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Pathogenicity and immunogenicity in mice of vaccinia viruses mutated in the viral envelope proteins A33R and B5R

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Abstract

The pathogenicity and immunogenicity in mice of WR.cl and WR.c3, two mutants of the Western Reserve (WR) strain of vaccinia virus, mutated in the A33R and B5R proteins of the outer envelope of the virus, respectively, were studied. WR.c1 was the most attenuated virus, WR.c3 was somewhat more pathogenic, while WR was the most virulent of the three. While the WR and the WR.c3 viruses, intranasally inoculated into mice, spread efficiently to the different internal organs of the animal, including the brain, WR.c1 was restricted to the lungs only. Mice, intranasally infected with 500 plaque forming units of the WR, WR.c1, or WR.c3 viruses, were protected against infection with a lethal dose of the WR strain.

Keywords: Vaccinia virus; Mutants; Pathogenicity; Immunogenicity

1. Introduction

Smallpox, the contagious and deadly disease caused by variola virus, was eradicated in 1980, due to the successful global immunization campaign led by the World Health Organization. The virus applied in this drive was vaccinia virus, which shares a high degree of immunological cross-reactivity with variola virus. However, the risk of re-appearance of the variola virus, due to biological warfare or a mutational/recombination event occurring in a genetically related virus, poses a serious threat for the human population even today. Furthermore, the recent outbreaks of monkeypox (Sejvar et al., 2004) raised also the possibility for future need for vaccination against this member of the poxvirus family. Several vaccinia viruses were used for immunization against smallpox, such as the Lister/Elstree, the New York City Board of Health (NYCBH) or Wyeth (Dryvax) strains (Fenner et al., 1988). These viruses, although highly protective, caused severe complications, such as postvaccinal encephalitis in some of the vaccinees, especially those suffering from immunodeficiency (Neff et al., 1963; Fenner et al., 1988). Another candidate virus for vaccination against smallpox is Modified vaccinia virus Ankara (MVA), a strain of vaccinia virus obtained after more than 500 passages in cultures of chicken embryo fibroblasts, and which consequently almost completely lost its capability to replicate in mammalian cells (Mayr et al., 1978; Carrol and Moss, 1997; Wyatt et al., 1998). The efficiency of immunization of human population with this virus for protection against smallpox, has not yet been demonstrated. However, it is expected that larger doses of MVA and multiple inoculations will be required in order to obtain sufficient protection when this virus will be applied (Earl et al., 2004).

Efforts to construct vaccinia virus mutants which will be more attenuated, yet highly protective against a lethal poxvirus, are currently underway in several laboratories. We isolated two mutants of the Western Reserve (WR) strain of vaccinia virus, which are mutated in genes coding for two of the proteins comprising the outer membrane of the extracellular enveloped virus (EEV) (Katz et al., 2002, 2003): WR.c1 has *C*-terminal truncation of 35 amino acids of A33R protein (Roper et al., 1996, 1998) and WR.c3 has proline instead of serine 189 in its B5R protein (Engelstad et al., 1992; Isaacs et al., 1992). These two mutants differ from their parent virus in their ability to release a higher proportion of EEV from infected cells in vitro (Katz et al., 2003). It was previously shown that EEV, which has an extra membrane and additional polypeptides to those of intracellular mature viruses (IMV), plays an important role in the spread of vaccinia virus from the infected cells (Appleyard et al., 1971; Payne, 1978; Moss, 2001). Preliminary studies we performed in mice (Katz et al., 2003) indicated that the two mutants, especially WR.c1, caused infected mice to lose weight only slightly,

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as compared to their parent virus. The present study aimed to follow the growth and spread of the two mutants WR.c1 and WR.c3 in the different internal organs of infected mice, and the protection they induce against a lethal poxvirus.

