HEPATITIS B VIRUS CORE-PRES2 PARTICLES EXPRESSED BY RECOMBINANT VACCINIA VIRUS

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Summary. – Vaccinia virus (VV) recombinants expressing hepatitis B virus (HBV) surface (HBsAg) or core (HBcAg) antigens (Kunke *et al.*, Virology 195, 132 – 139 (1993)] have been shown to raise specific antibodies in mice, nevertheless the levels of antibodies reactive with the preS2 and S antigens were low. In an attempt to enhance the immunogenicity of HBsAg-preS2, a fused C-preS2 gene was constructed. The fusion protein was expressed in *E. coli* and displayed both HBcAg and preS2 antigen as demonstrated by enzyme-linked immunosorbent assay (ELISA). The same gene was then expressed using recombinant VV and chimerical particles whose size and density were similar to those of native HBV core particles produced in CV-1 cells infected with recombinant VV. Unlike HBcAg, preS2 antigen could not be detected on these particles by ELISA but was revealed by immunoblot analysis only. The immunogenicity of the recombinant VV was evaluated in mice. Antibodies to HBcAg and VV antigen but not to preS2 antigen were found in sera of animals inoculated with 10^7 PFU of the recombinant VV. Presumably, HBcAg-preS2 particles produced in *E. coli* and in eukaryotic cells have a different conformation, and the presence of preS2 antigen on the surface of chimerical particle might be necessary for a pronounced antibody response.

Key words: hepatitis B virus; surface antigen; core antigen; preS2 antigen; recombinant vaccinia virus; fusion protein; chimeric particles; antibodies

Introduction

The nucleocapsid of HBV is built up of many copies of a 22 K core protein (for review see Neurath and Thanavala, 1990; Ganem, 1991). A production of core particles, bearing HBc antigenicity, has been achieved using several expressing systems including *E. coli* (Stahl *et al.*, 1982) and VV (Kunke *et al.*, 1993). These particles can serve as an efficient carrier for presentation of heterologous epitopes (for review see Schoedel *et al.*, 1994). Several sites have been found in the amino acid sequence of core protein, where foreign epitopes can be inserted without affecting the assembly of particles. However, it has been demonstrated that the position of the insertion determines the immunogenicity of the epitope and its accessibility for antibody binding (Schödel, 1992). Experimental HBV vaccines based on chimerical core particles comprise virus neutralization epitopes from the preS1, preS2, or S domains of HBsAg (Borisova *et al.*,1989; Stahl and Murray, 1989; Schoedel *et al.*,1992). Expression of the HBc-preS2 fusion gene in *E. coli* can result in formation of chimerical core-like structures exhibiting preS2 antigen on their surface, and capable of inducing preS2 antibodies in laboratory animals. In this study we report on the construction of a similar fusion gene and its expression in bacteria and in eukaryotic cells, when inserted in recombinant VV.

Abbreviations: CPE = cytopathic effect; ELISA = enzyme-linked immunosorbent assay; HBV = hepatitis B virus; HBcAg = HBV core antigen; HBsAg = HBV surface antigen; MoAb = monoclonal antibody; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; SDS = sodium dodecyl sulphate; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of SDS; VV = vaccinia virus

Recently, we constructed several recombinant VVs for expression of HBV envelope proteins (Němečková *et al.*, 1991), HBc or HBe antigens (Kunke *et al.*, 1993). The immunogenicity of these recombinant viruses was examined in laboratory animals. An administration of recombinants expressing HBsAg to mice elicited only low HBs- or preS2-specific antibody responses. However, the mice were primed, as was revealed by inducing antibodies to both antigens by subsequent administration of low doses of plasma-derived HBsAg. On the other hand, mice responded well to VV-expressed HBcAg (Kunke *et al.*, 1993). To increase the immunogenicity of VV-expressed preS2, we decided to construct a recombinant VV expressing the HBc-preS2 fusion protein.

Materials and Methods

Virus and cells. VV strain Praha, clone P13 (Kutinová *et al.*,1995) was propagated and titered on CV-1, LEP, and RAT-2 cell lines as described earlier (Kutinová et al., 1990).

