ます。私達の実験は，骨髄へも感染すると考えられる腹腔内感染や胎盤感染も行いましたが，潜伏感染の実験は脳へ直接感染させましたので造血幹細胞での潜伏感染の要因はあまり関与していないのではないかと考えます。

渡辺里仁（創価大学）：latent infection あるいは persistent infection についてお伺いします。確かに再活性化は脳室壁の細胞で起こっていると思います。しかし，遺伝子発現の残り方などから，多分 latent infection は感染して分化した神経細胞でも引き続き起こっているように思います。感染神経細胞で SSPE のように遺伝子が欠損したり mutation を起こしたりしているかどうかは観察されていますでしょうか？

筒井：神経細胞の MCMV な感染における遺伝子の欠損や mutation は調べておりません。神経細胞で早期遺伝子が比較的長期間発現しやすいけれど latency には移っていかないだろうと思います。大脳スライス培養での再活性化の部位と細胞マーカーの結果からは神経細胞からは再活性化してこないと考えました。CMV は増殖が静止状態にあるマクロファージで latency 起ることは文献的に明らかですので，神経細胞で起るかどうかはこれから検討する必要があります。

黒岩俊彦（東京医科歯科大学）：海馬の錐体神経細胞層の NMDA receptor の発現が強く落ちていたと思いますが，あのようになると強い記憶力の障害がでるのではないかと思いますが，実験の動物ではどうであったかということ，臨床症状との対応ではいかがでしょうか？

筒井：現在の研究は感染後 2 週間くらいまでしか調べてありませんので，臨床症状がどうなるかといった解析はまだやってありません。

長嶋（座長）：いまの質問に関連して，CMV にはたくさんの蛋白をコードしていますが，その中のどのような蛋白が NMDA receptor の down regulation に関与しているか分かりませんか？

筒井：まだわかりません。これからそのような問題に取り組んでいきたいと思います。

長嶋（座長）：会員を代表しまして筒井先生に一言お礼を申しあげたいと思います。先生は最初にお示しになりましたように，人体病理の症例からサイトメガロウイルス感染に関する疑問に関しまして非常に多数の実験系を組みまして，ひとつひとつをみごとに解明されました。そして今日はその結果を我々にお話し下さいました。このことは我々病理学者にとりましても，また多くのサエンテイストに深い感銘を受けました。また多くの若い研究者に対して多数の教訓そして勇気を与えて下さいました。先生がこれからもお元気で研究を続けて下さいますようお祈りします。

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Bacterial Expression of a Human Monoclonal Antibody-Alkaline Phosphatase Conjugate Specific for *Entamoeba histolytica*

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We previously produced human monoclonal antibody Fab fragments specific to *Entamoeba histolytica* in *Escherichia coli*. In order to use these Fab fragments for diagnostic purposes, an expression vector to produce a fusion protein of Fab and alkaline phosphatase (PhoA) in *E. coli* was designed and constructed. The *E. coli* PhoA gene was fused to the 3′ terminus of the gene encoding the heavy-chain Fd region. The kappa and Fd genes from a previously prepared antibody clone, CP33, which is specific for the 260-kDa lectin of *E. histolytica*, were used as human antibody genes. When the fusion protein of CP33 and PhoA was incubated with paraformaldehyde-fixed trophozoites of *E. histolytica* and developed with a substrate, the trophozoites appeared to be stained. These results demonstrate the feasibility of bacterial expression of a human monoclonal antibody-PhoA conjugate specific for *E. histolytica* and that the antibody can be used to detect *E. histolytica* antigen without the use of chemically conjugated secondary antibodies.

Amebiasis caused by infection with *Entamoeba histolytica* is one of the most important parasitic diseases not only in developing countries but also in developed countries. It has been estimated that 50 million people develop amebic colitis and extraintestinal abscesses, resulting in 40,000 to 100,000 deaths annually (3). Laboratory diagnosis of intestinal amebiasis is usually based on the microscopic detection of the organism in stool samples. However, nonpathogenic commensal *Entamoeba dispar*, which is morphologically identical with but genetically distinct from *E. histolytica*, has been identified recently as a separate species (9). Since treatment of *E. dispar* infection is not required, accurate diagnostic tools to discriminate between the two species are needed (3).

The application of monoclonal antibodies (MAbs) is one of several strategies for specific and sensitive diagnoses of infectious diseases. A number of MAbs which react specifically with *E. histolytica* or *E. dispar* have been produced by hybridoma technology (14, 16, 19–21). It has been reported that some MAbs were useful for detecting *E. histolytica* antigen in fecal and serum samples by sandwich enzyme-linked immunosorbent assay (1, 2, 11, 12). Recently, a new technology to produce a Fab fragment or single-chain Fv fragment in *Escherichia coli* has been established (4, 6, 15). The construction of vectors for the production of Fab in *E. coli* has also been reported (22, 24). When mouse immunoglobulin genes derived from a hybridoma producing *E. histolytica*-specific MAbs were expressed in this system, the specificity of the recombinant mouse Fab was comparable to that of the parent antibody (22). More recently, recombinant human MAb Fab fragments specific for *E. histolytica* have also been prepared from peripheral lymphocytes of a patient with an amebic liver abscess and of an asymptomatic cyst carrier (8, 18, 23). In order to use these human Fabs for diagnostic purposes, we report here the bacterial expression of a human Fab-alkaline phosphatase (PhoA) conjugate specific for *E. histolytica*.

The phagemid vector pRPLS/Fab1 (24) was digested with restriction enzymes *Not*I and *Eco*RI. A synthetic DNA linker consisting of two oligonucleotides (5′-GGCCGCAGGTGGC GGAGGTTCTGGTGGCGGAGGTTCTGGTGGCGGAGG TTCTAGACTCGAGTAAG-3′ and 5′-AATTCTTACTCGAG TCTAGAACCTCCGCCACCAGAACCTCCGCCACCAG AACCTCCGCCACCTGC-3′) was inserted into the *Not*I/*Eco*RI site of pRPLS/Fab1, thus creating a 15-mer Gly4Ser linker and recognition site for *Xba*I and *Xho*I. The resulting phagemid, pFab1-L, was digested with restriction enzymes *Xba*I and *Xho*I. To clone the *E. coli* PhoA gene, *E. coli* XL1-Blue was alkali lysed, neutralized, and subjected to PCR amplification. Two synthetic primers (5′-CCTCTAGAGGTACCCCAGAAATGCCTGTT CTAGAAA-3′ and 5′-GGCTCGAGTTTAAGCCCCAGAG CGGC-3′) were used to amplify 1.45 kb of the PhoA gene (17). The amplified gene was digested with *Xba*I and *Xho*I and subcloned into the *Xba*I/*Xho*I site of pFab1-L, resulting in phagemid pFab1-PhoA. This phagemid, pFab1-PhoA, was digested with *Xho*I and *Eco*RI. A synthetic DNA linker consisting of two oligonucleotides (5′-TCGAGGGTGGCGGAGGTTCT CATCACCATCACCATCACTAAG-3′ and 5′-AATTCATGG TGATGGTGATGGTGATGAGAACCTCCGCCACCC-3′) was inserted into the *Xho*I/*Eco*RI site of pFab1-PhoA, thus creating a 15-mer Gly_4Ser linker and a His_6 tag. The resulting plasmid was named pFab1-PhoA-H (Fig. 1).

As the source of human immunoglobulin genes, the kappa and Fd genes from a previously prepared antibody clone, CP33 (23), which is specific for the 260-kDa lectin of *E. histolytica*, were used for production of the fusion protein. The DNA fragment containing light- and heavy-chain genes was obtained by *Nhe*I/*Not*I digestion of pFab1-His2. The fragment was ligated with pFab1-PhoA-H and then introduced into compe-

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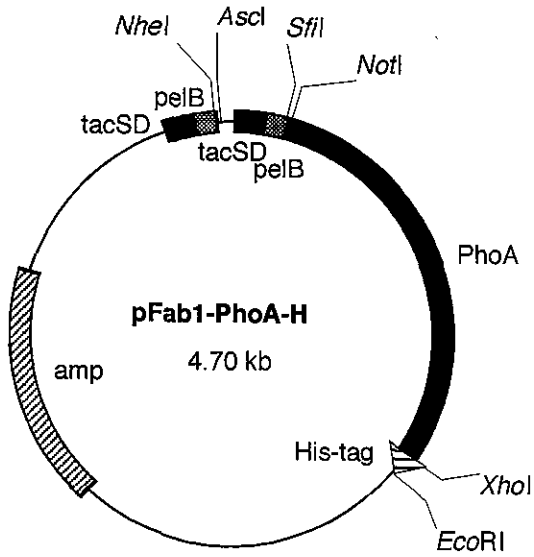

