Development of immunogenic recombinant Oka varicella vaccine expressing hepatitis B virus surface antigen

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Recombinant Oka varicella vaccine expressing hepatitis B virus (HBV) surface antigen (HBs) was constructed by inserting the HBs gene into the viral thymidine kinase (TK) gene and was examined for its immunogenicity in guinea-pigs. The HBs gene encoding 25 amino acids of preS2 and the whole of the S region was inserted into the TK gene of the cloned plasmid. The chimeric plasmid DNA and Oka varicella vaccine DNA were cotransfected and recombinant virus was isolated after immunofluorescence screening using a monoclonal antibody to HBs and a fluorescein-conjugated anti-mouse antibody. Expression of viral HBs was detected in the cytoplasm of infected cells and was stable over several repeated passages *in vitro*. The recombinant virus expressed 26K and 30K HBs molecules in infected cells and the culture supernatant contained 30K and 35K HBs molecules. HBs was purified at a density of 1·20 g/ml from the culture supernatants. The recombinant virus induced an antibody response to HBs as well as to varicella-zoster virus (VZV) in guinea-pigs, and the antibody titre to HBs was comparable to that induced by a recombinant HBs subunit vaccine produced in yeast. Thus a single dose of live recombinant Oka varicella vaccine could induce good immunity to VZV and HBs. The recombinant Oka varicella vaccine expressing HBs may be a good candidate for a combined HBV and VZV vaccine.

Introduction

About 200 million people are chronically infected with hepatitis B virus (HBV), which causes acute and chronic hepatitis, cirrhosis and primary hepatocellular carcinoma. Vaccination with blood-derived and genetically engineered subunit HBV surface antigens (HBs) has been developed and used in high risk groups to prevent HBV infection. Recently, recombinant vaccinia viruses expressing HBs have been constructed (Smith *et al.*, 1983; Paoletti *et al.*, 1984) and their immunogenicity as HBs vaccines has been well characterized in animal models (Moss *et al.*, 1984). However, vaccinia virus has not been used as a vaccine for smallpox in humans since 1980. Adenovirus, simian virus 40 and papillomavirus have also been used as expression vectors for HBs (Davis *et al.*, 1985; Hsiung *et al.*, 1984; Moriarty *et al.*, 1981).

Oka varicella vaccine has been successfully used to confer active immunity against varicella-zoster virus (VZV) in immunocompromised and healthy subjects in Japan, the United States and Europe, and its immunogenicity and safety have been well documented (Takahashi *et al.*, 1975; Andrè, 1985; Takahashi, 1987; Straus *et al.*, 1988; Gershon *et al.*, 1990). VZV is a DNA virus encoding 71 proteins (Davison & Scott, 1986) which can be used as a live vector to express foreign genes. For example, the Oka varicella vaccine virus has been used to express the Epstein–Barr virus membrane glycoprotein (gp350/220) (Lowe *et al.*, 1987). One of the advantages of the Oka varicella vaccine virus as an expression vector for vaccine use is that it is the only DNA virus vaccine currently licensed and used in humans and has few clinical side-effects. In this study we have constructed a recombinant Oka varicella vaccine expressing HBs by inserting the HBs gene into the viral thymidine kinase (TK) gene (Shiraki *et al.*, 1985; Mori *et al.*, 1988) and examined its immunogenicity as a varicella vaccine as well as an HBV vaccine in guinea-pigs.

Methods

Cells and virus. Human diploid fibroblasts, MRC-5 cells, were grown and maintained in MEM (Gibco) supplemented with 10% or 3% foetal bovine serum. Oka varicella vaccine strain (Takahashi *et al.*, 1975) was used as a recombinant expression vector.

