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TWO VACCINIA VIRUS RECOMBINANTS EXPRESSING HBsAg WITH DIFFERENT CONCENTRATION OF A- AND PRE-S2 ANTIGENIC DETERMINANTS

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Summary. – Comparative studies of two vaccinia virus (VV) recombinants expressing the hepatitis B virus (HBV) surface antigen (HBsAg) including the pre-S2 region (M-protein) showed that the L-pre-S2/15 recombinant expressed 5-fold more HBsAg as determined by the content of a-determinant than the recombinant vl37. However, both recombinants expressed comparable amounts of the pre-S2 antigenic determinant as assessed by enzyme immunoassay with monoclonal antibodies. According to our calculations, one HBsAg unit expressed by the recombinant vl37 contained 7–9 times more pre-S2 antigen than did one HBsAg unit expressed by the L-pre-S2/15 recombinant. Binding of pre-S2 region to polymerized human serum albumin was shown not to be an efficient assay at low pre-S2 concentration. HBsAg expressed by the vl37 recombinant was less extensively secreted from cells as compared to that expressed by L-pre-S2/15 recombinant. Both recombinants induced the production of antibodies to the pre-S2 antigenic determinant in rabbits. L-pre-S2/15 induced anti-HBsAg a-determinant antibody as well.

Key words: vaccinia virus; hepatitis B virus; recombinants; HBsAg; a-and pre-S2 antigenic determinants; synthesis dynamics; antigenic properties

Introduction

Preparation of vaccinia virus (VV) recombinants expressing the hepatitis B virus (HBV) surface antigen seems of importance for manufacturing of a live vaccine to hepatitis B. The first VV recombinants expressing HBsAg were produced on the basis of the WR strain (Smith *et al.*, 1983; Paoletti *et al.*, 1984)

or of the Lister strain (Altstein *et al.*, 1985; 1986). One of the recombinants (L-HB32) served for production of live dermal smallpox-hepatitis B vaccine which had been tested in laboratory animals and volunteers (Chernos *et al.*, 1990). This vaccine was proved to be of low neurovirulence and highly immunogenic for rabbits. Although the virus induced anti-VV antibody response in man, its immunogenicity eliciting anti-HBsAg antibody production was poor. In this context it seems necessary to continue the work to enhance the immunogenic capacity of recombinants towards the foreign gene products.

One of the important approaches in enhancing immunogenicity of the VV recombinants expressing HBsAg implies insertion of HBV gene coding for the 33–36 kD M-protein (Tiollais *et al.*, 1985) into the recombinant. This polypeptide contains at its NH_2-end the highly immunogenic pre-S2 region (Milich *et al.*, 1985). Such recombinants have been produced in several laboratories (Cheng and Moss, 1987; Pashvykina *et al.*, 1990; Kutinová *et al.*, 1990). The present communication is focused on comparative studies of two recombinants expressing HBsAg M-protein. It has been demonstrated that HBsAg expressed by these recombinant viruses significantly differs in the expression of the major (a-determinant) and pre-S2 antigenic determinant.

Materials and Methods

Viruses and cells. Table 1 summarizes the characteristics of the Lister strain of VV (LIVP variant) and three used pox-virus recombinants expressing HBsAg. The recombinant viruses were propagated in CV-1 continuous monkey kidney cell culture (Jensen *et al.*, 1964), or in the primary culture of chick embryo cells (CEC) in Eagle's MEM medium supplemented with 2 % foetal bovine serum (FBS). The virus was titrated using the plaque assay with or without agar.

Preparation of HBV antigen. CV-1 cells grown in 24-well plates (Flow Lab., England), were inoculated with 2–3 PFU/cell of the virus at 37 °C for 1 hr. After removal of the non-adsorbed virus by washing (two times with Dulbecco phosphate-salt buffer, pH 7.2), and icubation of cells in a 5 % CO_2 atmosphere at 37 ° until complete CPE appeared (48–72 hr), several specimens were prepared: 1) total material (culture fluid + cells) – the plates with cells were exposed to 3 freezing-thawing cycles, the debris was removed by centrifugation, and the supernatant stored at -20 °C until use; 2) the culture medium; and 3) cells were processed separately – the culture medium was removed and clarified by centrifugation; the pellet was transferred back to the well; substrate-adherent cells were fed with fresh medium in the same volume with subsequent 3 cycles of freezing-thawing; 0.2 % Tween-20 and 1 mmol/l phenylmethylsulphonylfluoride (PMSF) were added, the cell debris was removed by centrifugation, the supernatant fluid was collected. To prepare the cell concentrate and HBsAg from the culture medium, the virus was grown in 1 l-flasks on CEC. The culture fluid was collected when CPE reached confluency, centrifuged at 30,000 rev/min for 24 hr and the pellet containing HBsAg and VV particles was resuspended in 10 mmol/l Tris-buffer comprising 1 % of the initial volume. Glass-adherent cells were washed with 10 mmol/l Tris-buffer with 2 mmol/l EDTA and 0.1 mol/l NaCl, removed from glass, centrifuged and resuspended in the same buffer to 1 % of initial volume. The cells were disrupted by freezing, thawing and ultrasound, the cell debris was removed by centrifugation and the final product was considered to be a 100-fold cell extract (1 mmol PMSF was added both to the culture fluid and buffer solutions).