Monoclonal antibodies (MoAbs). PreS2-specific MoAb F124 (Budkowska *et al.*, 1986) was obtained by courtesy of Dr. A. Budkowska (Institut Pasteur, Paris). Anti-preS2 mouse MoAb S26 (Sominskaya *et al.*,1992) was obtained by courtesy of Dr. V. Bichko (Riga, Latvia). HBcAg-specific MoAb (Hložánek *et al.*, 1987) was a gift from Dr. J. Škvor (Sevac, Prague).

Plasmid vectors. To construct plasmid pCS2 (Fig. 1), the previously described plasmid pC95 (Kunke *et al.*, 1993) was digested with *Bam*HI and *Hpa*II. The 449 bp fragment coding for the 144 N-terminal amino acids of HBcAg was ligated to the 180 bp *Hpa*II fragment coding for the 55 preS2 amino acids. The preS2 fragment was prepared by polymerase chain reaction (PCR) amplification using oligonucleotide primers preS2-1 (5'-ACT-TCCGGAGATGCAGTGGAATTCTACTAC-3') and preS2-2 (5'-ATGGGATCCTAGTTCAGCGCAGGGTCC-3'), and plasmid 1.7 HEP (Kutinová *et al.*, 1990), which contains the preS2/S gene of HBV, ayw subtype (Pourcel *et al.*, 1982), as template DNA. The ligated C-preS2 fragment was digested with *Bam*HI and inserted in the *Bam*HI site of pUC18. The ligated DNA mixture was transfected in *E. coli* -K802 and transformed colonies were screened for synthesis of the HBc and preS2 antigens. One of the positive clones contained a plasmid which was denoted p24. This plasmid was cleaved with *Bam*HI and the C-preS2 coding sequence was ligated to pSN8 digested with *Bam*HI. The resulting plasmid was denoted pCS2. Plasmid pSN8, described by Kunke *et al.*(1993), is a VV insertion vector similar to pGS20 (Mackett *et al.*, 1984).

Generation of VV recombinants. Plasmid pCS2 was used for generation of a VV recombinant expressing chimerical HBc-preS2, denoted VV-CS2. Recombination and selection of recombinants was performed by standard procedures (Mackett, 1984; Paoletti, 1984; Perkus, 1986). Recombinant VV-C1 expressing HBcAg was prepared using the plasmid pC1 described by Kunke *et al.* (1993) and recombinant VV-M1 expressing HBsAg was described by Němečková *et al.* (1991).

HBcAg and preS2 antigen ELISA. The synthesis of HBV antigens was monitored in bacterial extracts or extracts of virus-infected cells. Briefly, *E. coli* K802 transformed with plasmid p24 or with control plasmids were grown overnight in 4 ml of LB broth containing 100 μg/ml ampicillin. The bacterial pellet was resuspended after washing in 0.5 ml TNE and disintegrated by sonication. Samples were centrifuged at 9700 x g for 20 mins and HBV antigens were measured in the supernatants. Recombinant VV-expressed antigens were detected as follows. Virus infected CV-1 cells at the stage of complete cytopathic effect (CPE) were frozen and thawed three times. Samples were clarified by centrifugation at 540 x g for 30 mins and HBV antigens in the supernatant were quantified. The synthesis of HBcAg was measured by ELISA as described previously (Kunke *et al.*, 1993). PreS2 ELISA was described by Němečková *et al.* (1991). Briefly, microtitration plates were coated with glutaraldehyde-polymerized human serum albumin. Washed wells containing the material tested were then incubated at 37°C for 2.5 hrs. After washing, the plates were incubated with the preS2-specific MoAbs F124 diluted 1:10,000 or S26 diluted 1:1000. The plates were washed again and incubated with peroxidase-labelled swine anti-mouse IgG (SwAM/Px, Sevac, Praha) diluted 1:2000. O-phenylene diamine was used as chromogen.