2. Materials and methods

2.1. Viruses and cells

BS-C-1 cells, a green monkey kidney cell line, was grown in Dulbecco modified Eagle medium (DMEM, Biological Industries, Beit-Haemek, Israel) supplemented with 7% Newborn calf serum (NBCS, Biological Industries). A seed stock of the Western Reserve (WR) strain of vaccinia virus was kindly donated by Dr. B. Moss, Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD. Crude stocks of the WR strain and of the WR.c1 and WR.c3 mutants were prepared in BS-C-1 cells in DMEM supplemented with 2% NBCS. When an extensive cytopathic effect was visible, usually within 2 days after infection, the cells were centrifuged and suspended in 1 ml DMEM supplemented with 2% NBCS and kept frozen at −80 °C until use. Purified virus was prepared from the cytoplasmic fraction of the infected cells, obtained after Dounce homogenization and separation from the nuclei, by centrifugation in Homef LC centrifuge at 2000 rpm for 5 min. The cytoplasmic fraction was then centrifuged through 36% sucrose in 10 mM Tris pH 9.0 in TST 28 rotor at 24,000 rpm for 45 min at 4 °C. The virus pellet was suspended in 1 ml of Tris buffer and stored at −80 °C, until use.

2.2. Determination of virus infectivity by the plaque assay

Ten-fold dilutions of vaccinia virus stocks were added to monolayers of BS-C-1 cells in 35 mm diameter plastic dishes (Nunc, Rosklide, Denmark) for 1 h at 37 °C. Then MEM-Eagle medium (Biological Industries), containing 5% NBCS and 0.7% Agar Noble (Difco Laboratories, Detroit, MI) was added. After incubation for 4–5 days at 37 °C, the cultures were fixed with formaldehyde (20% in 0.15 M NaCl), the semi-solid overlay was removed and the cells were stained with 0.1% crystal violet in 0.1 M citric acid. After washing the cells with water and air-drying, virus plaques were counted.

2.3. Virulence for mice

The virulence of vaccinia virus for mice was determined essentially as previously described (Turner, 1967; Williamson et al., 1990; Moore and Smith, 1992). Five to 6-week-old female BALB/c mice were anesthetized by inhalation of isoflurane (Rhodia Organique Fine, Bristol, UK). Then various concentrations of purified virus in total volume of 20 μl were intranasally applied using an Eppendorf pipette and a plastic tip. When the virus was introduced intracranially, after isoflurane inhalation, a volume of 30 μl was injected, using 1 ml glass syringe and 27 gauge hypodermic needle. The mice were weighed daily and those that reached humane end point (loss of more than 25% of their initial body weight) were euthanized. All animal experiments were done according to the guidelines of the Authority for Animal Facilities of the Hebrew University.

2.4. Virus in the internal organs of the mouse

The internal organs were removed from euthanized mice. They were weighed in a small plastic tube and 0.9 ml of DMEM containing 5% NBCS was added. The tube was then frozen and thawed three times and its contents transferred to 1 ml glass homogenizer. Following homogenization, the material was sonicated for 30 s and centrifuged at 1000 rpm for 7 s in an Eppendorf 5415 centrifuge. The supernatant was collected and virus titer was determined by plaque assay, as described above.

2.5. Determination of vaccinia virus antibodies by ELISA

One hundred microliters of a lysate of BS-C-1 cells infected with the WR strain of vaccinia virus and diluted 1:50 in coating buffer (Boehringer Mannheim, Germany), were added to each well of a round bottom 96-well plate (Nunc). The plate was then incubated overnight at 37 °C. The antigen was fixed by 2% formaldehyde at room temperature for 30 min. The plate was washed twice with 0.1% Tween 20 (Sigma, Chemical Co. St. Louis, MO) in saline. Blocking buffer (5% nonfat dry milk, 0.2% Tween 20 in phosphate buffered saline) was added and the plate was incubated at 37 °C for 1 h and then washed. Serial two-fold dilutions of the sera in blocking buffer were prepared and 100 μl were added to each well containing the fixed antigen. The plate was incubated at 37 °C for 1 h and washed with blocking buffer. Anti-mouse IgG-POD Fab fragments (Boehringer Mannheim) diluted 1:1000 in blocking buffer were added and incubated at 37 °C for 1 h. After another wash with blocking buffer, ABTS (Boehringer Mannheim) was added and incubated for 30 min at room temperature. The plate was read at A_{492} with a Organon Teknika microwell system (Austria) and the endpoint titer of the serum was determined.