Antigen assays. HBsAg was assessed by enzyme immunoassay for the a- and pre-S2 determinante in 96-well polystyrol plates (Flow Lab.). To establish the presence of HBsAg a-determinant

the wells were coated with 0.1 ml of guinea pig anti HBsAg IgG (10 μg/ml, 50 mmol/l bicarbonate buffer pH 9.6) for 1 hour. After two hours incubation with 0.1 ml of studied material horse radish peroxidase conjugated anti HBsAg a-determinant monoclonal antibody (MAb) (clone 3H2, A. Vilnius, Institute of Microbiology of the Latvian AS, Riga) was added to washed wells for 2 hours. The colour reaction was achieved by 0.02 mg/ml orthophenylenediamine in 40 mmol/l acetate buffer, pH 5.6 with 0.01 % H_2O_2 for 30 min. All incubations were carried out at 37 °C. For all dilutions IFA buffer was used (20 mmol/l natrium phosphate, 150 mmol/l LiCl, 0.2 % Tween-20, 1 % FBS, 1 mmol/l PMSF). The results were evaluated using Uniscan spectrophotometer (Flow Lab.). The mean value of 4–6 parallels was calculated. The results were compared with values of standard of known concentration of HBsAg. As negative control, uninfected cells and Lister strain inoculated cells were used. HBsAg titre was expressed as ng/10^6 of infected cells.

Pre-S2 antigen concentration was established by two methods. 1) Using MAb to pre-S2 determinant, clone F124 (Budkowska *et al.*, 1986). Briefly, 0.1 ml volume of studied material was added to the wells coated with above mentioned anti HBsAg antibodies for 2 hours. Washed wells were then incubated with anti-pre-S2 monoclonal antibodies for 1 hour, washed and then horse radish peroxidase conjugated swine anti-mouse IgG was added for 2 hours. The colour reaction was achieved as described. 2) Based on the pre-S2 region ability to bind to human polymerized albumin. The wells were coated with 0.1 ml of HPA prepared according to Hanson and Purcell (1979) and dried (Bluger *et al.*, 1985). After 30 min incubation with IFA buffer 0.1 ml of the studied material was added to each well for 2 hours and after washing the wells were incubated with horse radish peroxidase conjugated anti HBsAg monoclonal antibody for 2 hours. The colour reaction was achieved as described. As positive control pre-S2 antigen containing HBsAg preparation was used, negative controls were mentioned above. Pre-S2 antigen activity in the given material was expressed as mean optic density characterizing the undiluted material minus the optic density of the negative control.

Immunoblotting of recombinant-infected 100 fold cell concentrates was performed as previosly desctibed (Chelyapov *et al.*, 1988).

Assays for immunogenic activity of recombinants. Chinchilla rabbits were infected on scarified skin (5 × 5 cm), or using a bifurcation needle. VV neutralizing antibody titres were determined on chick embryos (Boulter, 1957). HBsAg antibodies were tested by radioimmunoassay using

Table 1. Characterization of the VV recombinants used

Recombinant	Vector virus	HBV genome fragment[6]			
		Length (kb)	Presence of pre-S2	Presence of S-gene	Length from transcription initiation site to the lst ATG (n.p.)
L-HB32[1]	LIVP[2]	1.28	−	+	67
L-pre-S2/15[3]	LIVP	1.44	+	+	46
vl37[4]	Praha[5]	1.7	+	+	303

[1]Altstein *et al.*, 1986; [2]Lister strain variant used for smallpox vaccine manufactured in the U.S.S.R.; [3]Pashvykina *et al.*, 1991; [4]Kutinová *et al.*, 1990; [5]Slonim and Hulenová, 1969; [6]HBV genome fragment is included in tk-gene of VV and controlled by the promotor of 7.5kD protein poxvirus gene (Mackett *et al.*, 1982); fragment sources: for L-HB32 and L-pre-S2/15 - pHBV320 (Pumpen *et al.*, 1981), for v137 - pAC2 (Pourcel *et al.*, 1982).

a commercial kit ("Radioisotope", Tashkent). Pre-S2 antibodies were tested in ELISA using a synthetic peptide (14-33 amino acid sequence of pre-S2 region (Hamšíková *et al.*, 1990).