Construction of a recombinant varicella vaccine strain. The recombinant Oka varicella vaccine virus expressing HBs was constructed as

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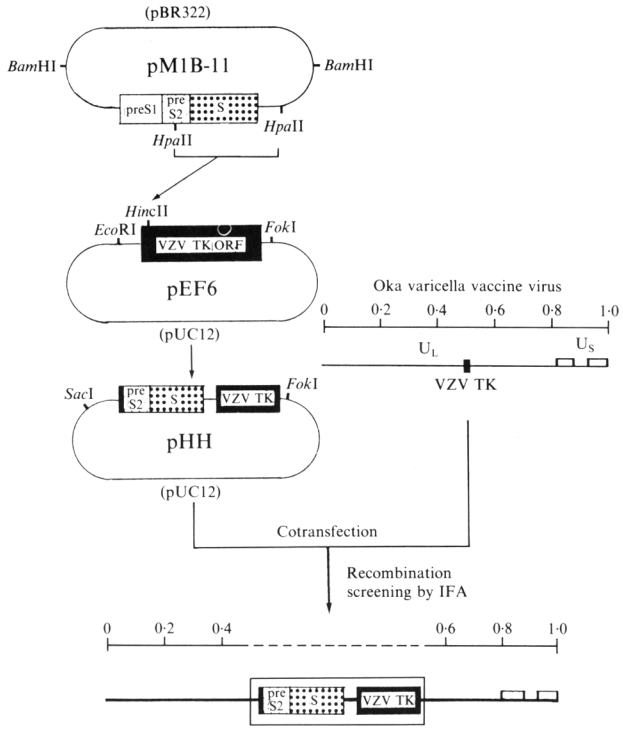

Fig. 1. Schematic representation of the construction of the recombinant Oka varicella vaccine virus expressing HBs. The H fragment of VZV DNA digested by *Sac*I was cloned into pUC12 (Mori *et al.*, 1988), and the *Eco*RI–*Fok*I fragment containing the TK gene was cloned into pUC12 (pEF6). The HBs gene was cloned into the *Bam*HI site of pBR322 (pM1B-11) (Yosida *et al.*, 1991) and a 1080 bp DNA fragment of the HBs gene was excised between the *Hpa*II sites for insertion into the *Hinc*II site of the TK gene. The final chimeric plasmid (pHH) contained the 4·8 kb *Sac*I–*Fok*I fragment of the VZV genome in pUC12. After cotransfection of VZV DNA and chimeric plasmid DNA (pHH), VZV foci were cloned and examined by the IFA test with monoclonal antibody to HBs.

shown in Fig. 1. The H fragment of VZV DNA digested by *Sac*I (*Sst*I) was cloned into pUC12 (Mori *et al.*, 1988), and its *Eco*RI–*Fok*I fragment containing the TK gene was recloned into pUC12 (pEF6). The HBs gene, from the HBV *adr* strain, was cloned in the *Bam*HI site of pBR322 (Yosida *et al.*, 1991) and the HBs gene in the plasmid (pM1B-11) was excised at its *Hpa*II sites for insertion at the *Hinc*II site of the TK gene. The final chimeric plasmid (pHH) contained a 1080 bp DNA fragment of the HBs gene between the 4·8 kb *Sac*I–*Fok*I fragment of the VZV gene, and its insertion site was sequenced. Plasmid pHH was designed to use the authentic initiation codon of the TK gene, utilizing the authentic promoter and enhancer of the TK gene, and express 25 amino acids of the preS2 and all of the S peptide.

Oka varicella vaccine virus was grown in MRC-5 cells. When extensive c.p.e. appeared, the cells were harvested and virus DNA was isolated based on the procedure described previously (Asano *et al.*, 1981). VZV DNA (2·5 μg/ml) and the chimeric plasmid DNA (pHH; 20 μg/ml) were cotransfected into MRC-5 cells in 60 mm plastic dishes by the calcium phosphate method (Graham & van der Eb, 1973). VZV foci were picked by cylinder cloning of infected cells and propagated in 25 cm^2 plastic flasks. A portion of the infected cells was transferred onto coverslips and examined by an immunofluorescent antibody (IFA) test following reaction with a monoclonal antibody to HBs (MBL). The cells on coverslips were washed with PBS, dried and fixed with a mixture (1 : 1) of methanol and acetone at −20 °C for 5 min, followed by staining with monoclonal antibody and anti-mouse IgG FITC conjugate. The infected cells from HBs-positive plaques were propagated, and cell-free virus stocks were prepared and frozen at −70 °C until use (Shiraki *et al.*, 1982). After infection with cell-free VZV, plaques were cylinder-cloned from 60 mm plastic dishes in which one to three VZV plaques had appeared. After examination by the IFA test a candidate recombinant virus clone expressing HBs was selected.

