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ANTIGENIC PROPERTIES OF VACCINIA VIRUS AND OF THE VIRUS RECOMBINANT STRAINS EXPRESSING HETEROLOGOUS GENES

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Summary. — Immunologic properties of vaccinia virus (VV), strain LIPV, and VV recombinant strains containing the gene of hepatitis B virus surface antigen and the TK gene of herpes simplex virus (HSV) have been studied. Production of antibodies against the majority of VV structural proteins, including nucleocapsid internal proteins was demonstrated in rabbits. Insertion of heterologous genes into the VV genome was without effect on the spectrum of antibodies produced against VV virion proteins. The data obtained in volunteers indicate that not only virus-neutralizing antibodies but also antibodies against most VV structural proteins are preserved in humans over many years. Reimmunization of volunteers with VV recombinant stimulates synthesis of antibodies against virion proteins whereas the spectrum of antibodies remains unchanged. Humans and rabbits did not differ in the spectrum of antibodies to VV virion proteins.

Key words: vaccinia virus (VV); genetically engineered VV recombinants; virus-specific antibodies; immunoblotting

Introduction

Prevention of smallpox with VV is not longer actual after the successful completion of the smallpox eradication campaing. Recent developments, along with the appearance of genetically engineered recombinant viruses offer new applications for VV as a vector for a new generation of vaccines (Mackett *el al.*, 1982; Panicalli, Paoletti, 1982; Chernos, Altshtein, 1984; Altshtein *et al.*, 1985, 1986). The possibility of using VV as a vector for insertion of genes expressing alien virus antigens such as herpes virus (Paoletti *et al.*, 1984), hepatitus B virus (Smith *el al.*, 1983; Moss *et al.*, 1984), vesicular stomatitis virus (Mackett et al., 1985), influenza virus (Bennink *et al.*, 1984) and others (Smith *et al.*, 1984) has been convincingly demonstrated. These data have laid the foundation for a new approach to vaccine design.

VV is one of the largest and most complex DNA-containing animal viruses. Its DNA contains about 187 thousand base pairs and encodes up to 200 proteins. Its virion structure consists of more than 100 polypeptides (Essani and Dales, 1979) of which at least 10 are present in the virus envelope (Oie and Ichihashi, 1981*a*). Immunologic properties of VV have been thoroughly studied with respect to both humoral and cellular immune responses (Chernos *et al.*, 1975; Dales *et al.*, 1983; Ueda *et al.*, 1969, 1972; Koszinovski and Ertl, 1975, 1976; Tagaya *et al.*, 1977; Hapel *et al.*, 1978; Esposito *el al.*, 1977; McFarland *et al.*, 1980; Oie and Ichihashi, 1981*b*; Dales and Oldstone, 1982; Malion and Holowczak, 1985). However, antigenic and immunogenic properties of the individual proteins of this complex virus were extensively and in full detail studied only recently owing to the development of immunoblotting method (Towbin *et al.*, 1979). Using immunoblotting the VV determinants were described reacting with antibodies (polyclonal and monoclonal ones) produced in the course of infection in mice (Wilton *et al.*, 1986), as well as with anti-vaccinia virus-neutralizing monoclonal antibodies (Rodriguez *et al.*, 1985) and specific monoclonal antibodies against monkey pox virus (Roumillat *el al.*, 1984). No reports on the spectrum of VV antigenic determinants for humans are available.

Considering the importance of prevention of human infection such as hepatitis B, we constructed a VV recombinante xpressing the hepatitis B virus surface antigen (HBsAg) employed as a vaccine (recombinant smallpox-hepatitis vaccine, SHV) for humans (Altshtein *et al.*, 1985; 1986). For the design of SHV, gene S of hepatitis B virus coding for HBsAg was inserted into TK gene of VV so that it blocked the expression of TK gene yielding the LIOGEN-HB/C2-TK$^-$ virus (LG-TK$^-$). To restore the TK$^+$-phenotype, the TK gene of herpes simplex virus type 1 was incorporated into the DNA of the recombinant VV. The resulting virus was referred to as LIOGEN--HB/C2-TK$^+$. (Altshtein *et al.*, 1985, 1986).

The possibility to employ genetically engineered recombinants for the prevention of some human and animal diseases necessitates to study the effects of insertion and expression of heterologous genes on the production of antibodies to VV virion proteins. Experiments were carried out with sera collected from immunized volunteers and also from rabbits immunized with VV and genetically engineered recombinants LG-TK$^-$ and LG-TK$^+$.

Materials and Methods

Virus. The VV strain LIVP and the following genetically engineered recombinant strains of smallpox-hepatitis vaccine (SHV) were used: LIOGEN-HB/C2-TK$^-$ (LG-TK$^-$) in which gene S coding for HBsAg was inserted into the region of VV DNA *Hind III* J fragment containing the TK gene and LIOGEN-HB/C2-TK$^+$ whose genome was identical with the LG-TK$^-$ VV genome except for the TK gene of herpes simplex virus inserted into the *HindIII* J fragment. Preparation of recombinant viruses was described by Altshtein *et al.* (1985, 1986).