3. Results

3.1. Pathogenicity of the virus mutants for mice

The pathogenicity of the viruses for mice, after intranasal inoculation with different doses of virus, was followed. Five- to 6-week-old BALB/c female mice were infected with 20 μl of virus suspension, containing 5×10^5, 5×10^4, 5000 and 500 p.f.u. of the WR, WR.c1 and WR.c3 viruses. The weight of the mice ($n = 4$) was followed daily for 2 weeks and the average weight, is presented (Fig. 1). When mice reached the humane end points (loss of 25% of their initial body weight), they were euthanized. Their blood was then collected, the sera was separated and the internal organs were removed and stored at −80 °C. At a dose of 5×10^5 p.f.u., both WR and WR.c3 viruses caused all mice to lose more than 25% of their initial body weight after 5 and 6 days, respectively (Fig. 1a). At this virus dose, the four WR.c1 infected mice lost weight significantly, but neither of them reached their critical low weight (Fig. 1a). At 5×10^4 p.f.u., all mice infected with the WR strain lost weight and reached their critical value,

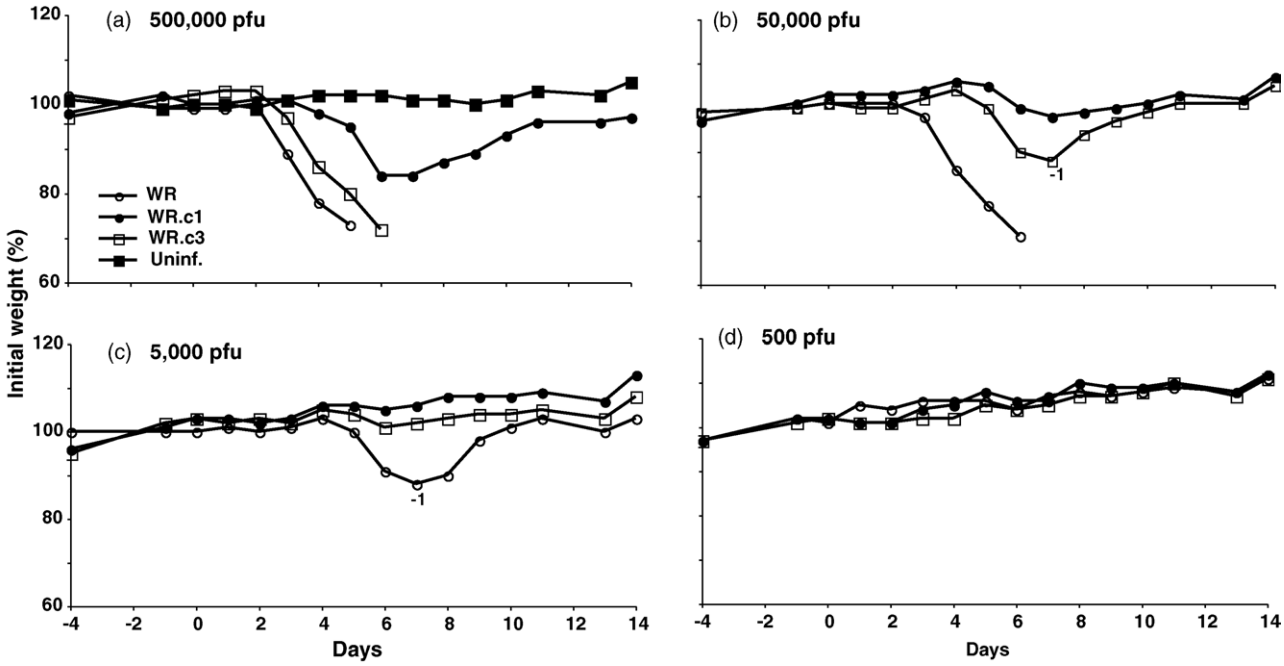

Fig. 1. Virulence for mice. The WR.c1 and WR.c3 mutants and the WR strain of vaccinia virus, at different doses, were intranasally inoculated into 5–6-week-old female BALB/c mice. The weight of four mice in each group ($n = 4$) was determined daily and those that lost more than 25% of their initial weight were euthanized; when a single mouse reached its critical low weight, it was euthanized and marked in the figure as: −1. The percentages of the weight relative to the initial average body weight are plotted. Standard deviation between mice in each group was in general less than 2%.

