference. Considering the progress in technologies for transgenic plants, many monoclonal antibodies would be produced by plant. Actually, several antibodies produced in plants are now undergoing phase II clinical trials [Fischer et al., 2003].

In this report, we showed that the B303 HBs antibody produced in plants had almost the same affinity and physiological activity as the virus neutralizing parental human monoclonal antibody CL4Mab. The MAb B303 has potential for both prevention and therapy of HBV infection. Characterization of the B294 and B303 MAbs supports the conclusion that these recombinant MAbs derived from the CL4MAb will be a clinical immunotherapeutic for preventing HBV transmission. However, data for CL4MAb or B303 have been obtained using sdr subtype HBV or HBs, and adw subtype HBs expressing cells. The problem of HBV escape mutants following HBV passive immunotherapy points to the need for further analysis of the MAbs against escape mutants. The most common mutations in the HBs protein were at amino acid positions 143–145. These positions are usually essential for recognition of antigen and neutralization by HBs antibodies [Mahoney, 1999]. In vitro studies of the HBV mutants indicated that some polyclonal antibodies more effectively inhibit the secretion of a mutant HBV from infected cells than MAbs [Schilling et al., 2003]. Preparations of polyclonal antibodies are composed of antibodies to single epitope determinants, much like MAbs. It is likely that the combination of some selected MAbs will be an effective means of neutralizing HBV in vivo. At this time, a combination of two MAbs to HBV is being developed as an anti-HBV drug [Galun et al., 2002]. It is necessary to obtain the cDNAs of other MAbs to HBs to produce the MAb cocktail, including CL4MAb, which will be a real substitute of HBIg. It would be desirable to produce the MAb cocktail in the GalT transformed plants. If this is possible, a safe alternative to HBIg will become available. We are confident that the transgenic plant derived biopharmaceuticals will become both safe and economical for promotion of global health.

REFERENCES

An G. 1985. High efficiency transformation of cultured tobacco cells. Plant Physiol 79:568–570.

Bakker H, Bardor M, Molthoff JW, Gomord V, Elbers I, Stevens L, Jordi W, Lommen A, Faye L, Lerouge P, Bosch D. 2001. Galactose-extended glycans of antibodies produced by transgenic plants. Proc Natl Acad Sci USA 98:2899–2904.

Beasley PR, Hwang LY, Stevens CE, Lin CC, Hsieh FJ, Wang KY, Sun TS, Szmuness W. 1983. Efficacy of hepatitis B immune globulin for

perinatal transmission of the hepatitis B virus carrier state: Final report of a randomized double-blind, placebo-controlled trial. Hepatology 3:135–141.

Borisjuk N, Sitailo L, Adler K, Malysheva L, Tewes A, Borisjuk L, Manteuffel R. 1998. Calreticulin expression in plant cells: Developmental regulation, tissue specificity, and intracellular distribution. Planta 206:504–514.

Borisjuk N, Borisjuk L, Logendra S, Petersen F, Gleba Y, Raskin I. 1999. Production of recombinant proteins in plant root exudates. Nat Biotech 17:466–469.

Breedveld FC. 2000. Therapeutic monoclonal antibodies. The Lancet 355:735–740.

Burnouf T, Radosevich M. 2000. Reducing the risk of infection from plasma products: Specific preventative strategies. Blood Rev 14: 94–110.

Burton DR, Barbas CF III, Persson MAA, Koenig S, Chanock RM, Lernar RA. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc Natl Acad Sci USA 88:10134–10137.

Casadevall A, Scharff MD. 1994. Serum therapy revisited: Animal models of infection and the development of passive antibody therapy. Antimicrob Agents Chemother 38:1695.

Centers for Disease Control and Prevention. 2002. Hepatitis B. In: Atkinson W, Wolfe C, editors. Epidemiology and prevention of vaccine-preventable diseases. 7th edition. Atlanta, GA: Public Health Foundation. pp 169–189.

Chargelegue D, Vine N, van Dolleweerd C, Drake PM, Ma J. 2000. A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. Transgenic Res 9:187–194.

Conrad U, Fiedler U. 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: An essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. Plant Mol Biol 38:101–109.

Datla RS, Hammerlindl JK, Panchuk B, Pelcher LE, Keller W. 1992. Modified binary plant transformation vectors with the wild-type gene encoding NPTII. Gene 122:383–384.

Davidov E, Holland J, Marple E, Naylor S. 2003. Advancing drug discovery through systems biology. Drug Discov Today 8:175–183.