The recombinant virus was propagated for 10 passages by cell-to-cell infection at a ratio of one infected : 5 to 10 uninfected cells and then cell-free virus was prepared. Expression of HBs was examined by the IFA test in 20 clones isolated after inoculation of cell-free virus.

DNA restriction analysis of recombinant virus. VZV DNA fragments derived from parental virus and the recombinant were cleaved by *Sac*I, electrophoresed through a 0·8% agarose gel and stained with ethidium bromide. DNA fragments were transferred to nylon filters and probed with [α-^{32}P]dCTP-labelled pM1B-11 DNA and pHH DNA based on the procedure reported previously (Mori *et al.*, 1988).

Detection of HBs in the culture supernatant and infected cells. The culture supernatant of recombinant virus-infected cells was centrifuged at 3000 r.p.m. for 20 min and then at 27000 r.p.m. for 3 h at 4 °C. The pellet was suspended in 0·2 ml of PBS and used as the culture supernatant fraction. Approximately 10^6 infected cells were suspended in 1 ml of PBS and disrupted by sonication followed by centrifugation at 3000 r.p.m. for 20 min. The HBs contents of both supernatants were titrated by the reversed passive haemagglutination (RPHA) test (Green Cross) using cells coated with anti-HBs serum and twofold dilution series of the samples.

HBs was purified from the culture media of recombinant virus-infected cells. The media were centrifuged at 5000 r.p.m. for 20 min and the supernatants precipitated with 10% polyethylene glycol (PEG) by centrifugation at 5000 r.p.m. for 20 min. The pellets were suspended in TEN (20 mM-Tris–HCl pH 7·4, 1 mM-EDTA, 150 mM NaCl) and subjected to two cycles of CsCl equilibrium centrifugation on discontinuous gradients with densities of 1·15, 1·25 and 1·4 g/ml in an SW27 rotor at 23000 r.p.m. for 20 h at 4 °C. HBs-containing fractions were assayed by RPHA test. The HBs fraction was centrifuged in TEN (density of 1·2 g/ml CsCl) in an SW41 rotor at 33000 r.p.m. for 40 h at 4 °C. The resulting HBs fraction was diluted and centrifuged onto a 10% sucrose cushion in an SW41 rotor at 40000 r.p.m. for 3 h. The pellet was suspended in PBS and observed under the electron microscope.

Identification of HBs induced in recombinant virus-infected cells. The parent Oka varicella vaccine virus- and recombinant virus-infected cells, or uninfected cells were labelled with 50 μCi/ml [^{35}S]methionine and [^{35}S]cysteine in medium without methionine for 4 h and lysed with RIPA buffer (20 mM-Tris–HCl pH 8·0, 1% Triton X-100, 0·5% sodium deoxycholate, 10 mM-NaCl and 1 mM-PMSF) and centrifuged at 35000 r.p.m. for 1 h (Shiraki *et al.*, 1982; Shiraki & Hyman, 1987). Culture supernatants of labelled cells were centrifuged at 3000 r.p.m. for 20 min. The supernatants were mixed with anti-VZV guinea-pig serum (Shiraki & Hyman, 1987) and anti-HBs rabbit serum (Konobe *et al.*, 1991), and the immune complexes were separated on Protein A–Sepharose CL-4B (Pharmacia) for analysis by SDS–PAGE.