Immunoblot analysis. CV-1 cells were infected with VVs at a multiplicity of 1 PFU/cell. Cell monolayers with 75-90% CPE were harvested with a rubber policeman and centrifuged. The pellets were lysed in 0.15 ml of lysis buffer containing 50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 1% sodium deoxycholate and 1.25 mmol/l phenyl methyl sylphonyl fluoride. After 20 mins of incubation at 0°C the lysates were clarified at 9600 x g for 10 mins at 4°C. Samples, unheated, were separated by electrophoresis in 15% polyacrylamide gel in the presence of SDS (SDS-PAGE) under reducing conditions according to Laemmli (1970). Proteins were electroblotted to BA85 nitrocellulose membrane. PreS2 antigen was detected with MoAb S26 and with a horseradish peroxidase-labelled second antibody SWAM/Px (Sevac, Prague). HBcAg was visualized by goat anti-HBe IgG labelled with horseradish peroxidase, obtained by courtesy of Dr. J.König (National Institute of Public Health, Prague).

Immunization of mice. Four-week-old non-inbred mice (strain ICR) were inoculated intraperitoneally with 10^7 PFU of VV-CS2, VV-C1, or VV-M1. For this purpose, viruses were propagated on chorioallantoic membranes of 11-day-old chick embryos, concentrated and partially purified according to Joklik (1962). Four weeks later all mice received intraperitoneally 0.5 ml of suspension containing 93 ng of purified plasma HBsAg absorbed to alum in phosphate-buffered saline (PBS). As shown previously, this amount of plasma HBsAg was not able to raise significant preS2 or HBs antibody levels in unprimed mice but elicited preS2 and HBs antibodies in mice that had been primed with VV-HBV recombinant (Hamšíková *et al.*,1990). The mouse sera were collected after another four weeks and the amounts of VV-, preS2-, and HBcAg-specific antibodies in individual sera were measured by ELISA.

HBc and preS2 antibody assays. HBc antibodies were determined by ELISA as described by Kunke *et al.* (1993). The test uses competition between the HBc antibody present in the sera tested and goat HBc antibody labelled with peroxidase.