FIG. 1. Structure of plasmid vector pFab1-PhoA-H used for expression of the fusion protein of Fab and alkaline phosphatase. Genes encoding the light chain and the Fd region of the heavy chain are ligated into the *NheI*/*Asc*I and *Sfi*I/*Not*I sites, respectively. tacSD, tac promoter Shine-Dalgarno sequence; pelB, signal sequence of pectate lyase of *Erwinia carotovora*; PhoA, gene for alkaline phosphatase; His-tag, gene for hexahistidine tag; amp, gene for ampicillin resistance.

tent *E. coli* JM109. The bacteria were spread on Luria-Bertani plates containing 50 μg of ampicillin per ml, and the vector with the inserts was selected. The positive clone was cultured in 1 liter of super broth (30 g of tryptone, 20 g of yeast extract, 10 g of MOPS [morpholinepropanesulfonic acid] per liter [pH 7]) containing ampicillin at 37°C until an optical density at 600 nm of 0.5 was achieved. Isopropyl-β-D-thiogalactopyranoside was added to the cultures to a final concentration of 100 μM, and the cultures were then incubated at 30°C for 12 h to achieve optimal expression. The bacteria were pelleted by centrifugation at 6,000 × *g* for 20 min, suspended in 20 ml of phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The lysates were centrifuged at 12,000 × *g* for 30 min, and the supernatant was filtered through 0.2-μm-pore-size syringe filters (Iwaki, Tokyo, Japan). Purification of the fusion protein from the supernatant was performed by affinity chromatography with His•Bind resin (Novagen, Madison, Wis.) in accordance with the manufacturer's instructions. Purified fusion protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (22). Western immunoblot analysis was also performed as previously described (22). The horseradish peroxidase (HRP)-conjugated goat immunoglobulin G (IgG) fraction specific to the human kappa chain (Organon Teknica, Durham, N.C.) and HRP-conjugated rabbit IgG fraction specific to alkaline phosphatase (Rockland, Gilbertsville, Pa.) were used for detection.

Approximately 2 × 10^5 trophozoites of *E. histolytica* HM-1:IMSS cultured axenically in BI-S-33 medium (10) were incubated on acetone-washed coverslips at 37°C for 30 min. The trophozoites were fixed with 4% paraformaldehyde in PBS for 30 min and then washed three times with PBS. After blocking with 5% bovine serum albumin was conducted for 15 min, the cells were incubated with the recombinant protein (50 μg/ml) for 30 min. After the cells were washed with PBS, development was conducted with a Vector red alkaline phosphatase substrate kit I (Vector Laboratories, Burlingame, Calif.) for 30 min in accordance with the manufacturer's instructions. Microscopic observation of the cells was performed under bright-field and fluorescent conditions by using a Nikon (Tokyo, Japan) XF-EFD2 fluorescence microscope.

SDS-PAGE analysis of the purified fusion protein of CP33 and PhoA revealed the expected sizes of two bands with apparent molecular masses of 25 and 75 kDa, although minor bands with apparent molecular masses of 50 kDa were also present (Fig. 2A). With Western immunoblot analysis, the 25-kDa band was recognized by an anti-human kappa chain goat antibody (Fig. 2B, lane 1). On the other hand, the 75-kDa band was detected by an anti-PhoA rabbit antibody, indicating that the molecule was a fusion protein of the Fd fragment and PhoA (Fig. 2B, lane 2). When the fusion protein of Fab-PhoA was incubated with paraformaldehyde-fixed trophozoites of *E. histolytica* and developed with the substrate, the surfaces of the trophozoites were stained clearly under both bright-field and fluorescent conditions (Fig. 3).

Recombinant human antibodies have been developed re-

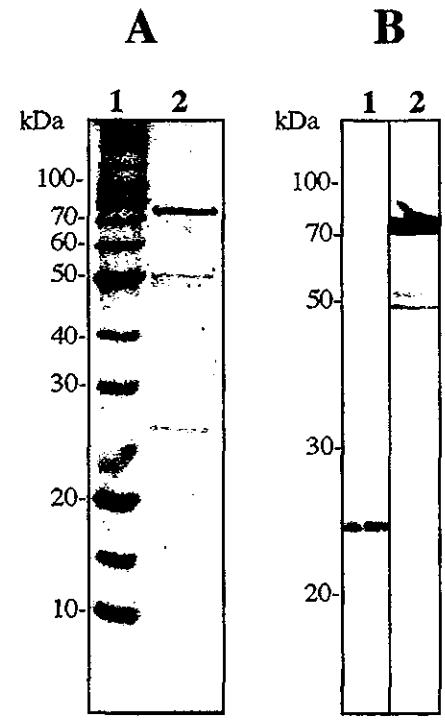

FIG. 2. SDS-PAGE (A) and Western immunoblot (B) analyses of a purified fusion protein of human Fab CP33 and alkaline phosphatase. (A) Two micrograms of the protein was subjected to analysis in 10% gel under reducing conditions and then stained with Coomassie brilliant blue. Lane 1, molecular size markers (BenchMark protein ladder; Life Technologies, Gaithersburg, Md.); lane 2, purified CP33-PhoA. Numbers to the left indicate molecular masses of the markers (in kilodaltons). (B) Protein bands were transferred to a polyvinylidene difluoride membrane. Lane 1 was treated with HRP-labeled anti-human kappa chain goat antibody. Lane 2 was treated with HRP-labeled anti-PhoA rabbit antibody.

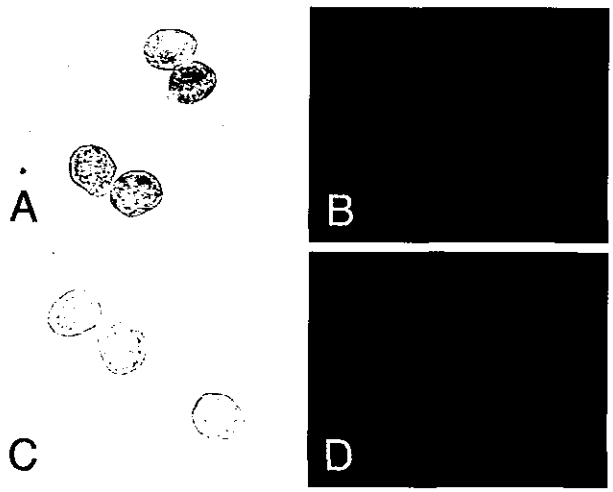

FIG. 3. Immunocytochemistry of *E. histolytica* with a fusion protein of human Fab CP33 and alkaline phosphatase. Paraformaldehyde-fixed trophozoites were treated with the fusion protein (A and B) and then with the substrate Vector red. As the controls (C and D), trophozoites were treated with a supernatant of *E. coli* lysates (vector control). (A and C) Bright-field microscopy; (B and D) fluorescence microscopy with a green filter. Magnification, ×360.

cently for application in immunoprophylaxis, or the treatment of infectious diseases. Although such human antibodies would also be useful for diagnostic purposes, one of the disadvantages of the use of human antibodies for the detection of pathogens in human samples might be reactivity of endogenous immunoglobulins with the secondary anti-human antibodies used in indirect methods. Therefore, direct labeling of the human antibody with enzymes is needed to reduce nonspecific binding of the second antibody. It was reported recently that immunoglobulin genes derived from murine hybridoma cells could be expressed in *E. coli* as fusion protein Fab-PhoA (7, 25) or scFv-PhoA (5, 7, 13, 17). The present study demonstrates that the bacterial expression of a human MAb-PhoA conjugate specific for *E. histolytica* is also possible. In addition to the advantage of using the antibody to detect the *E. histolytica* antigen without the need for chemically conjugated secondary antibodies, there is no requirement for experimental animals or reagents and equipment for the culture and cryopreservation of hybridoma cells. Accordingly, the use of this human recombinant antibody also provides an economic benefit.

The antigen recognized with CP33 was the heavy subunit of the galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin of *E. histolytica* (23). It is well known that this lectin molecule is suitable as a target antigen for the detection of *E. histolytica* in fecal and serum samples (1, 2, 11, 12). In conclusion, we propose here that the human Fab-PhoA fusion protein can be used in the diagnosis of amebiasis.