Immune response to the recombinant virus in guinea-pigs. The immunogenicity of HBs expressed by the recombinant virus was examined in guinea-pigs. Three-week-old guinea-pigs weighing approximately 250 g were immunized subcutaneously with 3000 p.f.u/dose of the parent Oka varicella vaccine virus, the recombinant virus or control antigen made from uninfected cells. Immunogenicity of the recombinant Oka varicella vaccine was compared with that of

recombinant HBs subunit vaccine (10 μg/dose:alum precipitate; Biken, Merck & Co.) produced in yeast. Antibody titres to VZV and HBs were determined by a neutralization test (Shiraki *et al.*, 1982) and by passive haemagglutination (PHA) using cells coated with HBs antigen (Kaketuken), respectively.

Results

Construction of recombinant Oka varicella vaccine expressing HBs

As shown in Fig. 1, the chimeric plasmid (pHH) was produced by inserting the HBs gene into the VZV TK gene and its construction was verified by DNA sequencing. The insertion site was the second codon of the open reading frame (ORF) of the VZV TK gene. The HBs gene product would be expected to initiate at the ATG of the VZV TK gene and be followed by a serine codon created at the insertion site, 25 amino acids of preS2 and the whole S peptide. Construction of the chimeric plasmid was designed to replace the expression of the VZV TK gene with that of the HBs gene, utilizing the authentic enhancer and promoter of VZV TK.

After cotransfection of the chimeric plasmid DNA and VZV DNA, plaques were isolated and screened for the expression of HBs by the IFA test. Recombinant viruses expressing HBs were again plaque-purified after inoculation of cell-free virus and a candidate recombinant virus clone was selected by comparison of the immunofluorescence of the isolated clones. Five recombinant clones were selected by the IFA test from 35 plaques isolated after cotransfection. The recombination rate was 14·3%.

Cell-free virus was prepared from infected cells at the 10th cell-to-cell passage and 20 clones were isolated and examined for the expression of HBs by the IFA test. All clones examined expressed similar levels of HBs. This suggested good stability of expression of HBs in the recombinant virus.

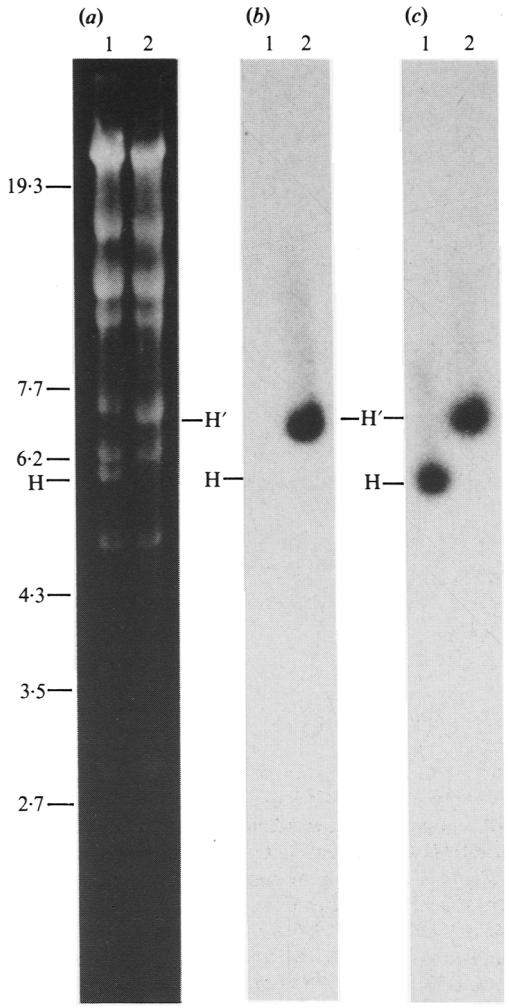

Fig. 2. DNA restriction analysis of the recombinant and parent viruses. VZV DNA of the parent (lanes 1, 3 and 5) and recombinant (lanes 2, 4 and 6) viruses cleaved by *Sac*I (*Sst*I) was electrophoresed through a 0·8% agarose gel and stained with ethidium bromide (*a*). Digested DNA fragments were transferred to a nylon filter and probed with [α-^{32}P]dCTP-labelled pM1B-11 DNA (*b*) and pHH DNA (*c*). Fragment sizes (kb) are indicated. H and H′ are *Sac*I-H and *Sac*I-H′ fragments, respectively.