The viruses administered to the volunteers were produced using the conventional technique for propagation of VV and preparation of the smallpox vaccine.

Immunization. SHV was tested in 13 volunteers immunized with VV at least 10 years ago. Peripheral venous blood was collected from volunteers one day before inoculation and 1 and 3

months thereafter. Chinchilla rabbits weighing 2—3 kg were immunized according to the technique described elsewhere (Chernos *et al.*, 1975). The titres of virus-neutralizing (VN) antibodies in human and rabbit sera were determined according to a modified technique of Boulter (1957).

Preparation of antigens, electrophoresis, immunoblotting. Cultivation and purification of viruses as well as gel-electrophoresis were carried out as described previously (Chelyapov *et al.*, 1984). The proteins were transferred from 11% polyacrylamide gel (PAG) to nitrocellulose membrane Trans Blot (Bio Rad, U.S.A.) in a device described in Nielsen et al. (1982). One liter of the transfer buffer contained 14. 4 g of glycine, 3 g of Tris, 0.01% sodium dodecyl sulphate and 20% ethanol. The transfer was carried out at 20 V and 0.5 A for 3.4—5 hr at room temperature. Nitrocellulose was treated with 3% bovine serum albumin solution in 0.15 mol/l NaCl, 25 mmol/ lTris-HCl, pH 7.2 for 1 hr and then with sera (at 1/50 and 1/100 dilution for human and rabbit sera, respectively) in the same buffer for 15—17 hr. Nitrocellulose strips were washed for 4× 10 min in the buffer with NaCl and Tris containing 0.05% Tween-20 to remove the serum and incubated for 2 hr with antispecies immunoglobulins, conjugated with peroxidase (at dilution 1/250). The excess of the second antibody was removed by washing and nitrocellulose was treated with 0.5% solution of chloronaphthol.

Electron microscopy. Concentrated and purified virus was applied on formvar-coated grids (carbon spraying), fixed with 2.5% solution of glutaraldehyde, washed with deionized water and contrasted with 2% phosphotungstic acid, pH 7.0. The preparations were then examined in electron microscope JEM-100 CX-II with instrumental magnification of 36,000 x.

Results

Properties of the vaccinia virus recombinants

The genome of the genetically engineered VV recombinants contains an 1.5 kilobase (kb) (LG-TK$^-$) and a 4.3 kb (LG-TK$^+$) fragment of heterologous DNA which comprises 0.8 and 2.3% of VV DNA, respectively. In spite of the small-sized inserts, it seemed interesting to find out whether or not it will influence the antigenic characteristics of VV structural proteins and the physicochemical or morphological properties of the virus. The viruses were compared in terms od sedimentation in sucrose gradient and polypeptide composition of virion proteins labelled with ^{14}C-amino acids and analysed in 11% PAGE (Fig. 1). The viruses proved to be identical both in this test and according to their sedimentation parameters (data not shown). Fig 2 shows the electronmicroscopic findings obtained with purified and concentrated preparations of viruses LIVP and LG-TK$^+$. It can be seen that virions of the indicated viruses do not differ. The LG-TK$^-$ virus was also morphologically identical to LIVP virus (data not shown).

Virus-neutralizing antibodies in volunteers and rabbits

The level of VN antibodies appeared to be still rather high in humans pre-inoculated at least 10 years ago (Table 1): minimal titre was 20, the maximum amounted 320. Titre of 640 was observed in a volunteer who was continuously in contact with VV. Vaccination of humans with SHV of TK$^+$ or TK$^-$ phenotype increased the titre of VN antibodies (tested within 1 month) up to values as high as 320 to 2560 exceeding previous titres 2 to 64-fold. After 3 months, the antibody titre remained either unchanged or further increased to 2 to 4 times higher values. The rise in the titre of VN antibodies appeared unrelated to the TK-phenotype of the virus used for vaccination. Thus, for example, in the volunteer No. 13 with origi-

Table 1. Titres of neutralizing antibodies to vaccinia virus in volunteers and rabbits vaccinated with the VV strain LIVP and with recombinant viruses LIOGEN-HB/C2-TK⁻ (LG-TK⁻) and LIOGEN-HB/C2-TK⁺ (LG-TK⁺)