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Transgenic Tobacco Cells Producing the Human Monoclonal Antibody to Hepatitis B Virus Surface Antigen

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The recombinant human monoclonal antibody (MAb) against hepatitis B virus (HBV) surface antigen (HBsAg) was expressed in tobacco suspension cultures. The parental CL4MAb was produced by the Epstein–Barr virus (EBV) transformed human cell line TAPC301-CL4. The CL4MAb cDNA was introduced into tobacco suspension cells by *Agrobacterium* mediated transformation. The monoclonal antibodies (MAbs), B294 and B303, which were derived from CL4 and subsequently produced in plant cells were selected for study. After purification on Protein A columns, B294 and B303 MAbs had anti-HBs relative affinity constants similar to the parental CL4MAb. B303 MAb interacted with cell surface HBsAgs and showed complement-dependent cytotoxicity in a manner that was similar to anti-HBs human immunoglobulins (HBIg) that are used clinically. The results of this study point to the feasibility of producing MAbs to HBsAg in plants as an alternative to HBIg. ***J. Med. Virol. 73:208–215, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: recombinant antibody; transgenic plant; HBV; HBs

INTRODUCTION

Immune serum has been an important therapeutic agent for many infectious diseases since early in the 20th century [Casadevall and Scharff, 1994]. The administration of antibodies to rabies virus and respiratory syncytial virus are typical examples of passive immune therapy [Keller and Stiehm, 2000]. High titer hepatitis B virus (HBV)-neutralizing serum is also administered prophylactically against hepatitis B viral infection. The transmission of HBV occurs on sexual contact, parenteral exposure, such as accidental exposure to HBV in clinical settings, or maternal–neonatal transmission [Centers for Disease Control and Prevention, 2002]. Among the different transmission scenarios, it is the most important to prevent maternal–neonatal transmission for control of HBV and a vaccination program for newborns is ongoing [Kane and Brookes, 2002]. The use of a combination of anti-HBs human immunoglobulins (HBIg) and subunit HBV vaccine effectively prevented the transmission and subsequent infection of infants [Beasley et al., 1983]. HBIg is also used prophylactically for prevention of hepatitis B recurrence in hepatitis B-seropositive liver transplantation recipients [Muller et al., 1991]. HBIg is prepared from the sera of HBsAg antibody positive donors and the safety is guaranteed by rigorous product standards. The pharmaceuticals derived from human plasma or animal cell cultures are produced by new technologies in developed countries, however, production standards have increased both manufacturing costs and prices to the consumer. The high-sensitive viral detection system is recommended for selection of healthy donors for human blood derived pharmaceuticals [Burnouf and Radosevich, 2000]. Donor and production controls HBIg of that use costly technologies are not practical for developing countries. Genetically modified plants, capable of producing recombinant protein therapeutics, such as human monoclonal antibodies (MAbs), would be good choices for a number of reasons. Plant cells have fewer human infectious agents than human plasma and mammalian cells. Only sucrose, minerals, and some plant hormones are needed to grow the plant tissue cultures. Plants grown in either the field or greenhouses require only light, water, and a few agrochemicals. Because plants do not need animal serum or other animal-derived nutrients, they are safe and inexpensive hosts for production of the recombinant pharmaceutical proteins such as MAbs [Fischer et al., 2003]. The steps

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that are required in mammalian cells for expression of full-length antibodies, such as protein folding, assembly, endoplasmic reticulum (ER)-mediated glycosylation and Golgi-mediated glycan processing, also occur in plants [Ma and Hein, 1995; Rayon et al., 1998; Sanderfoot and Raikhel, 1999; Vitale and Denecke, 1999]. Many recombinant biopharmaceuticals, including antibodies, have been developed in recent years [Breedveld, 2000; Raskin et al., 2002; Davidov et al., 2003]. In theory, the production of these new drugs could be shifted from animal to plant cells and scaled to the desired level. However, the transgenic technology for generation of biopharmaceuticals requires additional research before it becomes acceptable in our society.

The human cell line TAPC301-CL4 originated from Epstein–Barr virus (EBV) transformed B-cell lines that were derived from peripheral blood of a healthy human volunteer with high titer anti-HBs (adr subtype). The CL4MAb (IgG1/kappa) produced by the TAPC301-CL4 cells neutralized the HBV (adr subtype) activity in vivo [Matsui, 1982]. However, the MAbs from EBV transformed cells are not recommended for human use. In this report, the cDNA of the whole CL4MAb was cloned and introduced into the genome of suspension cultures of tobacco cells. The CL4MAb was expressed in tobacco plant cultures, purified, and the properties were compared with those of the original CL4MAb secreted by the TAPC301-CL4 cells.

MATERIALS AND METHODS

Binary Vector Constructions

The three constructs used in this study are shown in Figure 1. Only the signal sequences differ among the three plasmids. The p29 contained the original leader sequences (LS) of CL4MAb gene. In p30, the LSs were replaced with the synthesized DNA coding for the secretion sequence (SS) of calreticulin of *Nicotiana plumbaginfolia* [Borisjuk et al., 1998, 1999] that sorts proteins to the apoplastic spaces. In p31, the LSs were synthesized DNA coding for the SS of hordothionin of barley [Florack et al., 1994] that directs proteins to the ER pathways. All immunoglobulin chains were cloned between the cauliflower mosaic virus 35S promoter (35S) with the omega sequence (Ω: translational enhancer) and nopaline synthase terminator (NosT) [Luehrsen et al., 1992; Sheen et al., 1995]. The translation initiation sequences (IS) were changed from GTCGACATG to AACAATG for enhancement of antibody expression [Guerineau et al., 1992]. The heavy- and light-chain expression cassettes, arranged in tandem, constituted the antibody expression cassette (Ab cassette). The β-glucuronidase-NosT of pBI101 vector [Datla et al., 1992] was replaced with the Ab cassettes.

Transformation of Tobacco Suspension Culture

Recombinant binary vectors, p29, p30, and p31 were used to transform ElectroMAX™ *Agrobacterium tumefaciens* LBA4404 cells (Invitrogen Corp., Carlsbad, CA) by electroporation. Suspension cultures of BY-2 tobacco cells [Nagata et al., 1981] were transformed by standard methods [An, 1985] using co-cultivation with *A. tumefaciens* that had been transformed by p29, p30, and p31. Kanamycin resistant cells were selected on Murashige and Skoog (MS) plates containing 100 μg/ml kanamycin [Nagata et al., 1981]. Between 10 and 20 colonies from each of the transgenic lines, designated as the B29, B30, or B31 series, were inoculated into the MS medium. The transgenic cells were propagated for 7 days at 26°C in the dark, under the same conditions used for BY-2 cells [Nagata et al., 1981].

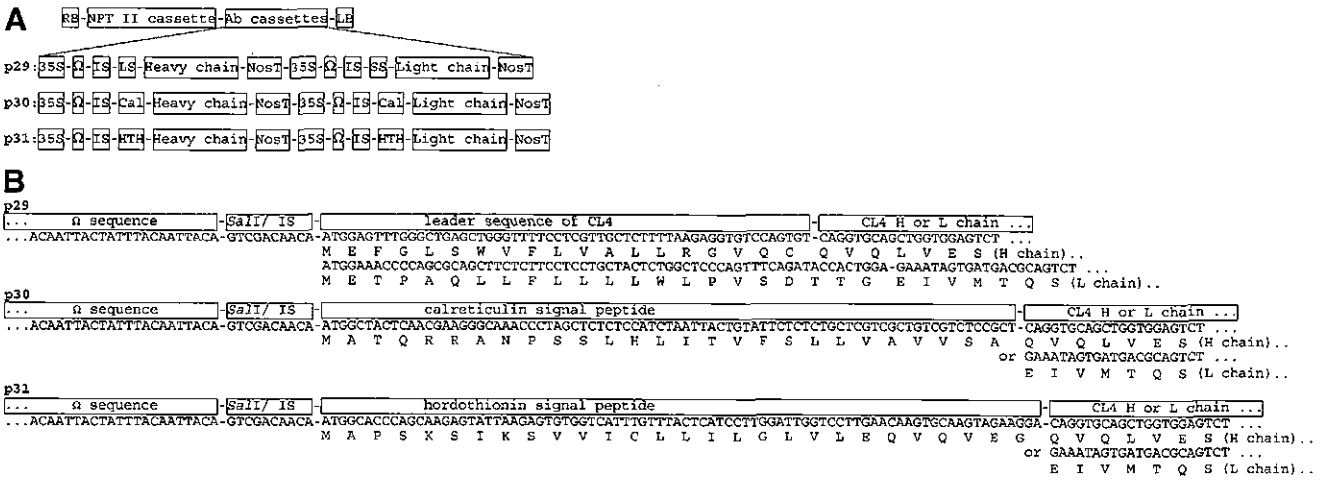

Fig. 1. Schematic representation of expression cassettes of the CL4 antibody. **A**: The antibody cassettes (Ab cassettes) were cloned into the T-DNA region separated from the binary vector, pBI101, by the right border (RB) and left border (LB) sequences. The Ab cassette of p29 binary vector contained original leader sequences (LSs) in the light and heavy chain of CL4 cDNA. Those LSs were replaced with secretion sequence (SS) of plants (p30: calreticulin (Cal) of *Nicotiana plumbaginifolia*, p31: hordothionin (HTH) of barley. The Ab cassettes contained cauliflower mosaic virus 35S promoter (35S) with omega sequence (Ω), restriction endonuclease *SalI* recognition site, initiation sequence (IS: AACA), nopaline synthetase terminator (NosT). **B**: DNA and deduced amino acid sequences of N-terminal region of the antibodies are indicated.