Detection of the HBs gene in recombinant virus

Insertion of the HBs gene into the VZV TK gene was confirmed by DNA analysis of the recombinant virus as shown in Fig. 2. Digestion of the parental VZV DNA by *Sac*I yielded the expected fragments including *Sac*I-H. Digestion of the recombinant virus DNA by *Sac*I also yielded the expected fragments including the modified *Sac*I-H fragment (*Sac*I-H′) (Fig. 2). The HBs gene was detected in the H′ fragment of recombinant viral DNA by Southern blot analysis using pM1B-11 as a probe. Southern blots probed with pHH revealed both the parental *Sac*I-H fragment and the recombinant fragment, *Sac*I-H′. This result confirmed that the HBs gene was inserted correctly into the TK gene of the recombinant virus genome.

Expression of HBs in recombinant virus-infected cells

IFA using monoclonal antibody to HBs revealed cytoplasmic immunofluorescence in recombinant virus-infected cells as shown in Fig. 3. The amount of HBs expressed in recombinant virus-infected cells and the culture supernatant was measured by RPHA and was estimated to be 10 μg/ml and 4 μg/ml (i.e. 23 ng/ml in the culture supernatant), respectively, by comparison with control antigen produced in yeast (Yosida *et al.*, 1991)

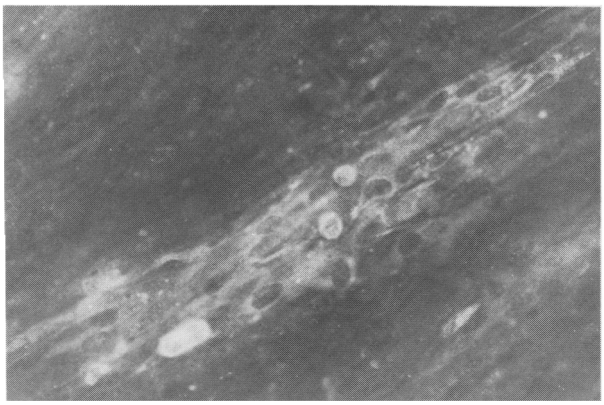

Fig. 3. IFA staining of the recombinant virus-infected cells using monoclonal antibody to HBs.

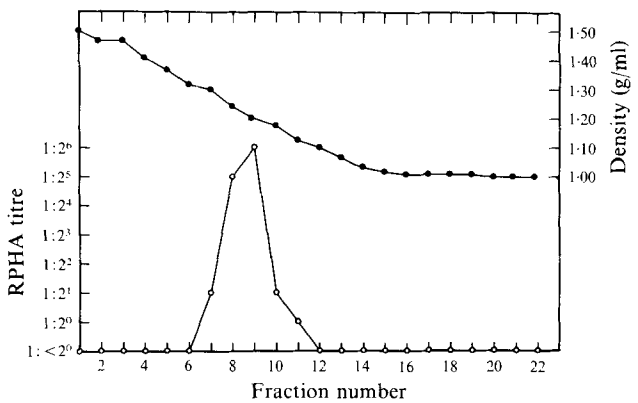

Fig. 4. Purification of HBs secreted from cells infected with the recombinant virus. HBs secreted from infected cells was concentrated and subjected to equilibrium centrifugation in CsCl at 33000 r.p.m. for 40 h. The density (●) of the fraction was monitored by measuring the volume and weight and the titres of HBs antigen (○) were assayed by the RPHA test.