Results

Fig. 1 depicts HBsAg formation in L-pre-S2/15 and vl37-infected CV-1 cell culture as detected by a- and pre-S2-determinants in the culture fluid and cell fraction. Table 2 summarizes the data on comparison of HBsAg expression of three recombinants as detected by the a-determinant content. Table 3 shows comparative analysis of L-pre-S2/15 and vl37 recombinants in terms of the relative content of a- and pre-S2-determinants in HBsAg. As evident from the Figure 1 and Tables 2 and 3, L-pre-S2/15 recombinant proves to produce 5–10 times more HBsAg than the vl37 recombinant, being somewhat superior to the L-HB-32 recombinant. However, the assays with the use of MAb did not reveal any significant differences of pre-S2 antigen concentration in L-pre-S2/15 and vl37 recombinants. HPA assays demonstrated a marked superiority (5–7-fold)

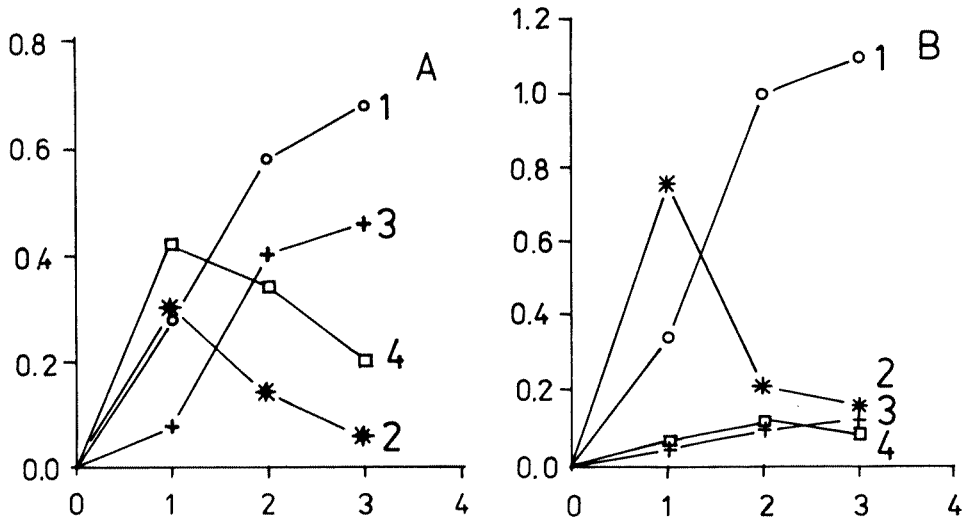

Fig. 1.
The dynamics of HBsAg formation detected by a- and pre-S2 antigenic determinants in VV recombinant-inoculated CV-1 cells
Abscissa: days after inoculation (2-3 PFU/cell): Ordinate: index of absorption (A_{492}) in the course of examining the material diluted 1:2 by enzyme immunoassay using MAbs to a- or pre-S2 antigenic determinants (see *"Materials and Methods"*).
A - pre-S2 determinant; B - a-determinant
1 - L-pre-S2/15 (culture fluid)
2 - L-pre-S2/15 (cells)
3 - vl37 (culture fluid)
4 - vl37 (cells)

Table 2. HBsAg formation in recombinant-inoculated CV-1 cells

Recombinant	No of expts	HBsAg formation (a-determinant) ng/10^6 cells[1)]	Relative formation of HBsAg[2)]
L-HB32	4	277±12	4.4
L-pre-S2/15	5	339±36	5.4
vl37	5	63±15	1.0

[1)]Total content of HBsAg in the culture fluid and cells 72 hr after inoculation of 2-3 PFU/cell.
[2)]HBsAg formation with vl37 recombinant was defined as 1.0.