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

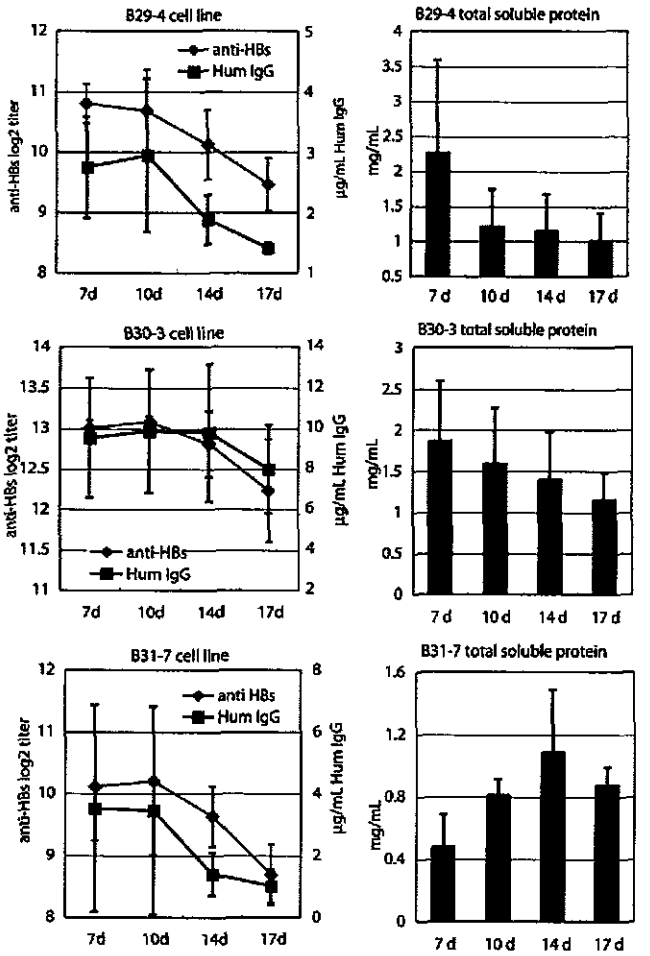

Fig. 2. Time course of antibody production in transgenic cell lines. B29-4, B30-3, and B31-7 cultures were sampled from 7 to 17 days after inoculation. Both anti-HBs titers and human IgG in samples were measured by ELISA. The amounts of total soluble proteins (TSP) were measured by Bradford assay. The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

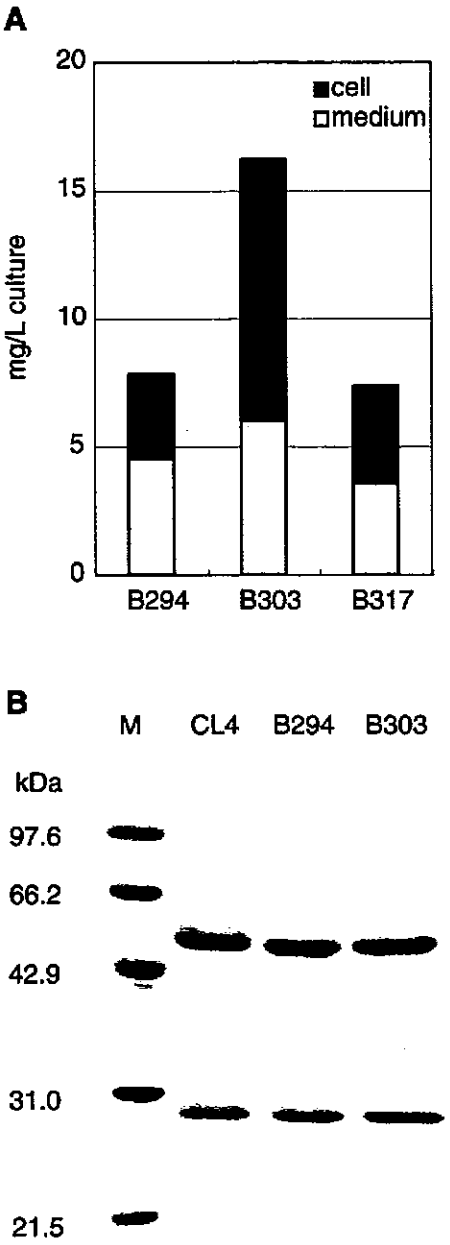

Fig. 3. Antibody purification from transgenic cell lines. **A**: IgG protein yields from B29-4 and B30-3 cultures. IgGs were purified from both cells and media of 10 day cultures with protein A columns. The B294 and B303 MAbs were quantified by ELISA. The maximum yield of the six times preparative scale 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

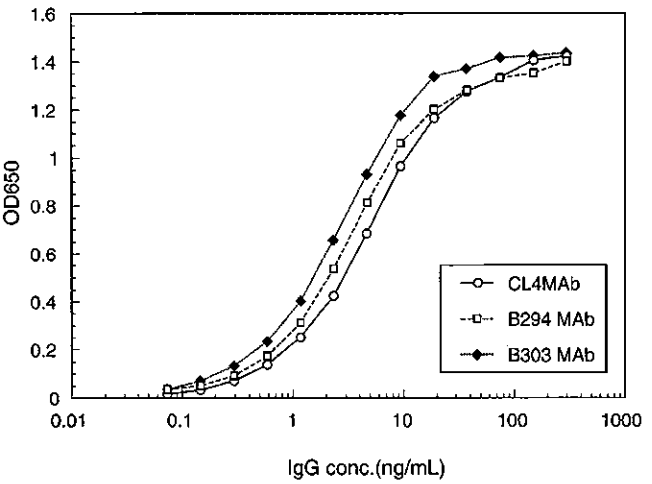

Fig. 4. Quantitative analysis of purified antibodies. Serially diluted solutions of the CL4, B294, and B303 MAbs were analyzed using the anti-HBsAg ELISA. The typical result of five experiments are shown.

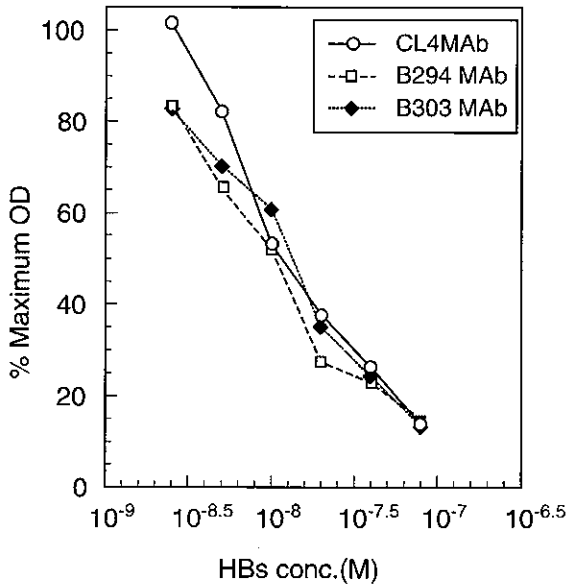

Fig. 5. Inhibition ELISA assays of purified antibodies. Affinities of the CL4, B294, and B303 MAbs were estimated by inhibition ELISA. The HBsAg was used as solid-phase antigen and serially diluted soluble HBsAg was used as inhibitor. The concentration of HBsAg, which gave 50% inhibition of maximum binding 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

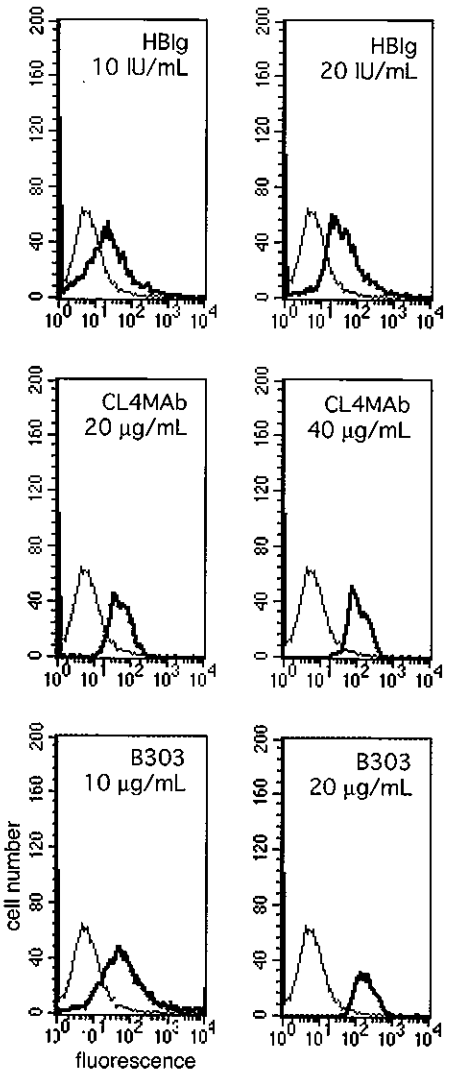

Fig. 6. Binding assay of the antibodies to cell surface HBsAg. The Alexander cells with surface HBsAgs were treated with HBIg, CL4, B303, and TI23 MAbs. Cell-antibody complexes were detected with FITC-conjugated goat anti-human IgG specific antibody and analyzed by flow cytometry. The number of cells 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