while those infected with the WR.c1 virus lost some weight but fully recovered afterwards (Fig. 1b). Of those infected with such a dose of the WR.c3 virus, only one mouse reached its critical weight 7 days after infection, while the others were partially affected but completely recovered afterwards (Fig. 1b). When infected with 5000 p.f.u. of the WR strain, the mice lost weight significantly, but only one mouse reached its critical low weight 7 days after infection (Fig. 1c), while mice infected with a similar dose of the WR.c3 lost very little weight and then recovered. Mice infected with the same dose of WR.c1 kept gaining weight (Fig. 1c). A virus dose of 500 p.f.u. did not effect the weight of any of the infected mice (Fig. 1d). The results show that the WR.c1 and WR.c3 mutants are clearly less pathogenic for mice than the WR strain. Furthermore, the attenuation of the WR.c1 mutant is significantly greater than that of the WR.c3 mutant. These weight loss data confirm in general our previously published results (Katz et al., 2003). We then proceeded with this experiment further, by titration of the infectious virus in the different organs of the mice (Fig. 2). Virus was found only in the nine mice infected with the WR strain and in three mice infected with the WR.c3 mutant that were euthanized prior to the end of the experiment (Fig. 2). However, virus could not be detected in all other mice infected with these two viruses that survived the 2 weeks of the study, nor in all those infected with WR.c1 (data

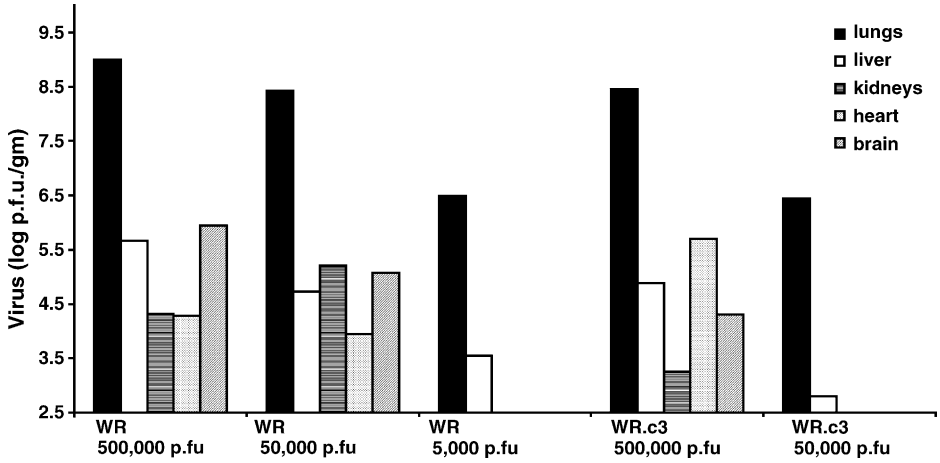

Fig. 2. Distribution and growth of the viruses in the internal organs of mice. When the mice, infected with different doses of the WR strain and the mutant WR.c3 strain, as described in Fig. 1, reached their critical low weight, they were euthanized, their internal organs were removed and the virus titer in the different organs was determined by the plaque assay. When more than one mouse was examined, the titers presented are average of four values (for WR) or two (for WR.c3).

Table 1
ELISA titer of antibodies in sera of infected mice surviving 2 weeks

Virus	Virus dose (p.f.u.)	ELISA titer
WR	5×10^3	3200
	5×10^2	3200
WR.c1	5×10^5	12800
	5×10^4	1600
	5×10^3	800
	5×10^2	800
WR.c3	5×10^4	1600
	5×10^3	1600
	5×10^2	1600
–	–	<100

not presented). In mice infected with the two largest doses of the WR strain (5×10^5 and 5×10^4 p.f.u.), the higher titers of the virus were observed in the lungs, while lower titers were found in the liver, kidneys, heart and brain. In the mouse infected with 5000 p.f.u. of WR, virus was found at lower titers and just in the lungs and liver (Fig. 2). In mice infected with the WR.c3 strain at a dose of 5×10^5 p.f.u., virus was observed in all the internal organs that were examined, while in the mouse infected with 5×10^4 p.f.u., virus was found mainly in the lungs and very little also in the liver (Fig. 2). The results indicate that when high virus doses are used for intranasal inoculation, and when the virus is more pathogenic (WR), high titers of virus are found in all the internal organs examined. However, when lower virus dose or less pathogenic virus (WR.c3) infect the mice, virus can be detected in the lungs at high titer and in the liver, at a significantly lower titer (Fig. 2).