Table 3. Correlation of a- and pre-S2 antigenic determinants in HBsAg induced by two VV recombinants

Recombinants	No of expts	Relative content			Pre-S2 ratio*	
		a-determinant	Pre-S2 determinant			
			MAb	HPA	MAb	HPA
L-pre-S2/15	3	4.8	-	0.13	-	36.9
vl37		1.0	-	1.0	-	1.0
L-pre- S2/15	2	8.0	1.1	-	7.3	-
vl37		1.0	1.0	-	1.0	-
L-pre-S2/15	1	10.3	1.13	0.19	9.1	54.2
vl37		1.0	1.0	1.0	1.0	1.0

*pre-S2 antigenic determinant content and ratio in vl37 recombinant-induced HBsAg was arbitratily defined as 1.0
MAb - the method based on the application of monoclonal antibodies;
HPA - the method based on the application of human polymerized albumin;
- not studied

of the vl37 recombinant as compared with the L-pre-S2/15 recombinant. These data show that the amount of pre-S2 antigenic activity in form of HBsAg expressed by vl37 recombinant is 7–9 times higher than in the case of L-preS2/15 recombinant.

A similar observation was revealed when other approaches were used in the studies of recombinants in CEC.(Table 4, Fig. 2). In these cells L-pre-S2/15 recombinant induced 3 times more HBsAg then the vl37 recombinant, but, on the other hand, the pre-S2 determinant content is 2.5 times lower. In other words, vl37 recombinant induced HBsAg contained 7.5-fold amount of pre-S2 determinant as compared to that induced by L-pre-S2/15 recombinant. The vl37 recombinant induced HBsAg had stronger cell association than that induced by L-pre-S2/15 recombinant (the same tendency was detected in CV-1 cells, Fig. 1). Immunoblot technique (Fig. 2) showed a significantly higher amount of pre-S2 polypeptide in vl37 recombinant infected cells. The studies

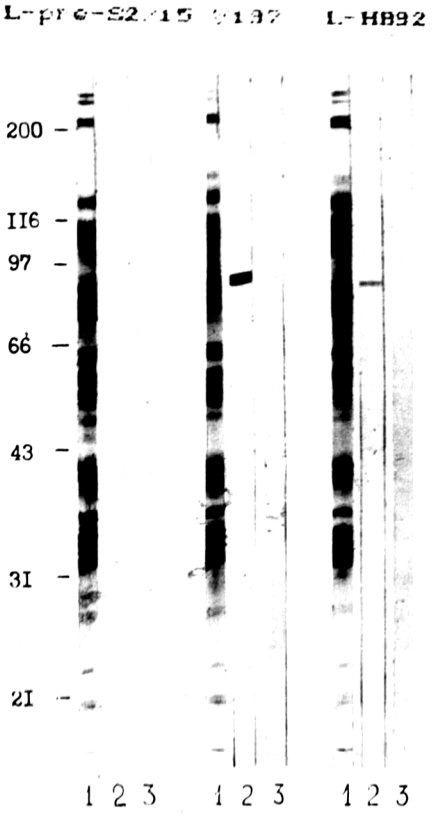

Fig. 2.
Pre-S2 antigen detection in VV recombinant-inoculated chick embryo cells by immunoblot technique
1 - anti-a (HBsAg) MAb
2 - anti-pre-S2 Mab
3 - anti-VV serum

of VV antigens in the same experiments showed that the differences detected between L-pre-S2/15 and vl37 recombinants were associated neither with their different reproductive activity nor with their different ability to be secrected from cells.

The immunogenic activity of L-pre-S2/15, vl37 and L-HB-32 recombinants was studied in rabbits (Table 5). As evident from the Table, L-pre-S2/15 and vl37 recombinants in contrast to L-HB-32 recombinant induced antibody response towards the pre-S2 antigen. Antibodies to the HBsAg a-determinant were detected only in L-pre-S2/15 and L-HB-32 recombinant inoculated rabbits. All three recombinants induced virus-neutralizing antibodies to VV in similar titres indicating the lack of significant differences in their capacity to replicate in the rabbit skin.