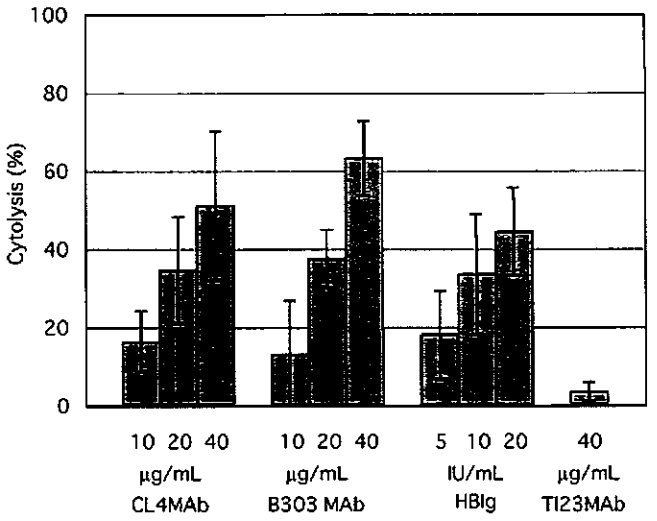

Fig. 7. Antibody induced complement-dependent cytotoxicity. The Alexander cells were treated with CL4 and B303 MAb. Hepatitis B immunoglobulin (HBIg) was used as positive control, and TI23 MAb was used as negative control. Columns and vertical bars represent the mean cytotoxicity values ± SD (n = 3).

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.

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Expert Opinion

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General

Transgenic plant-derived pharmaceuticals – the practical approach?

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Production of biopharmaceuticals in transgenic plants would involve the creation of a new industry. Those transgenic plants, including staple food crops, could provide many benefits to people all over the world. However, the new industry might require a strict regulation system. It is probable that such a strict system would not be acceptable to Japan or to most developing countries. Many countries should use non-food crops for production of biopharmaceuticals and take on more simple systems. The new industry must develop strategies for promoting the benefits of transgenic plant-derived biopharmaceuticals on both the domestic and worldwide scales.

Keywords: antibody, biopharmaceutical, developing country, GM crop, transgenic plant

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1. Introduction

Recombinant DNA technology has been available for ~ 30 years, and from it developed the biotechnology industry, which has grown steadily since its early days. As part of these developments, we have seen many associated scientific activities, such as the mapping of the human genome and, subsequently, postgenome projects [1,2]. The plant biotechnology industry has been a major player in this field and has created many transgenic plants, more popularly known as genetically modified (GM) crops. These crops could have an influential role to play in the present and future of biotechnology. One of the reasons behind this thinking is that GM crops have the potential to improve people's daily lives and health by providing a stable supply of food, materials and even pharmaceuticals, which would all be hallmarked by a consistent and reliable quality [3-5]. If we can steer plant biotechnology in the right direction, create effective GM crops and use them wisely, we will be able to solve one of the more difficult problems of 21st century – feeding the world's growing population.

2. Molecular farming

The biotechnology industry in industrialised countries is producing many biopharmaceuticals through the application of recombinant DNA technologies. In particular, > 200 monoclonal antibody (mAb)-based products are in clinical development, with many more in the preclinical stage [6]. If these trends continue, they could lead to new problems. Many biopharmaceuticals are bulk-produced in mammalian cell culture facilities. However, as the worldwide capacity of this means of manufacturing biopharmaceuticals is limited, plant biotechnology could provide an alternative [7]. Recent advances in plant biotechnology have made the production of human protein in plants a realistic possibility. However, it is thought that human-style glycosylation might be necessary for the successful development of therapeutic glycoproteins. Therefore, the role that plant-specific glycosylation may play in the development of recombinant mammalian glycoproteins in transgenic plants has raised a few questions

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hunger in these regions of the world [4,5,24,25], their food crops must be protected, as they are often the only source of food. Obviously, adverse contamination of their foods by transgenic introgression will increase the starvation in these impoverished countries. Nevertheless, the introduction of acceptable guidelines is necessary for even the majority of these countries, and, therefore, the utilisation of non-food crops for the production of biopharmaceuticals has a wide appeal. The production of pharmaceuticals using medicinal plants (e.g., antibodies from tobacco [11]), the retrieval of industrial materials from material plants (pulp obtained from trees with altered lignification processes [26]), and improving the nutritional qualities of existing and well-established crop plants (e.g., 'designer' nutrition in golden rice [27]) may provide a simple and easily acceptable starting point. If developing countries could export such raw materials for modern biopharmaceuticals, it will provide them with a good source of revenue to improve the lives of their general population.

5. Conclusion

The world is facing difficult times, with growing populations and dwindling resources, coupled with climate change, affecting many of the poorer, but often more heavily populated areas. Analysis of world trends has shown that greatly improved technologies will be necessary to avoid conflicts and for a transition to a sustainable system [24,25]. As mentioned in the introduction, plant biotechnology offers a great potential in this respect. We must use these technologies in order to realise a sustainable way of life for all the people of the world. The production of biopharmaceuticals by transgenic plants may well be the trend of the next decade, and industrialised countries will reap the rewards. However, it is important that developing countries also benefit from the introduction of these new technologies. Therefore, we must develop strategies that pay full attention to promoting the benefits of bioengineering and biopharmaceuticals, not only on the domestic front, but also worldwide. Of particular relevance in this respect is the production of pharmaceuticals, including edible vaccines [28,29], which are undergoing development, and by which the parallel contamination of food crops must be prohibited. In order to realise the benefit of an edible vaccine, excellent and feasible strategies will also be necessary to ensure that it is distributed to the large numbers of people in need. We must advance, but at the same time keep our focus firmly on the future of the world and the needs of all its people.

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5. Conclusion

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Simvastatin induces apoptosis of Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines and delays development of EBV lymphomas

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Simvastatin and pravastatin are inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, and are used as antihypercholesterolemia drugs. Simvastatin, but not pravastatin, binds to the inserted domain of leukocyte function antigen (LFA)-1 and inhibits the function of LFA-1, including adhesion and costimulation of lymphocytes. Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) express high levels of LFA-1 on their surface and grow in tight clumps. Here we show that simvastatin (2 μM) inhibits clump formation and induces apoptosis of EBV-transformed LCLs. The apoptosis-inducing effect of simvastatin depends on binding to the inserted domain of LFA-1. Simvastatin, but not pravastatin, dissociates EBV latent membrane protein 1 from lipid rafts of LCLs, resulting in down-regulation of nuclear factor κB activity and induction of apoptosis. Analysis of multiple EBV-positive and -negative cell lines indicated that both LFA-1 and EBV latent membrane protein 1 expression were required for simvastatin's effects. Administration of simvastatin to severe combined immunodeficiency mice followed by inoculation with LCLs resulted in delayed development of EBV lymphomas and prolonged survival of animals. To our knowledge, this is the first report in which a drug that targets LFA-1 has been used to treat B cell lymphoma. These data suggest that simvastatin may have promise for treatment or prevention of EBV-associated lymphomas that occur in immunocompromised persons.

Infection of primary B cells with Epstein–Barr virus (EBV) results in transformation with growth of the cells in tight clumps and immortalization of the cells. These immortalized B cells have an immunoblastic morphology and express each of the EBV nuclear antigens (EBNAs) and latent membrane proteins (LMPs) (1, 2). EBNA-2 is a transactivator that up-regulates expression of cellular genes and LMPs. LMP-1 is an oncoprotein that constitutively activates nuclear factor κB (NF-κB) to induce B cell proliferation (3). LMP-1 also induces expression of adhesion molecules leukocyte function antigen (LFA)-1, LFA-3, and intercellular adhesion molecule 1 (ICAM-1) on the surface of EBV-transformed B cells (4, 5). The high level expression of adhesion molecules contributes to clumping of EBV-infected B cells *in vitro* (6).

EBV-associated immunoblastic lymphomas occur in immunocompromised patients such as those with AIDS or transplant recipients (7, 8). Because these EBV-associated immunoblastic lymphomas express each of the EBNAs and LMPs (8) that induce proliferation of B cells, the virus is thought to be directly responsible for the pathogenesis of these tumors (3). LMP-1 in EBV-associated lymphoma cells binds to tumor necrosis factor receptor-associated factors, and the tumors show activation of NF-κB (9). Many immunocompromised patients with EBV-associated immunoblastic lymphoma have tumors at extranodal sites such as the brain, lung, or gastrointestinal tract. The high-level expression of LFA-1 and other cellular adhesion molecules in these tumors may contribute to their extranodal location (10). The prognosis of these lymphomas is often poor for patients with irreversible immunosuppression, and treatment options are limited.