Vaccinated subjects	Viruses	Titres at various intervals			
		before vaccination	after vaccination (months)		
			1	3	7
Volunteers					
1	LG-TK⁻	1/20	1/160	1/640	—
2	the same	1/40	1/320	—	—
3	the same	1/40	1/320	—	—
4	the same	1/40	1/1280	1/1280	—
5	the same	1/160	1/640	1/640	—
6	the same	—	1/1280	—	—
7	the same	1/320	1/1280	—	—
8	LG-TK⁺	1/20	1/1280	—	—
9	the same	1/160	1/1280	—	—
10	the same	1/320	1/640	1/2560	—
11	the same	1/320	1/1280	—	—
12	the same	1/320	1/1280	1/2560	—
13	the same	1/640	1/2560	1/2560	—
Rabbits					
2	LIVP	<1/10	1/640	1/640	1/160
11	the same	<1/10	1/1280	1/1280	1/320
24	the same	<1/10	1/1280	1/640	1/160
9	LG-TK⁻	<1/10	1/2560	1/1280	1/160
3	LG-TK⁺	<1/10	1/2560	1/640	1/320

Note: All volunteers were previously vaccinated with VV at least 10 years before. (—) indicates the absence of data.

nal titre of 640, inoculation of the virus LG-TK⁺ resulted in a rise up to 2560 within 1 month whereas in the volunteer No. 4 the LG-TK⁻ virus caused a rise in the titre from 40 to 1280. On the average, viruses LG-TK⁺ and LG-TK⁻ caused an equal increase of virus-neutralizing titres in humans 1 month post-vaccination.

The results obtained in 37 rabbits appeared to be rather homogenous. Table 1 shows typical results in 5 rabbits inoculated with different virus strains. One month after single vaccination the titres of VN antibodies reached 640 to 2560 to be followed by a monotonic fall. Original virus LIVP and its recombinants LG-TK⁺ or LG-TK⁻ did not differ in eliciting VN antibodies in the rabbits either, as evident from given data.

Antigenic composition of viruses LIVP, LG-TK⁻ and LG-TK⁺

All 3 viruses tested were identical in terms of polypeptide composition according to the data of SDS-PAG electrophoresis (Fig. 1). Reciprocal treatment of viruses with hyperimmune rabbit sera did not reveal antigenic differences that could occur because of gene-engineering manipulations (data not shown). Therefore, virus LIVP was used as antigen for immuno-

blotting experiments. Fig. 3 shows typical results obtained in volunteers and in rabbits. Noteworthy, antibodies were produced against most virion proteins regardless of their localization in the virion. Thus, for example, surface proteins 42kD, 35kD and 11kD are as potent antigens as the nucleoid proteins with molecular weights 135kD, 88kD, 62kD, 60kD and 26kD.

All volunteers were previously vaccinated at least 10 years ago, the volunteer No. 8 (Fig. 3, lane 19) at least 15 years ago. Yet, the spectrum of antibodies circulating in their blood was very broad and essentially unchanged at revaccination, the basic change being a quantitative increase. On the average, immune response to VV was the same in different individuals according to the immunoblotting data, yet some individual variations were still observed. Thus, for example, in volunteer No. 4 antibody level to proteins 35 kD and 12.5 kD was very high (Fig. 3, lanes 9—11, protein 12.5kD is marked with a dot). Volunteer No. 8 had a very strong immune response to protein 26kD. It can be seen from Fig. 3 and from Table 1 that TK-phenotype of SHV was without effect on the character of human immune response to VV virion proteins.

The spectrum of antibodies against VV virion proteins synthesized in rabbits was quite similar to the spectrum of antibodies produced in humans (Fig. 3, lanes 28—32). Rabbits were characterized by a more marked response to polypeptides 15.5kD and 23 kD which were presumably glycoproteins (Oie and Ichihashi, 1981*a*). Obviously, some VV structural proteins are antigenically important polypeptides with respect to antibody formation both in humans and in rabbits. These are the surface polypeptides 42kD and 35kD and the major histone-like polypeptide 11kD, as well as the core proteins 135kD, 88kD, 62kD, 60kD and 26kD. The level of antibody production against proteins 35kD and 26kD (Fig. 3, lanes 9—11, 19—20, 29 and 30) displayed individual variations.

Discussion

Construction of genetically engineered recombinant vaccines of new generation is an essentially new step in the development of vaccine research. This imperatively calls for thorough investigations in the properties of recombinant viruses as compared to the original strain. Vaccinia virus is one of the most promising vectors for the design of new vaccine preparations. SHV LIOGEN-HB/C2 is one of the first preparations of this type. For preparation of SHV, the gene of hepatitis B virus surface antigen (S gene) was inserted into the TK gene of VV which resulted in a TK^- phenotype of the recombinant virus. Since the modification of TK-phenotype of VV could change the biologic properties of the virus, another recombinant strain LIOGEN-HB/C2-TK^+ with the inserted TK gene of herpes simplex virus was prepared, in which the TK-phenotype of VV was restored. The task of the present research was to study the influence of insertion of heterologous genes (S gene of hepatitis B virus and TK gene of herpes simplex virus) on the expression of antigenic properties of virion proteins of the vector virus.