Discussion

The comparative studies of two VV recombinants expressing HBsAg with pre-S2 region have revealed marked differences. The L-pre-S2/15 recombinant proved to produce a significantly higher amount of HBsAg than did the vl37 recombinant. This seemed to be associated with the observation that the

Table 4. S- and pre-S2 antigenic determinants of extra- end intracellular HBsAg induced by VV recombinants in chick embryo cell culture

Recombinants	Antigens								
	S			pre-S2			VV		
	cf	cells	cf/cell	cf	cells	cf/cell	cf	cells	cf/cell
L-HB32	10 240	10 240	1.0	<10	<40	-	5 120	20 480	0.25
L-pre-S2	10 240	10 240	1.0	5 120	5 120	1.0	5 120	20 480	0.25
vl37	1 280	5 120	0.25	5 120	20 480	0.25	5 120	20 480	0.25

100-fold concentrated antigens from the culture fluid (cf) and cells (48 hr after inoculation, 10–20 PFU/cell) were prepared as described in *"Materials and Methods"*. The antigen titres presented in the Table were defined as the end-point dilution of the preparation which in enzyme immunoassay had the optic density index increased 2.1-fold and in radioimmunoassay – radioactivity index increased 3-fold as compared to negative control. S-antigen was determined using the commercial kit for immunoradiometric assays for HBsAg ("Radioisotope", Tashkent), pre-S2 antigen was detected using the MAb test (*"Materials and Methods"*). VV antigens were determined with the use of a commercial immunoenzyme kit for orthopoxvirus antigen assays (Tomsk Research Institute for Vaccines and Sera).

Table 5. Immunogenic activity of HBsAg-expressing VV recombinants

Virus	Route of inoculation	Virus dose (PFU)	Antibody titre to: HBsAg		pre-S2		VV	
	Bifurcation needle	6×10^6	160 20	[2/2]	80 20	[2/2]	160 160	[2/2]
L-pre-S2/15	Scarification	3×10^7	80 80	[2/2]	10 320	[2/2]	2560 1280	[2/2]
vl37	Scarification	4×10^6	<10 <10	[0/2]	20 20	[2/2]	640 160	[2/2]
L-HB32	Scarification	8×10^7	80 80	[2/2]	<10 <10	[0/2]	640 1280	[2/2]
LIVP	Scarification	10^8	<10 <10	[0/2]	<10 <10	[0/2]	640 1280	[2/2]

For blood tests, samples were collected 2 months after inoculation; in the [brackets] - the number of animals with antibodies (the numerator) total number inoculated of animals (the denominator).

nontranslated insertion between promotor and ATG codon of the expressing gene is significantly shorter in the former recombinant; a fact substantially affecting expression (Mackett *et al.*, 1984). Unexpectedly, vl37 recombinant induced HBsAg appeared to contain 7–9-fold higher amount of pre-S2 determinant. This finding can be explained by the assumption that in the case of vl37 recombinant translation of mRNA coding for HBV M-protein starts predominantly on the first ATG codon which is typical for the eukaryotic translation system. HBsAg is built up mainly on the basis of M-protein containing both the pre-S2 antigenic determinant and the major product of S-gene participating in the formation of the a-determinant. In the case of L-pre-S2/15 recombinant, due to the peculiarity of the secondary structure of mRNA, translation starts predominantly on the second ATG codon and that is why this recombinant induced HBsAg consists mainly of the S-gene product devoid of pre-S2 deter-

minant; M-protein in such HBsAg comprises no more than 1/7–1/9. Interestingly, the considerable insertion between the promotor and 2nd ATG codon (about 200 b.p.) does not reduce S-gene expression in L-pre-S2/15 recombinant as compared to L-HB32 (62 b.p. insertion).

According to our data, HPA assay proves to detect inodequately small amounts of the pre-S2 polypeptide in L-pre-S2/15-induced HBsAg as compared to MAb tests . Apparently, binding of the HBsAg particles with low concentration of pre-S2 antigenic specifities on the surface to HPA is weak; that's why they are removed during the washing procedure. Consequently, the result of HPA test are rather the picture of pre-S2 epitope concentration on the surface of particles produced than of its absolute amount.

Vl37 induced HBsAg was noted to be bound to cell more closely than L-pre-S2/15 induced HbsAg. Conceivably it is associated with a different concentration of pre-S2 polypeptide in these antigens. The role of HBsAg pre-S2 region in delaying its secretion from cells was demonstrated previously (Cheng and Moss, 1987).

The results of evaluating recombinant immunogenic activity in rabbits well agree with those of a- and pre-S2 determinant detection in HBsAg induced by three recombinants. Only L-pre-S2/15 which induced as much HBsAg as L-HB32 and as much pre-S2 antigen as vl37 did, was capable of inducing antibodies to both a- and pre-S2 determinants. The lack of antibodies against a-determinant in vl37 inoculated rabbits might be explained not only by weaker HBsAg expression, but by somewhat lower dose of the virus used in these experiments.

We hope that the obtained results will be helpful for constructing VV recombinants with optimal immunogenicity in respect to HBV protective determinants.

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