Simvastatin is a member of the statin family of drugs that inhibit 3-hydroxy-3-methylglutaryl CoA reductase (11). Statins lower plasma cholesterol levels, resulting in reduction of the risk of cardiovascular disease (12). Weitz-Schmidt *et al.* (13) demonstrated that certain statins, including simvastatin and lovastatin, bind to the I (inserted) domain of LFA-1 and inhibit its function (13). In contrast, other statins such as pravastatin do not bind to LFA-1. LFA-1 is expressed on the surface of various leukocytes and plays an important role in cell adhesion and costimulation of T cells. The I domain of LFA-1 is the binding site for ICAM-1, a ligand of LFA-1 (14, 15). The binding of simvastatin or lovastatin to the LFA-1 I domain induces a conformational change in LFA-1, resulting in inhibition of the interaction of LFA-1 with ICAM-1 (13). As a result of their binding to LFA-1, these statins inhibit the costimulatory activity of LFA-1 and suppress the inflammatory response in a murine model of peritonitis (13).

Here, we investigate the ability of simvastatin to inhibit EBV-positive B cell proliferation. Because simvastatin binds to and inhibits the function of LFA-1, we postulated that the drug would inhibit the growth of these cells both *in vitro* and *in vivo*. Inoculation of EBV-transformed lymphoblastoid cell lines (LCLs) into severe combined immunodeficiency (SCID) mice results in the formation of EBV-associated immunoblastic lymphomas that contain EBV genomes and express EBNAs, LMPs, and adhesion molecules including LFA-1 (16, 17). In the present study, we treated EBV-transformed LCLs *in vitro* with simvastatin, and administered the drug and inoculated SCID mice with LCLs to assess development of B cell lymphomas.

Materials and Methods

Cell Culture and Viability Assay. Three EBV-transformed LCLs, 12A1, 6B10, and 295H, EBV-positive Burkitt lymphoma cell lines [P3HR-1 (18), Akata (19), Mutu-1 (20), and Mutu-3 (20)], a human herpesvirus-8-positive EBV-negative primary effusion lymphoma cell line (BCBL-1) (21), an EBV-negative Burkitt lymphoma cell line (BJAB) (22), and EBV-negative T cell lines [Jurkat (23) and II-23 (24) cells; obtained from Carl Ware, La Jolla Institute for Allergy and Immunology, San Diego] were tested. For cell proliferation and viability assays, 2×10^4 cells per ml were cultured in 12- or 24-well plates for 5–7 days. Cell viability was assessed with XTT (Cell Proliferation Kit II, Roche Molecular Biochemicals), trypan blue, or propidium iodide (PI) staining. For PI staining, cells were washed with PBS, PI (5 μg/ml) was added, cells were washed, and fluorescent intensity was assessed with flow cytometry. Percent cell death was determined by the ratio of PI-positive cells to all gated cells.

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Abbreviations: EBV, Epstein–Barr virus; ICAM-1, intercellular adhesion molecule 1; I domain, inserted domain; LCL, lymphoblastoid cell line; LFA, leukocyte function antigen; LMP, latent membrane protein; PI, propidium iodide; SCID, severe combined immunodeficiency.

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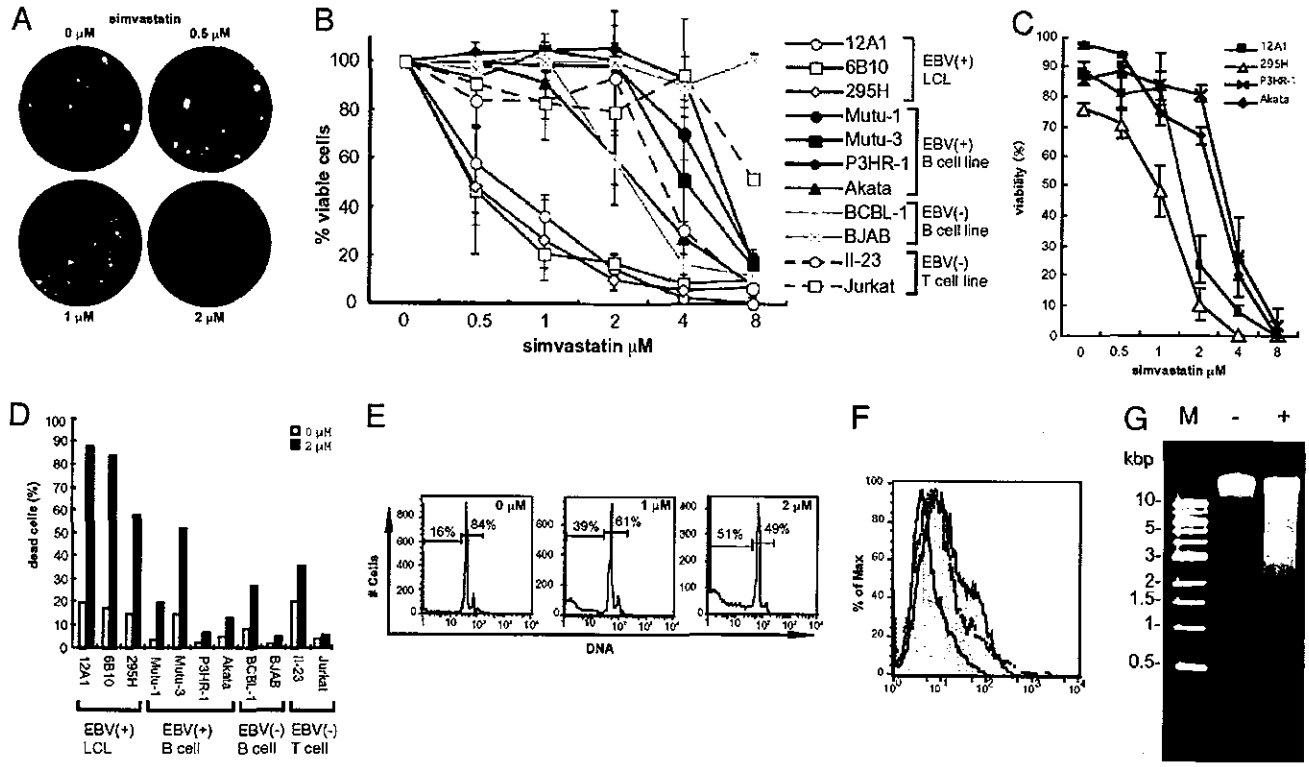

Fig. 1. Simvastatin inhibits clump formation and induces apoptosis in LCLs. (*A*) Cell clumping of LCLs was observed after simvastatin was added at 0–2 μM for 5 days. (*B*) XTT cell proliferation assay was performed after addition of simvastatin (0–8 μM) to various cells lines for 7 days. Error bars indicate standard deviations for four independent experiments. (*C*) Cell viability was assayed by trypan blue staining after cell lines were cultured with 0–8 μM simvastatin for 7 days. Error bars indicate standard deviations for three separate experiments. (*D*) Percentage of dead cells in the absence (open bars) or presence (filled bars) of 2 μM simvastatin for 7 days as determined by PI staining and flow cytometry. PI-positive cells were counted as dead cells. (*E*) PI staining. Cells were treated with or without simvastatin for 5 days. Cell populations in sub-G_0–G_1 and G_0–G_1–S–M phase are indicated. (*F*) TUNEL assays were performed for cells treated with 2 μM simvastatin for 5 days (gray area with solid line), no simvastatin for 5 days (white area with solid line), or serum starvation for 72 h (white area with dotted line). (*G*) DNA ladder formation for cells cultured with (+) or without (−) 2 μM simvastatin for 5 days.

Reagents and Antibodies. Simvastatin and pravastatin (Calbiochem) were converted to their open acid forms before use *in vitro* (25). Soluble ICAM-1 was purchased from R & D Systems. LFA-1 antibodies TS1/22 (American Type Culture Collection) (26) and G25.2 (BD Pharmingen) were used as primary antibodies, and fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat anti-mouse Ig (Caltag, Burlingame, CA) was used as the secondary antibody for immunofluorescence and flow cytometry. TS1/22 antibody was obtained from hybridoma cells.

Lipid Raft Studies. Detergent extraction and flotation assay for lipid rafts were performed as described (27). Immunoblotting was performed by using anti-LMP-1 monoclonal antibody (S-12, BD Pharmingen), anti-CD71 monoclonal antibody (Zymed), and anti-Lyn monoclonal antibody (Santa Cruz Biotechnology).