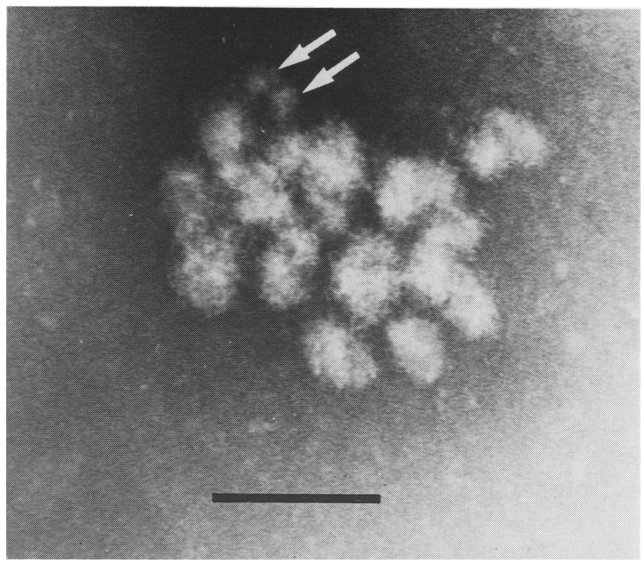

Fig. 5. Electron microscopy of the purified fraction of HBs secreted from cells infected with the recombinant virus. Arrows indicate putative monomer HBs particles. The bar represents 100 nm.

Table 1. *Measurement of HBs expressed in the recombinant virus-infected culture by the RPHA test*

Antigen	RPHA titre
Non-infected cell fraction*	1:<1
Parent vaccine virus-infected cell fraction*	1:<1
Recombinant virus-infected culture supernant†	$1:2^5$
Recombinant virus-infected cell fraction*	$1:2^6$
Control antigen made in yeast (250 μg/ml)	$1:2^{12}$

* Low speed supernatant of the sonic lysate at a concentration of 10^6 cells/ml.

† Culture supernatant was concentrated 175 times.

(Table 1). Thus HBs was produced in infected cells and was secreted into the culture medium.

HBs secreted from infected cells was concentrated by precipitation with PEG and purified by three cycles of equilibrium centrifugation in CsCl followed by a sucrose step gradient. The HBs fractions were identified by RPHA as shown in Fig. 4 and their density was estimated to be 1·20 g/ml. The HBs fraction was distinct from the VZV particle fraction. The final purified HBs fraction was rich in 20 to 25 nm particles as shown in Fig. 5. These results suggest that HBs was secreted from the infected cells in a particulate form.

Identification of HBs in recombinant virus-infected cells

Recombinant virus-infected cells were labelled with [^{35}S]methionine and [^{35}S]cysteine, and HBs was immunoprecipitated with anti-HBs serum as shown in Fig. 6. HBs molecules with M_rs of 26K and 30K were detected only in recombinant virus-infected cells. HBs secreted from infected cells detected as in Table 1 and Fig. 4 was identified as 30K and 35K polypeptides in the labelled culture supernatant, which contrasts with the 26K and 30K polypeptides detected in the infected cells as shown in Fig. 6. The results suggest that the 26K and 30K HBs molecules synthesized in infected cells may be modified or processed to 30K and 35K during secretion into the culture supernatant.

Immune response to HBs in guinea-pigs inoculated with the recombinant virus

Immunogenicity of the recombinant virus expressing HBs was examined in guinea-pigs. The parental vaccine