Fischer R, Twyman RM, Schillberg S. 2003. Production of antibodies in plants and their use for global health. Vaccine 21:820–825.

Florack DEA, Dirkse WG, Visser B, Heidekamp F, Stiekema WJ. 1994. Expression of biologically active hordothionins in tobacco. Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. Plant Mol Biol 24:83–96.

Galun E, Eren R, Safadi R, Ashour Y, Terrault N, Keeffe EB, Matot E, Mizrachi S, Terkieltaub D, Zohar M, Lubin I, Gopher J, Shouval D, Dagan S. 2002. Clinical evaluation (phase I) of a combination of two human monoclonal antibodies to HBV: Safety and antiviral properties. Hepatology 35(3):673–679.

Guerineau F, Lucy A, Mullineaux P. 1992. Effect of two consensus sequences preceding the translation initiator codon on gene expression in plant protoplasts. Plant Mol Biol 815–818.

Kane MA, Brookes A. 2002. New immunization initiatives and progress toward the global control of hepatitis B. Curr Opin Infect Dis 15: 465–469.

Keller MA, Stiehm ER. 2000. Passive immunity in prevention and treatment of infectious Diseases. Clin Microbiol Rev 13:602–614.

Luehrsen KR, Wet JR, Walbot V. 1992. Transient expression analysis in plants using firefly luciferase reporter gene. Methods Enzymol 216:379–414.

Ma JK-C, Hein MB. 1995. Immunotherapeutic potential of antibodies produced in plants. Trends Biotechnol 13:522–527.

Mahoney FJ. 1999. Update on diagnosis, management, and prevention of hepatitis B virus infection. Clin Microbiol Rev 12:351–366.

Masuho Y, Matsumoto Y, Sugano T, Fujinaga S, Minamishima Y. 1987. Human monoclonal antibodies neutralizing human cytomegalovirus. J Gen Virol 68(5):1457–1461.

Matsui K. 1982. Safety and neutralizing activity against HBV of a human monoclonal anti-HBs antibody. J Nihon Univ Med Assoc 41(12):1163–1171.

Muller R, Gubernatis G, Farle M, Niehoff G, Klein H, Wittekind C, Tusch G, Lautz HU, Boker K, Stangel W, Pichlmayr R. 1991. Liver transplantation in HBs antigen (HBsAg) carriers: Prevention of hepatitis B virus (HBV) recurrence by passive immunization. Hepatology 13:90–96.

Nagata T, Okada K, Takebe I, Matsui C. 1981. Delivery of tobacco mosaic virus RNA into plant protoplast mediated by reverse-phase evaporation vesicles (Liposomes). Mol Gen Genet 184:161–165.

Palacpac NQ, Yoshida S, Sakai H, Kimura Y, Fujiyama K, Yoshida T, Seki T. 1999. Stable expression of human β1,4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns. Proc Natl Acad Sci USA 96:4692–4697.

Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I, Fridlender B. 2002. Plants and human health in the twenty-first century. Trends Biotechnol 20:522–531.

Rayon C, Lerouge P, Faye L. 1998. The protein *N*-glycosylation in plants. J Exp Bot 49:1463–1472.

Sanderfoot AA, Raikhel NV. 1999. The specificity of vesicle trafficking: Coat proteins and SNAREs. Plant Cell 11:629–641.

Schilling R, Ijaz S, Davidoff M, Lee JY, Locarnini S, Williams R, Naoumov NV. 2003. Endocytosis of hepatitis B immune globulin into hepatocytes inhibits the secretion of hepatitis B virus surface antigen and virions. J Virol 77(16):8882–8892.

Sheen J, Hwang S, Niwa Y, Kobayashi H, Glbraith DW. 1995. Green-fluoresent protein as a new vital marker in plant cells. Plant J 8:777–784.

Shouval D, Wands JR, Zurawski VR, Jr., Isselbacher KJ, Shafritz DA. 1982. Selecting binding and complement-mediated lysis of human hepatoma cells (PLC/PRF/5) in culture by monoclonal antibodies to hepatitis B surface antigen. Proc Natl Acad Sci USA 79:650–654.

Sojikul P, Buehner N, Mason HS. 2003. A plant signal peptide-hepatitis B surface antigen fusion protein with enhanced stability and immunogenicity expressed in plant cells. Pro Natl Acad Sci 100: 2209–2214.

Vitale A, Denecke J. 1999. The endoplasmic reticulum: Gateway of the secretory pathway. Plant Cell 11:615–628.