Table 1. Protein expression, activation of NF-κB, and cell death induced by simvastatin in cell lines

Cell line	Description	Clumping formation	LFA-1*	ICAM-1*	EBV†	LMP-1‡	NF-κB‡	Cell death by simvastatin§
12A1	LCL	++	++	++	+	+++	++	+
6B10	LCL	++	++	++	+	+++	+++	+
295H	LCL	+	+	++	+	+++	+++	+
Mutu-1	B cell line (BL)	−	−	−	+	−	−	−
Mutu-3	B cell line (BL)	+	+	++	+	+	+	+
P3HR-1	B cell line (BL)	−	++	++	+	−	+	−
Akata	B cell line (BL)	−	−	−	+	−	+	−
BCBL-1	HHV-8-positive cell	−	−	+	−	−	−	−
BJAB	B cell line (BL)	−	+	++	−	−	−	−
II-23	T cell line	−	++	+	−	−	+	−
Jurkat	T cell line	−	−	+	−	−	−	−

*Expression levels of LFA-1 and ICAM-1 were determined with flow cytometry (Fig. 5).
†EBV-positive cells lines are indicated in refs. 18–24.
‡Expression level of LMP-1 and constitutive activation of NF-κB were determined by immunoblot and gel shift assay, respectively (Fig. 5).
§Cell death induced by 2 μM simvastatin is defined as >50% cell death by PI staining (Fig. 1*D*). BL, Burkitt lymphoma; HHV-8, human herpesvirus 8.

Nuclear Extraction and Electrophoretic Mobility-Shift Assays. Nuclear extracts were prepared from 1×10^7 cells as described (28). Activation of NF-κB was determined by using 5 μg of nuclear extract in the gel-shift assay system (Promega) according to the manufacturer's instructions.

Apoptosis Assays. DNA fragmentation by apoptosis was detected by PI staining as described (29). Terminal deoxytransferase-mediated dUTP nick end labeling (TUNEL) assays were performed by using the *in situ* cell death detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. DNA ladder formation was performed by extracting DNA from cells with the genomic DNA purification kit (Gentra Systems), and electrophoresis was performed.

Animal Experiments. Simvastatin tablets (ZOCOR, Merck) were mixed with mouse food at a ratio of 160 mg of simvastatin per 65 g of powdered food. Untreated animals received powdered food without simvastatin. LCLs (0.25×10^6, 1×10^6, or 4×10^6) were inoculated i.p. into 8-week-old SCID mice. Simvastatin was given either 3 days before (pretreatment group) or 7 days after (treatment group) inoculation of cells and continued until 4–6 weeks after inoculation. Thereafter, food without simvastatin was given to all mice, because of the side effects of prolonged high-dose simvastatin. All dead mice were autopsied and examined for the presence of lymphomas.

Results

Simvastatin Inhibits Clump Formation and Induces Apoptosis of LCLs. LCLs and Burkitt lymphoma cells that express each of the EBNAs, LMPs, and LFA-1 grow in tight clumps. Binding of anti-LFA-1 antibody TS1/22 to the I domain of LFA-1 inhibits clumping of phorbol myristate acetate-stimulated LCLs after 18 h (6). To determine whether simvastatin affects clumping of unstimulated LCLs, we treated an LCL (12A1) with various concentrations of the drug. Five days after the addition of simvastatin, LCL clumps broke apart in wells treated with ≥2 μM simvastatin, whereas clumps remained in wells treated with ≤1 μM simvastatin (Fig. 1*A*). Dissociation of clumps was also observed in other LCLs (6B10 and 295H) and in Mutu-3 cells (data not shown). Cell viability assays were performed on various cell lines expressing different levels of LFA-1, ICAM-1, and LMP-1 (Table 1 and Fig. 5, which is published as supporting information on the PNAS web site). XTT cell proliferation assays showed that simvastatin decreased the number of viable cells in a dose-dependent manner (Fig. 1*B*). A loss in viability of LCLs was induced by 0.5 μM simvastatin, whereas ≥2 μM simvastatin was required to reduce viability of other cells. Trypan blue staining showed that LCLs 12A1 and 295H had >50% reduction in viability with 2 μM simvastatin, whereas Akata and P3HR-1 cells required higher concentrations of the drug (4 μM) to achieve >50% reduction in viability (Fig. 1*C*). The number of dead cells began to increase 5 days after addition of simvastatin at the time when clumps started to dissociate. PI staining followed by flow cytometry was also used to determine cell viability with 2 μM simvastatin. Cells expressing LMP-1 such as LCLs (12A1, 6B10, and 295H) and Mutu-3 showed low (<50%) viability after culture in 2 μM simvastatin for 7 days (Fig. 1*D*). Thus, incubation of cells with 2 μM simvastatin for 5 days inhibits clump formation and induces death in cells expressing LMP-1 (Table 1).

Some statins induce apoptosis of certain tumor cells *in vitro* (30). PI staining showed that treatment of LCLs with simvastatin for 5 days induced a dose-dependent increase in fragmented DNA that was smaller than G_0–G_1 DNA (2n), indicative of apoptosis (29) (Fig. 1*E*). TUNEL assay confirmed that simvastatin induced DNA fragmentation in LCLs (Fig. 1*F*). Gel electrophoresis showed that simvastatin induced DNA fragment in LCLs resulting in formation of a DNA ladder (Fig. 1*G*). Thus, simvastatin induces apoptosis in LCLs.

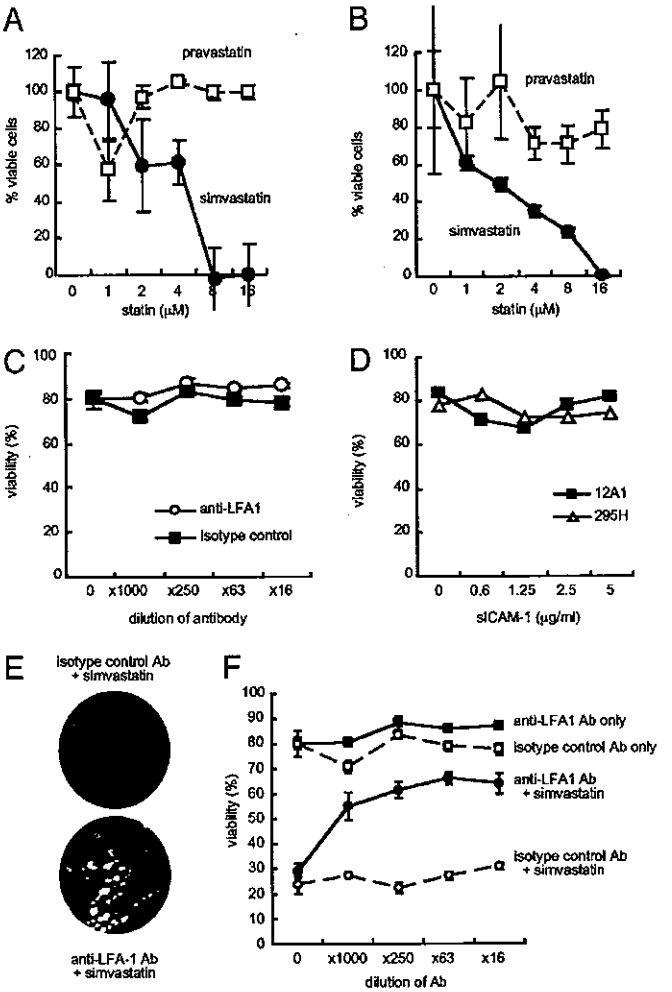

Fig. 2. Apoptosis-inducing effect of simvastatin depends on binding to the I domain of LFA-1. (*A* and *B*) XTT cell proliferation assay was performed for LCLs 12A1 (*A*) and 6B10 (*B*) in the presence of simvastatin or pravastatin after 5 days. (*C*) Cell viability was assayed by trypan blue staining for LCL 12A1 in the presence of anti-LFA-1 antibody TS1/22, which recognizes the I domain of LFA-1, or an isotype control antibody for 7 days. (*D*) Cell viability was measured by trypan blue staining of LCLs 12A1 and 295H cultured with soluble ICAM-1 (sICAM-1) for 7 days. (*E*) LCL 12A1 was cultured with anti-LFA-1 (TS1/22) or isotype control antibody for 1 h, simvastatin (2 μM) or no compound was added, and the cells were cultured for 7 days. Cell clumping is reduced with isotype control antibody and simvastatin (*Upper*) but not with anti-LFA-1 antibody and simvastatin (*Lower*). (*F*) Cell viability was assayed by trypan blue staining of LCLs cultured with an anti-LFA-1 or isotype control antibody. Error bars indicate standard deviations of three separate experiments.