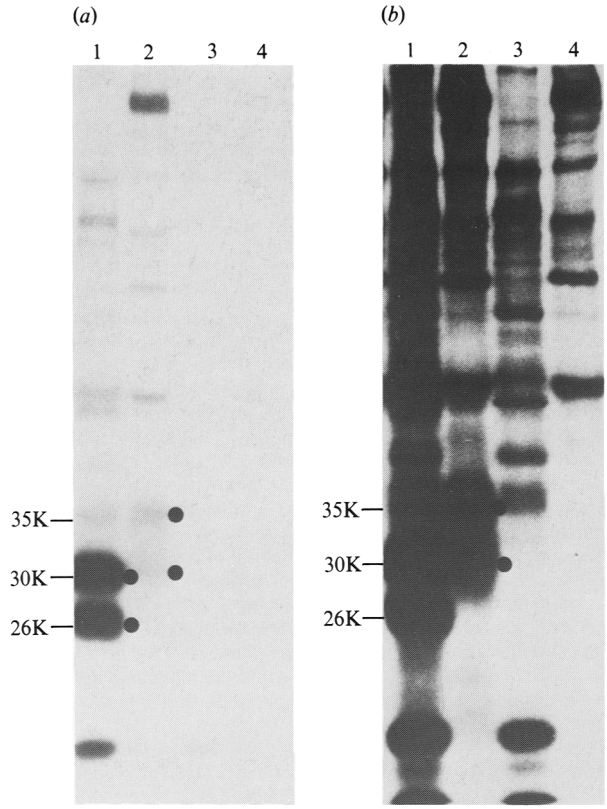

Fig. 6. Detection of HBs in cells and culture medium infected with recombinant virus. HBs synthesized in cells (lanes 1 and 3) and secreted into the culture supernatant (lanes 2 and 4) was labelled with [^{35}S]methionine and [^{35}S]cysteine, and immunoprecipitated with anti-HBs serum. Lanes 1 and 2, recombinant virus-, and lanes 3 and 4, varicella Oka vaccine-infected cells, respectively. Panel (*b*) is a longer exposure of (*a*).

Table 2. *Comparison of immune response to HBs in guinea-pigs after inoculation of the live recombinant varicella vaccine or recombinant HBs subunit vaccine*

Immunogen	Experiment 1*			Experiment 2*
	4 weeks	6 weeks	8 weeks	4 weeks
HBs subunit vaccine				
No. 1	1: 32	1: 32	1: 32	1:128
No. 2	1:128	1:128	1:128	1: 32
No. 3	1: 32	1: 32	1: 32	1: 64
Recombinant varicella vaccine†				
No. 1	1: 32	1: 64	1: 64	1: 32
No. 2	1: 32	1: 64	1: 64	1: 32
No. 3	1: 64	1: 64	1: 64	1:128
Parent varicella vaccine†				
No. 1	1:<1	1:<1	1:<1	NT‡
No. 2	1:<1	1:<1	1:<1	NT
No. 3	1:<1	1:<1	1:<1	NT

* HBs subunit vaccines used were 10 μg/dose produced by Biken (Konobe *et al.*, 1991) in experiment 1 and Merck & Co. in experiment 2.

† Cell-free virus preparations containing 3000 p.f.u. were used as the recombinant varicella or parent varicella vaccines.

‡ NT, Not tested.

virus and the recombinant virus induced an antibody response to VZV, but only the recombinant virus elicited a response to HBs (data not shown). Recombinant Oka varicella vaccine induced levels of antibody to HBs comparable to those elicited by the recombinant HBs subunit vaccine as shown in Table 2. The recombinant virus was as immunogenic as the recombinant HBs subunit vaccine currently used. The results show that the recombinant Oka varicella vaccine virus expressing HBs can induce a good immune response to VZV as well as to HBs.

Discussion

We have constructed an immunogenic recombinant Oka varicella vaccine expressing HBs. The HBs gene was inserted into the TK gene by linking the HBs gene to the translation initiation site of TK and utilizing the authentic regulatory unit of VZV. The preS1 region and a receptor region of preS2 for polymerized human serum albumin were not included in the recombinant virus because these regions are thought to have affinity for hepatocytes (Machida *et al.*, 1984; Neurath & Kent, 1988). Consequently, recombinant Oka varicella vaccine virus and infected cells should not exhibit liver tropism.