Antibodies against vaccinia virus in sera of infected mice which survived the 2 weeks duration of the study, were determined by ELISA. It was found that WR virus doses of 5000 and 500 p.f.u. induced two-fold higher titers of antibodies than WR.c3, while the latter induced higher titers of antibodies than WR.c1 (Table 1). The highest titer of antibodies (1:12 800) was obtained when a dose of 5×10^5 p.f.u. of the WR.c1, the most attenuated virus of the three, was applied (Table 1); such a dose of the other two viruses was lethal for the mice and they did not survive the 2 weeks duration of the study.

3.2. Growth and distribution of the three viruses in the different internal organs of the infected mice

The growth and spread of the viruses after intranasal infection of mice were followed at 2-day intervals. Five- to 6-week-old female BALB/c mice were infected with 5×10^5 p.f.u. of the WR strain of vaccinia virus and its two mutants, WR.c1 and WR.c3. The weight of the surviving mice was determined daily. The results obtained (not shown) were quite similar to those shown in Fig. 1a. While all mice infected with the WR strain had to be euthanized within a week after infection, only a single mouse infected with WR.c3 was euthanized, and this occurred on the ninth day after infection. WR.c1 affected only slightly the weight of the mice that completely recovered later on. At 2-day intervals (or when loosing more than 25% of their initial weight),

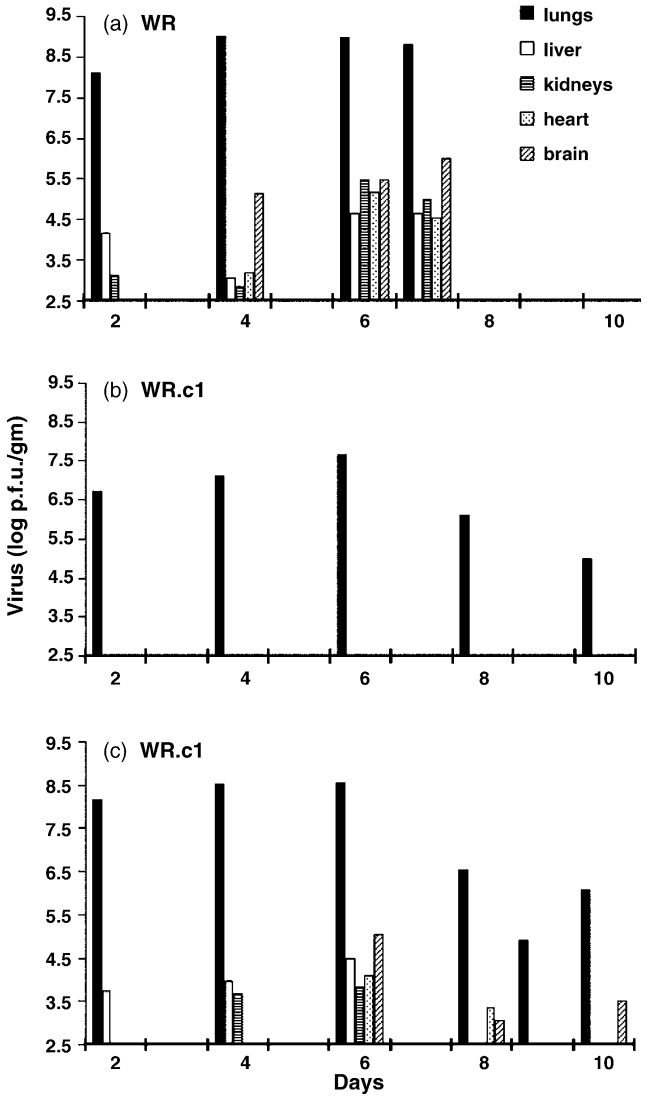

Fig. 3. Distribution and growth of the viruses in the internal organs of mice. Couples of mice of the groups infected intranasally, as described in Fig. 1, were euthanized every 2 days, unless they did not survive reaching their critical low weight previously. Their internal organs were removed and the virus titer in these organs was determined by the plaque assay. The average titer is presented.