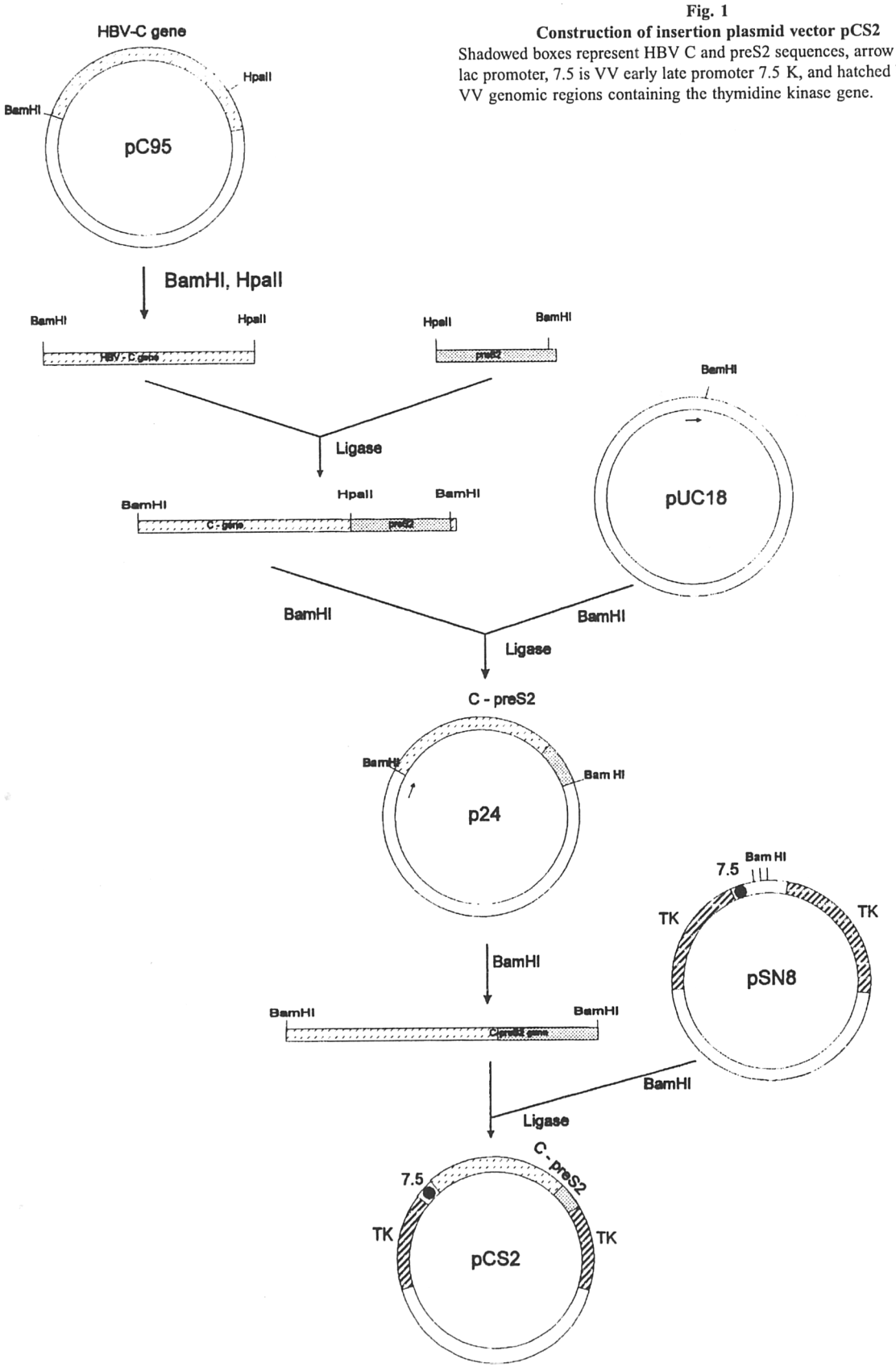

Fig. 1
Construction of insertion plasmid vector pCS2
Shadowed boxes represent HBV C and preS2 sequences, arrow indicates lac promoter, 7.5 is VV early late promoter 7.5 K, and hatched boxes are VV genomic regions containing the thymidine kinase gene.

Briefly, microtitration plates were coated with 2 μg of anti-HBc MoAb. Then 7 μg of *E. coli*-expressed purified HBcAg (gift from Dr. M. Janda, Sevac, Praha) was added to each well and incubated for 1 hr at 37°C. A tested serum diluted 1:4 with goat anti-HBc peroxidase-conjugated IgG diluted 1:800 was added and the plates were incubated for 1 hr at 37°C. A_{492} values of negative sera were about 1.00 The presence of HBc antibodies in sera was manifested by a decrease in absorbance values. ELISAs for preS2- and VV-specific antibodies were described by Hamšíková *et al.* (1990).

Results

Synthesis of HBV antigens in E. coli

The results summarized in Table 1 indicate that p24 was able to induce synthesis of both HBcAg and preS2 antigen in *E. coli*. Bacteria transformed with pC95 produced HBcAg. Plasmid pUC was used as negative control. The HBc-preS2 fusion gene from p24 was therefore used for constructing VV recombinants.

Detection of expressed chimerical protein by immunoblot analysis

Synthesis of HBc-preS2 chimerical protein in CV-1 cells infected with VV-CS2 was demonstrated by immunoblot analysis (Fig. 2). Among the transferred proteins only a single 22 K band (lane 4) reacted specifically with both anti-preS2 MoAb S26 and with goat HBe antibody (lanes 4, 5). To show the double antigenic specifity of this band, the membrane was cut in the middle of the lane 4. The band had similar weight as that of the HBV core protein expressed by VV-C1 (lane 6). The MoAb S26 reactivity was demonstrated by its ability to detect preS2 antigen of the 32 K middle envelope HBV protein (lane 2). Besides HBV proteins, also a non-specific high molecular mass band appeared in lanes 1-4. Thus, recombinant VV-CS2 induced synthesis of HBc-preS2 fusion polypeptide.