The recombinant Oka varicella vaccine induced the synthesis of HBs in the cytoplasm of infected cells and secreted HBs particles into the culture supernatant independently of VZV particles. Expression of HBs by the recombinant virus was stable for at least 10 passages *in vitro*. Guinea-pigs have been used as animal models for VZV infection (Myers *et al.*, 1980; Yamanishi *et al.*, 1980; Matsunaga *et al.*, 1982; Shiraki *et al.*, 1984*a*, *b*) and a single dose of the recombinant virus induced antibodies to both VZV and HBs. The antibody level to HBs was comparable to that induced by HBs subunit vaccine. These results suggest that the recombinant virus could be used as a varicella vaccine as well as an HBV vaccine.

HBs exists as large, medium and small antigens, which are encoded by the preS(1 and 2) and S genes, the preS2 and S genes, and the S gene, respectively (Stibbe & Gerlich, 1983; Laub *et al.*, 1983; Heermann *et al.*, 1984; Machida *et al.*, 1984; Neurath *et al.*, 1985; McLachlan *et al.*, 1987). Recombinant vaccinia viruses expressing large, medium or small HBs have been described (Cheng & Moss, 1987). The large surface antigen construct produced proteins of 39K and 42K which were not secreted from infected cells. Recombinant virus expressing medium surface antigen produced a protein of 33K which was found both in cells and in the supernatant. Recombinant virus expressing small surface antigen produced proteins of 24K and 27K which, again, were found in both cells and supernatants.

In this recombinant varicella virus, the HBs expressed was 26K and 30K in the infected cells, and 30K and 35K in the culture supernatant. The M_rs were intermediate between those of the small and medium surface antigens expressed in recombinant vaccinia viruses. This was to be expected because the recombinant Oka varicella vaccine virus was designed to express the partial preS2 (25 of 55 amino acids) gene and the whole S gene. HBs synthesized in infected cells might be modified or processed, resulting in slower electrophoretic mobilities (Fig. 6), and be secreted into the culture supernatant as HBs particles (Table 1, Fig. 4). The size of the particles observed in the purified HBs fraction (Fig. 5) is similar to that of the 22 nm particles found in the serum of HBV carriers.

VZV infection induced both humoral and cell-mediated immunity, including T cell-mediated cytotoxicity and delayed type hypersensitivity (Asano *et al.*, 1981; Shiraki *et al.*, 1984*a*, *b*; Takahashi, 1987; Hickling *et al.*, 1987; Diaz *et al.*, 1988). Oka varicella vaccine also induces humoral and cellular immunity, including delayed type hypersensitivity (Asano *et al.*, 1981, 1985; Shiraki *et al.*, 1984*a*, *b*; Andrè, 1985; Takahashi, 1987; Diaz *et al.*, 1988; Straus *et al.*, 1988; Gershon *et al.*, 1990). Vento *et al.* (1985, 1987) demonstrated that cellular immunity (T lymphocyte sensitization) to HBs is associated with virus clearance in chronic and acute hepatitis. Therefore, induction of cellular immunity as well as humoral immunity to HBV by recombinant varicella vaccine may occur and be advantageous for protection against HBV infection. Immunity to VZV induced by the Oka varicella vaccine is prolonged and protective (Asano *et al.*, 1985; Takahashi, 1987; Gershon *et al.*, 1990). These observations suggest that immunity to VZV and HBV induced by a single dose of the recombinant Oka varicella vaccine may also be prolonged.

The advantages of Oka varicella vaccine strain as a live expression vector have been discussed by Lowe *et al.* (1987). One is that it is the only live attenuated DNA virus vaccine currently licensed and used. Prevention of HBV infection has been achieved by vaccination with blood-derived or genetically engineered HBs subunit vaccine. In this study the recombinant varicella vaccine expressing HBs induced good immunity to both VZV and HBs in guinea-pigs. Therefore, immunogenicity of the recombinant Oka varicella vaccine virus expressing HBs can be evaluated as a candidate for a live HBV and VZV vaccine, or a polyvalent live vaccine.

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