mice were euthanized, their blood was collected and separated, and their internal organs were removed. The titer of the virus in the different internal organs of the mice was determined. From the samples of the second day onwards, all three viruses were found in the lungs of the infected mice at relatively high titers (Fig. 3); WR.c1 was absent from the lungs of the infected mice on day 12 (not shown). On the second day, the WR strain was present also in the liver and kidneys, while WR.c3 virus was found in the liver only (Fig. 3). On the fourth, sixth and seventh day, the WR strain was found in all the internal organs of the infected mice examined (Fig. 3a). WR.c3 was present in all the internal organs of the mice assayed on the sixth day, and except for the liver and kidney, on day 8 as well. On the 10th day it was also found in the brain of the mice (Fig. 3c). The results indicate that the WR strain is the most pathogenic virus of the three viruses examined and capable of spreading most extensively

Table 2
ELISA titer of antibodies in sera of mice infected with 5×10^5 p.f.u. of the different viruses

Virus	Day	ELISA titer
W.R.	2	200
	4	800
	6	800
	7	1600
WR.c1	2	<100
	4	<100
	6	200
	8	2400
	10	14400
	12	12800
WR.c3	2	400
	4	200
	6	400
	8	100
	9	100
	10	100

to the different organs. WR.c3 is less pathogenic than the WR strain, while WR.c1 is the most attenuated; found only in the lungs, the primary site of infection, and could not be detected anymore on day 12 after infection.

When antibodies against vaccinia virus were determined in mice infected with WR or with the WR.c3 strain, the titer of antibodies was quite low (Table 2). This may result from the high level of pathogenicity exerted by these viruses, which caused the mice to lose weight considerably and even to reach their critical low weight within 7–10 days. In contrast, in the mice infected with the WR.c1 virus which survived the 12 days of the study, a titer of 2400 was observed on day 8, reached 14 400 on day 10 and 12 800 two days later (Table 2), when the mice recovered and gained weight significantly.

3.3. Intracranial inoculation of mice with the WR strain and its two mutants

Since we observed that WR.c1 after intranasal inoculation is found in the lungs only, in contrast to the WR.c3 mutant and the WR strain of vaccinia virus that reached and proliferated even in the brain (Fig. 2), we wished to find out whether WR.c1 can grow in the mouse brain after intracranial inoculation. The three viruses were injected at different doses into the brain of 5–6-week-old BALB/c female anesthetized mice, in a volume of 30 μl. The weight of the mice was then followed daily (Fig. 4). All mice reached their critical low weight within 4–7 days after inoculation. Those infected with the higher dose of virus reached their critical low weight earlier than the other. No significant attenuation of the mutants WR.c1 and WR.c3, as compared with WR, the parent virus, was exhibited using this virus dose and site of inoculation. The results show that WR.c1 is capable of proliferating in the brain tissue once it arrives there. When the virus titer in the brain of the mice was measured, it was found that the values obtained with the three viruses ranged between 1.5×10^6 and 4.7×10^8 p.f.u./g. However, no significantly lower virus titers in the brains of the WR.c1 infected mice were observed than in those infected with the other two viruses (data not presented).