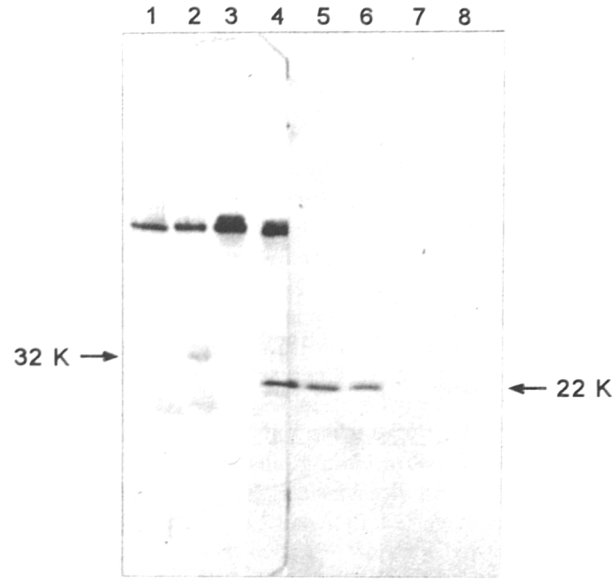

Fig. 2
Immunoblot analysis of VV-expressed HBV proteins
Samples were prepared from cells infected with parental VV (lane 1, 8), VV-M1 (lanes 2, 7), VV-C1 (lanes 3, 6), or VV-CS2 (lanes 4, 5). The membrane with blotted proteins was cut into two pieces in the middle of the lane 4. The left part (lanes 1-4) was stained with anti-preS2 MoAb, the right part (lanes 4-8) was stained with HBc antibody.

Table 1. Detection of HBV antigens expressed in *E. coli* and by VV recombinants in non-denatured extracts by ELISA

Expression vector	Inserted gene	Amount of HBV antigens (A_{492}) HBcAg	preS2	HBsAg
pUC18[a]	–	0.15	0.33	ND
pC95[a]	C	2.6	0.37	ND
p24[a]	C-preS2	1.1	0.80	ND
VV[b]	–	0.15	0.35	0.19
VV-M1[b]	preS2-S	0.16	>2.5	>2.1
VV-C1[b]	C	>2.7	0.38	0.24
VV-CS2[b]	C-preS2	>2.6	0.34	0.23

[a]Expression in *E. coli* K802.
[b]VV strain Praha, clone 13 and its recombinants.
ND = not done.

Detection of HBcAg and preS2 antigen by ELISA

The synthesis of HBcAg and pre-S2 antigen in cells infected with VV recombinants was tested by ELISA (Table 1). HBcAg was found in non-denatured extracts of CV-1 cells infected with VV-CS2. HBcAg was also produced in cells infected with VV-C1. PreS2 antigen, which could be detected in cells infected with VV-M1, was not found in extracts from cells infected with VV-CS2 by any of the ELISA modifications applied.

Physical properties of chimerical particles

The size and density of HBV antigen particles expressed by VV-CS2 were determined by centrifugation in sucrose and CsCl density gradients and compared with HBcAg particles expressed by VV-C1. HBcAg expressed by both recombinants was found in the same fraction after sedimentation in sucrose gradient (10-30% w/v, 4 hrs, 150,000 x g, data not shown). The buoyant density of both HBc-preS2