It is known that VN antibodies keep on circulating long after smallpox vaccionation (Downie *et al.*, 1961). It is also known that after revaccination the titres of VN antibodies increase several times (Downie *et al.*, 1969). Table 1 shows that the average increase in VN antibodies for TK^+ and TK^- variants is the same for humans and rabbits. Moreover, rabbits show no differences in the titre of virus -neutralizing antibodies between the parent strain LIVP and LG-TK^+ or LG-TK^-. Consequently, genetically engineered SHV LIOGEN-HB/C2 with different TK phenotypes had an equal capacity to produce antibodies neutralizing VV.

It was essential to find out against which VV proteins the antibodies circulating in the blood of vaccinated subjects were produced and how long they circulated. No such data were available in literature. Our data shown in Fig. 3 indicate that antibodies were produced against most virion proteins: both surface proteins (42kD, 35kD, 11kD) and nucleoid proteins (135kD, 88kD, 62kD, 60kD, 26kD). It is noteworthy that antibodies against these proteins circulate in the blood of vaccinated subjects over many years (at least 10 to 15 years). Revaccination generally results in quantitative changes of antibody content being essentially without effect on their spectrum (Fig. 3, lanes 3—27). It should be noted that antigenic spectra are similar in different individuals, though individual variations have been observed. For example, volunteers Nos. 4 and 8 were found to have an increased level of antibodies to 35kD and 26kD (Fig. 3, lanes 9—11 and 19—20).

According to the immunoblotting data the spectrum of antibodies to VV structural proteins produced in rabbits is hardly different from that in humans. Antibodies to polypeptides 135kD, 88kD, 62kD, 60kD, 42kD, 35kD, 26kD and 11kD were detected in both humans and rabbits. Samples with increased level of antibodies to 35kD and 26kD polypeptides were also found among many rabbit sera tested (Fig. 3, lanes 29 and 30).

The results obtained indicate that sera of humans vaccinated VV 10 to 15 years earlier contained antibodies not only to surface proteins responsible for induction of VN antibodies, but also to virion internal proteins. Such a long-lasting circulation of antibodies to nucleoid proteins suggests that they may be involved in the immune response of the organism.

Genetic engineering manipulations needed for the production of SHV on VV basis were without effect on the tested properties of the virus. Recombinant viruses were equally sedimented in sucrose gradient, had no morphological differences as evident from electron microscopic findings, had identical spectra of structural proteins: hyperimmune rabbit sera to these viruses contained antibodies to the same virion proteins. Furthermore, these viruses caused equal increase of virus-neutralizing antibodies in humans and in rabbits. Revaccination of previously inoculated humans with recombinant SHV strains with TK^+ or TK^- phenotypes led to an increase of the level of antibodies to virion proteins, their spectrum remaining essentially un changed. Inoculated humans showed similar antigenic spectra. Antibody spectra of rabbits and humans were also similar.

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Legend to Figures (Plates LXIII—LXV):

Fig. 1. Electrophoregram of virion proteins of the viruses: LIVP (1), LIOGEN-HB/C2-TK$^-$ (2) and LIOGEN-HB/C2-TK$^+$ (3) in 11% SDS-PAG (the proteins are labelled with ^{14}C-hydrolysate of amino acids). In the left the molecular weights of some virion proteins are indicated.
Fig. 2. Virions of LIVP (1,2) and LIOGEN-HB/C2-TK$^+$ (3,4) (negative contrast). 1,3 — magn. × 80,000; 2,4 — magn. × 250,000.
Fig. 3. Immunoblotting of antibodies to VV virion proteins produced in volunteers (3—27) and in rabbits (28—33) immunized with LIVP (28—30), LIOFEN-HB/C2-TK$^-$ (2—18, 31) and LIOGEN-HB/C2-TK$^+$ (19—27, 32).
1 — virion proteins of LIVP in 11% SDS-PAG stained with Coomassie 3—33 nitrocellulose strips containing VV proteins treated with sera of volunteers: No. 1 : 3, 4, No. 2 : 5, 6, N. 3 : 7, 8 No. 4 : 9—11, No. 5 : 12—14, No. 6 : 15, 16, No. 7 : 17, 18, No. 8 : 19, 20, No. 9 : 21, 22, No. 10:23—25, No. 11—26, 27 and rabbits: No. 2 : 28, No. 11 : 29, No. 24 : 30, No. 9 : 31, No. 3 : 32. The sera were collected before immunization (3, 5, 7, 9, 12, 15, 17, 19, 21, 23, 26, 33), 1 month after immunization (4, 6, 8, 10, 13, 16, 18, 20, 22, 24, 27—32) or 3 months after immunization (11, 14, 25). 2 — serum of nonimmunized child.