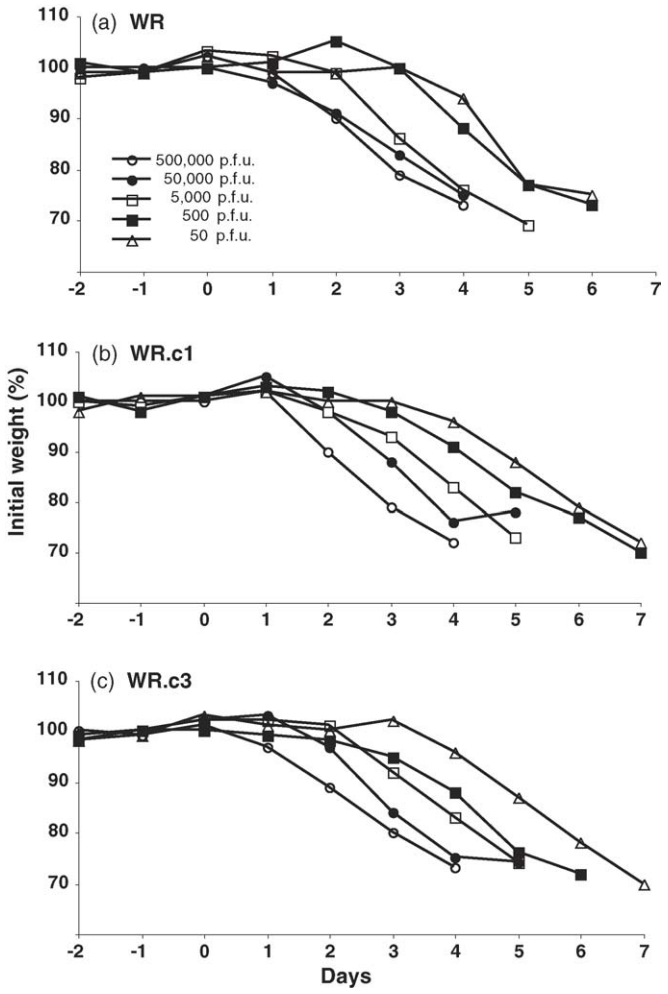

Fig. 4. Intracranial inoculation. Mice were intracranially injected with different doses of the WR.c1, WR.c3 and the WR strain of vaccinia virus. The weight of two mice in each group was determined daily and those that lost more than 25% of their initial weight were euthanized. The percentages of the weight relative to the average initial weight, are plotted against days before and after infection. Standard deviation between mice in each group was in general less than 2%.

3.4. Protection of mice immunized with the two mutants WR.c1 and WR.c3 and with the WR strain

In order to evaluate the protection achieved by the two mutants WR.c1 and WR.c3 and by the WR strain, to a lethal challenge, 5–6-week-old female BALB/c mice were intranasally inoculated with 500 p.f.u. of each virus in a volume of 20 μl. Two weeks later, the mice were challenged with a lethal dose (5×10^4 p.f.u.) of the WR strain. The weight of the mice was then followed for 2 weeks. Mice that lost 25% of their initial weight were euthanized and their blood and internal organs were removed, sera were separated and kept at −80 °C. All the surviving mice were euthanized at the end of the study and their blood and internal organs were analyzed, as well. The data of the weight of the mice followed daily, show that while all of the

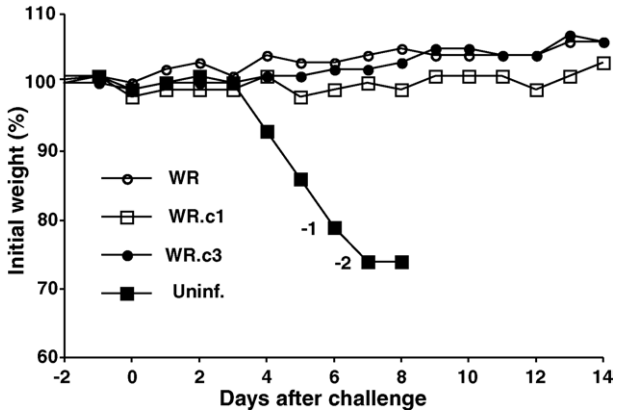

Fig. 5. Protection of immunized mice. Groups of four mice were infected with 500 p.f.u. of the WR.c1, WR.c3 and the WR strain of vaccinia virus. Two weeks later, the mice were challenged with 5×10^4 p.f.u. of the WR strain. The weight of the mice was determined daily and those that lost more than 25% of their initial weight, were euthanized; when one or two mice were euthanized, they are marked in the figure as: −1 and −2, respectively. The percentages of the weight relative to the initial average weight are plotted against days before and after challenge. Standard deviation between mice in each group was in general less than 2%.

unimmunized mice reached their critical weight between the sixth and eighth day after infection with the lethal dose of the WR strain, all the mice immunized with 500 p.f.u. of the WR, WR.c1 or WR.c3, kept gaining weight (Fig. 5). As expected, significant virus titers were observed in all the internal organs of the unimmunized mice examined but not in any of the immunized mice (data not shown).

When antibodies against vaccinia virus were analyzed by ELISA, it was found that the WR strain was the best inducer of antibodies; a titer of 1:800 was already observed 14 days after infection and increased to 1:3200 14 days after infection with the challenge virus (total of 28 days after the first infection). Of the three viruses, WR.c3 was the poorer inducer of antibodies of the three, reaching a titer of 1:600 only, 2 weeks after challenge, while WR.c1 induced antibodies at a titer of 1:1400. Unimmunized mice that reached their critical low weight within 6–8 days after infection with the challenge virus, as expected, did not exhibit any detectable antibodies by then.