The Apoptosis-Inducing Effect Is Specific for Simvastatin and Depends on Binding to the I Domain of LFA-1. To determine whether loss of cell viability of LCLs occurs with a statin with different binding properties than simvastatin, LCLs were incubated with pravastatin, which does not bind to LFA-1 (13). Pravastatin had little effect on viability of LCL 12A1 or 6B10 (Fig. 2 *A* and *B*). Anti-LFA-1 monoclonal antibody TS1/22 and soluble ICAM-1 (sICAM-1) bind to the I domain of LFA-1 (26, 31), which is the site on LFA-1 targeted by simvastatin (13). Anti-LFA-1 TS1/22 antibody bound to LFA-1 on the cell surface at a 1:100 dilution by flow cytometry (Fig. 5); however, the antibody did not affect viability of 12A1 cells at concentrations up to 1:16 (Fig. 2*C*). sICAM-1 used at concentrations (5 μg/ml) that block rhinovirus infection (31) did not affect viability of LCLs 12A1 and 295H (Fig. 2*D*). Thus, antibody or another ligand that binds to the I domain of LFA-1 does not induce death of LCLs.

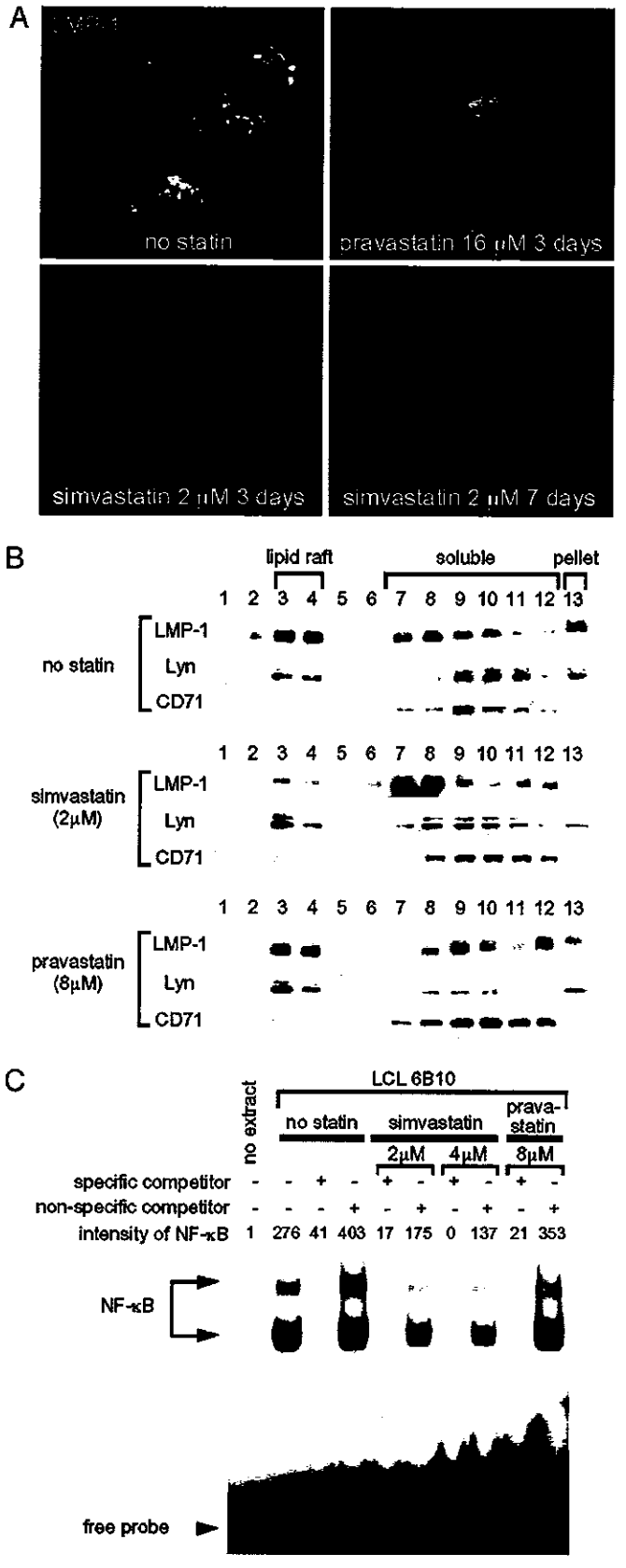

Fig. 3. Simvastatin alters the localization of LMP-1, displaces LMP-1 from lipid rafts, and inhibits NF-κB activation in LCLs. (*A*) Immunofluorescence of LMP-1 in LCL 6B10 in the absence or presence of simvastatin or pravastatin. Cells were fixed, permeabilized, and incubated with anti-LMP-1 antibody (CS1–4, DakoCytomation, Carpinteria, CA), followed by FITC-conjugated secondary antibody and PI. LMP-1 is green (FITC) and nuclei are red (PI). (*B*) Immunoblotting of LCL fractions. LCLs were cultured in the absence or presence of simvastatin or pravastatin for 3 days, and cell lysates were fractionated by sucrose gradient ultracentrifugation. Equal aliquots of each fraction beginning at the top of the centrifuge tube were probed with antibodies to LMP-1, Lyn (a tyrosine kinase that localizes in lipid rafts), and CD71 (a transferrin receptor that does not localize in rafts). (*C*) Electrophoretic mobility-shift assays for NF-κB. LCL 6B10 was cultured in the absence or presence of simvastatin or pravastatin for 3 days, and nuclear extracts were used in electrophoretic mobility-shift assays with a radiolabeled NF-κB probe. Specific or nonspecific nonradioactive competitor oligonucleotides were added to some assays. The intensity of NF-κB-specific bands was measured by using a phosphorimager.

Antibody to LFA-1 (TS1/22) has been shown to induce signal transduction in lymphocytes (32). Therefore, pretreatment of LCLs with the antibody might inhibit the effects of simvastatin. Pretreatment of cells with anti-LFA-1 antibody TS1/22 blocked the ability of simvastatin to dissociate clumps of LCLs (Fig. 2*E*) and to reduce viability of LCLs (Fig. 2*F*).

Simvastatin, but Not Pravastatin, Dissociates LMP-1 from Lipid Rafts and Reduces Activation of NF-κB. *In vitro* assays indicate that cholesterol depletion by high doses of statins disrupts lipid rafts and alters the localization and function of proteins in lipid rafts on the cell membrane (33–35). EBV LMP-1 localizes in lipid rafts and constitutively activates NF-κB via tumor necrosis factor receptor-associated factors (3, 27, 36). Therefore, we postulated that simvastatin may affect localization of LMP-1 in lipid rafts and impair signal transduction by LMP-1. To determine whether simvastatin alters localization of LMP-1, we treated LCLs with simvastatin and performed immunofluorescence assays for the viral protein. LMP-1 localized to large punctate structures in LCLs in the absence of simvastatin (Fig. 3*A*). After treatment with simvastatin for 3 or 7 days, LMP-1 showed a fine granular pattern in most of the cells that was much fainter and more diffuse than in untreated cells. In contrast, LMP-1 maintained its large punctate structures in cells treated with pravastatin. To further examine the effect of statins on LMP-1 localization, cell extracts were fractionated by using centrifugation and floatation in sucrose gradients (27). Lyn localizes to lipid rafts, whereas CD71 is in the soluble fraction (27). Immunoblotting showed that lipid raft fractions (fractions 3 and 4) contained the highest concentrations of LMP-1 in untreated or pravastatin-treated LCLs. At 2 μM simvastatin, the highest concentrations of LMP-1 shifted to soluble fractions (fractions 7–12), but Lyn remained in lipid rafts (fractions 3 and 4) (Fig. 3*B*). Quantitative analysis showed that 47% of LMP-1 was localized in lipid rafts of untreated cells; after treatment with simvastatin for 3 days, only 7% of LMP-1 was located in rafts. These data indicate that simvastatin, but not pravastatin, alters the localization of LMP-1 in the cell and dissociates LMP-1 from lipid rafts.

Each of the LCLs used in the present study showed constitutive activation of NF-κB (Fig. 5). Electrophoretic mobility-shift assays showed that treatment of LCLs with simvastatin (2 μM or 4 μM) for 3 days reduced the level of activated NF-κB, whereas pravastatin (8 μM) did not reduce NF-κB (Fig. 3*C*). Cell viability at 3 days ranged from 80–90% in cells treated with either statin or in untreated cells. Because inhibition of NF-κB induces apoptosis of LCLs (28), our results suggest that reduction of NF-κB by simvastatin results in induction of apoptosis in LCLs.

Simvastatin Delays the Onset of EBV Lymphomas and Prolongs Survival in SCID Mice Inoculated with EBV-Transformed LCLs. Because simvastatin induced apoptosis of LCLs, we tested the effect of the drug on SCID mice with EBV lymphomas. Simvastatin (250 mg/kg/day) was given orally to SCID mice beginning 3 days before i.p. inoculation with EBV-transformed LCLs. Control animals did