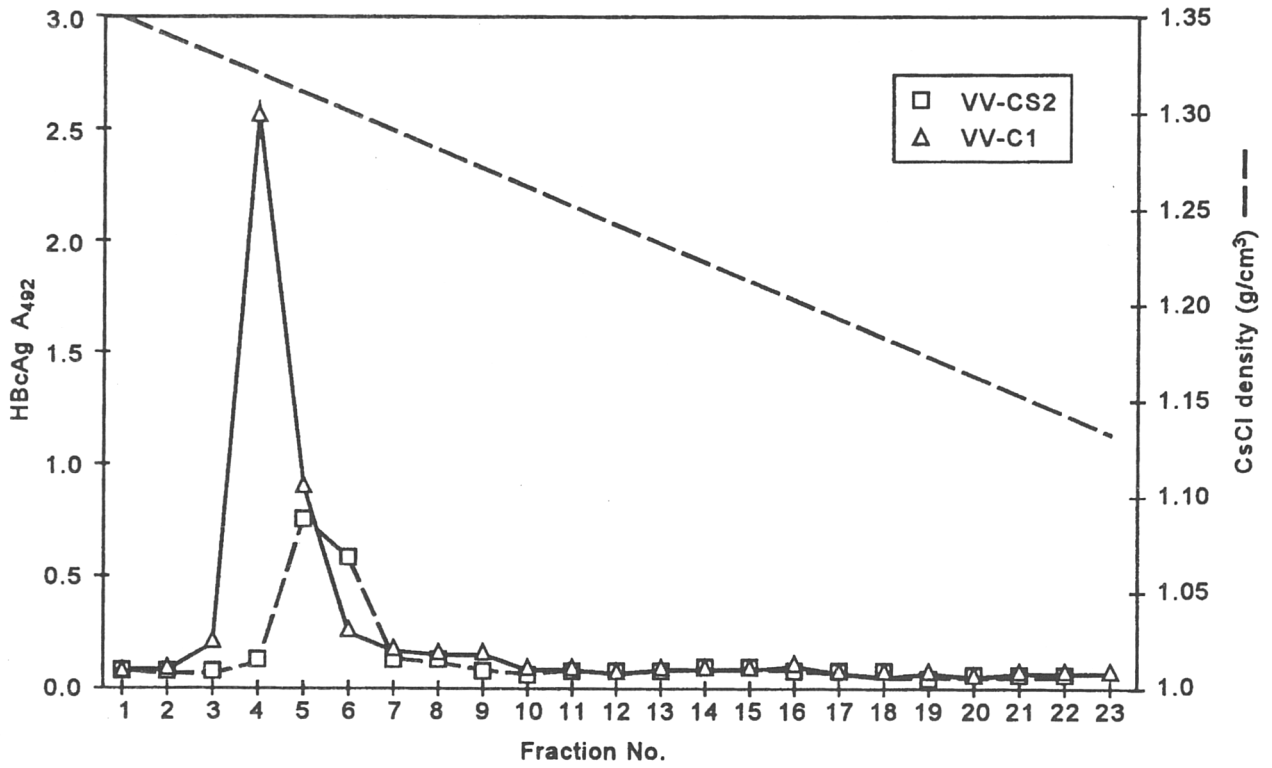

Fig. 3
Sedimentation profiles of HBcAg in extracts of cells infected with VV-CS2 and VV-C1
Isopycnic CsCl density gradient centrifugation at 220,000 x g for 62 hrs. HBcAg determined by ELISA (A_{492}).

antigen and HBcAg was about 1.32 g/cm³ (Fig. 3). From similar physical properties of VV-expressed HBc-preS2 antigen and HBcAg particles and from their HBc antigenicity, typical of nucleocapsid particles, we concluded that HBc-preS2 antigen can form nucleocapsid-like particles. We observed that the VV-expressed chimerical particles were less stable during the purification procedure than the nucleocapsid particles.

Immunogenicity of VV-CS2

The main purpose of this study was to evaluate the immunogenicity of HBV antigens of chimerical particles synthesized *in vivo* in cells infected with VV recombinants expressing HBc-preS2 fusion gene. Antibody responses in mice inoculated with the different recombinant viruses are shown in Fig. 4. The animals responded to all recombinants by producing similar levels of VV antibodies. Both VV-CS2 and VV-C1 induced HBc antibodies, which were not produced after inoculation of VV-M1. PreS2 antibodies were only found after administration of VV-M1, whereas neither VV-CS2 nor VV-C1 induced any preS2 response.