4. Discussion

WR.c1 and WR.c3, are mutated in A33R and B5R proteins, respectively, two components of the external envelope of the EEV (Katz et al., 2003). Reversion to the wild type strain could be achieved by transfection with the corresponding genes of the wild type strain (our unpublished results). The two mutants were isolated on the basis of their extensive release as EEV from infected cells in vitro (Katz et al., 2002, 2003). EEV has an important role in the spread of the virus in vitro and in vivo (Reviewed by Moss, 2001) and may affect the pathogenicity of the virus and the protection it induces against challenge with a lethal dose of a poxvirus. The WR strain of vaccinia virus, the parent virus of the two mutants, is relatively more pathogenic for mice than other strains of vaccinia virus, due to its previous adaptation to grow in mouse brain. We found that the two mutants, WR.c1 and WR.c3, are significantly attenuated for the mice, as compared to their parent virus. At a virus dose of 5×10^4 p.f.u., all mice infected with the WR strain lost weight and reached their humane low value within 6 days after infection. Of those mice infected with the WR.c3 virus, only one reached its critical weight 7 days after infection, while the other lost less than 15% of their initial weight and fully recovered thereafter (Fig. 1b). Furthermore, all mice infected with a similar dose of the WR.c1 virus lost very little (less than 5%) of their weight and completely recovered afterwards (Fig. 1b). The Lister strain of vaccinia virus, a strain used for vaccination against smallpox, did not affect the weight of the mice infected at such a dose (our unpublished results).

Although the results described above, using the weight loss criterion for the pathogenicity for the mice, confirm in general our previous findings (Katz et al., 2003), in the present study we also followed the fate of the virus in the infected mice. We found that the significant attenuation of the WR.c1 as compared to its parent virus is even more striking when the spread of the viruses to the different internal organs of the mouse was studied. The WR strain and the WR.c3 mutant, after intranasal inoculation and growth in the lungs, reached the liver, kidneys and heart, passed the blood/brain barrier and proliferated extensively in the brain (Fig. 2). In contrast, the WR.c1 mutant proliferated in the lungs only and could not reach the other internal organs examined, nor the brain (Fig. 2). Only when the viruses were injected directly into the brain, then even WR.c1 could proliferate there, and consequently was lethal for the mice, similarly to WR.c3 and the WR strain (Fig. 4). The results show that the restricted growth and spread of WR.c3, and especially WR.c1, to the different organs of the mice, as compared to their parent virus, is even more prominent than when just the effect on the weight of the infected mice, was followed (Fig. 1).

The two mutants and the WR strain of vaccinia virus, injected intranasally at a dose of 500 p.f.u., protected the mice against a lethal dose of a challenge poxvirus, introduced 2 weeks later (Fig. 5). A similar dose of the Lister strain of vaccinia virus protected only 50% of the immunized mice (our unpublished results). The results suggest that in spite of the failure of WR.c1 to spread from the lungs to the different internal organs of the mice, it is still capable to protect the mice against challenge virus, similarly to the WR.c3 mutant and the WR strain. The recent study of Kidokoro et al. (2005) showing that deletion of B5R from vaccinia LC16m8 strain resulted in attenuation of the virus, without affecting its ability to protect mice against pathogenic poxvirus, supports our findings with the WR.c3, which is also mutated in this gene.

Antibodies against vaccinia virus, determined by ELISA, at significantly high titers, were observed in mice intranasally infected with 5×10^5 p.f.u. of the WR.c1 mutant, on days 10 and 12 (Table 2). From the data obtained in the present study, we cannot conclude whether the high titer of antibodies observed in the mice on the 10th and 12th day after infection with the WR.c1 mutant, contributed to the improvement in their physiological condition or that their better conditions, as expressed in their gaining of weight, which was initiated a week following infec-

tion, was the trigger for the production of the high titers of the antibodies.

The results clearly show the great biological significance of the mutation that took place in WR.c1, involving truncation of the C-terminal domain of the A33R protein. While high degree of attenuation of the virus for mice was achieved, the capability to protect the mice against a lethal challenge of a poxvirus was still retained. Introduction of such mutation into vaccinia virus strains currently used for vaccination, may improve their efficiency by preventing complications that may occur, without significantly affecting their protective potential. The importance of the A33 protein or antibodies against this protein for protection of mice against infection with an orthopoxvirus was recently shown (Fogg et al., 2004; Lustig et al., 2005).

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