Discussion

The aim of the study presented here was to enhance the immunogenicity of VV-expressed preS2 antigen. We decided to make use of the capability of HBV nucleocapsid particles to function as immunogenic carrier of foreign epitopes. Moreover, the synthesis of chimerical particles directly *in situ* in VV-infected cells may help to avoid an additional purification of particles produced in bacteria otherwise. Therefore we constructed a VV recombinant that induced in infected cells the synthesis of a fusion protein formed by the 144 N-terminal amino acids of the HBV nucleocapsid protein and by the 55 amino acids of the preS2 region of the HBV envelope protein, which were positioned on the carboxyl terminus. This fusion gene has already been expressed in *E. coli* and purified particles had been shown to induce antibody response to preS2 antigen (Borisova *et al.*,1989; Stahl and Murray; 1989, Schoedel *et al.*, 1992). The preS2 epitope had been detected on the surface of such *E. coli*-produced HBc-preS2 particles by MoAbs. However, it had later been shown that certain other regions of HBcAg, e.g. around the 77th amino acid position are much more suitable for insertion (Schoedel *et al.*, 1992; Puschko *et al.*, 1994).

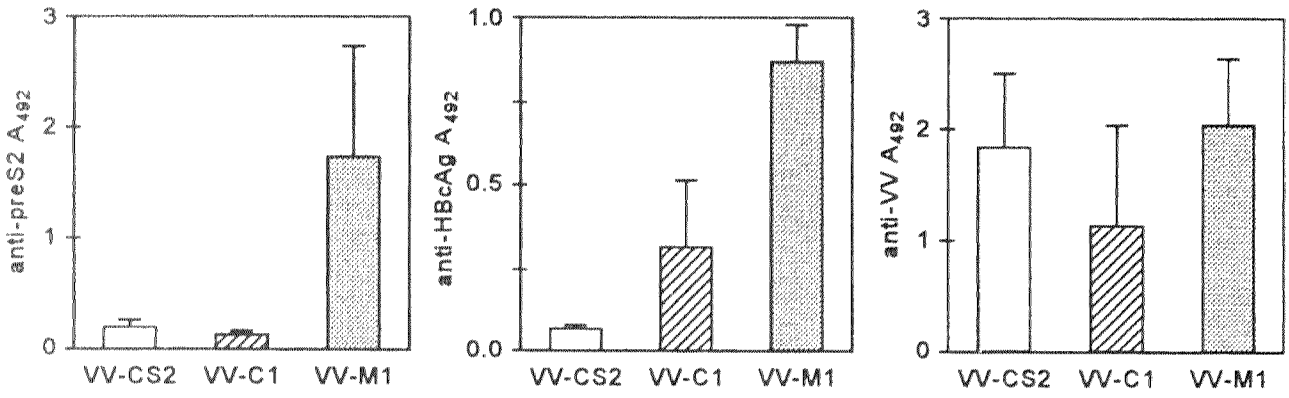

Fig 4.
Antibody response to preS2, HBc, and VV antigens in mice

Mice were inoculated with 10^7 PFU of recombinant VV, boosted with 93 ng of alum-adsorbed plasma HBsAg 4 weeks later and sera were collected after another four weeks. Each column represents an arithmetical mean of A_{492} values as detected in sera of five individual mice. Error bars indicate the confidence intervals at 0.05 level. The amounts of VV and preS2 antibodies were proportional to the A_{492} values. A_{492} values of about 1.00 were regarded as negative results of the HBc antibody assay; the presence of HBc antibodies in tested sera reduced the A_{492} values.

Also in our hands, the expression of plasmid p24 in *E. coli* gave particles with similar properties as described by others. In contrast to bacteria-expressed particles, the reactivity of particles extracted from VV-CS2-infected cells failed to react with both S26 and F-124 preS2-specific MoAbs. The original target to increase the immunogenicity of preS2 antigen was not achieved as VV-CS2 was such a poor immunogen that it was even unable to prime an anti-preS2 response. The conclusion from our study is that bacteria- and VV-expressed chimerical particles, in spite of their similar primary structure, have different antigenic properties, and this could reflect specific features of particle assembly in eukaryotic and bacterial cells.

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