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Original article

Brazilian *vaccinia virus* strains show a classical *orthopoxvirus* infection course and cross-protection

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Abstract

Objectives: The purpose of this work was to study the infection course and cross-protection in mice after intradermal injection of *Vaccinia virus* (VACV) strain Western Reserve and three Brazilian VACV strains: Araçatuba, Muriaé and BeAn58058 isolated from cow, human and rodent, respectively. **Methods:** Balb/c mice were inoculated by footpad and back scarification and daily monitored regarding lesion development and weight loss. To check cross protection after intradermal VACV inoculation, mice were subsequently infected with different VACV strains and monitored to check lesion development. Serum neutralization assays were performed to check for the presence of antibodies against *Orthopoxvirus*. **Results:** After VACV intradermal inoculation the lesion development pattern was similar in mice infected with the different virus strains. By using the footpad scarification model, cross-protection among VACV strains was observed. Moreover, neutralizing antibodies against *Orthopoxvirus* were detected in sera from mice infected with all VACV strains. **Conclusion:** Although it was not possible to observe virulence differences among VACV strains isolated from cow, rodent and human using the murine model, this inoculation route showed to be an appropriated model to study lesions development since it mimics natural infections by VACV in nature.

Keywords: *Orthopoxvirus*; Zoonoses; Balb/c mice; Intradermal injection; *Vaccinia virus*; Bovine vaccinia outbreaks

INTRODUCTION

The prototype member of the family *Poxviridae* and genus *Orthopoxvirus*, the *Vaccinia virus* (VACV) was used as a potent live vaccine against smallpox. It has been a quarter of a century since smallpox was declared eradicated by the World Health Organization and the vaccination programs ceased[1]. In the present-day scenario, the emergence of a pathogenic poxvirus with the ability to efficiently spread among humans and animals represents an immediate public health problem[2-4]. Several recent events illustrate the vulnerability of human and animal populations to poxvirus infections: (i) human *Monkeypox virus* (MPXV) outbreaks occurred in the United States[5-7]; (ii) human infection by *Cowpox virus* (CPXV) in Europe[8-10]; (iii) outbreaks of *Buffalopox virus* affecting buffaloes, cows and humans in India, Pakistan, Egypt, Nepal and Bangladesh[4, 11]; and (iv) VACV outbreaks in Brazil[3].

In Brazil, the *Vaccinia* outbreaks occurred on hundreds of small dairy farms, causing great economic losses[12, 13]. During those outbreaks, affected dairy cattle and humans presented typical poxvirus

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lesions[14, 15]. However, the natural reservoirs of Brazilian *Vaccinia* virus strains (BR-VACVs), possibly involving wild and domestic animal species, have not been identified thus far. In fact, some BR-VACVs were previously isolated from rodents and sentinel mice from the 1950s to 1970s in Brazil's northern and southeastern states[16, 17]. Importantly, in nature, BR-VACVs could be under different selection pressures imposed by successive infections across different host species, facilitating the emergence of new variants that could exhibit different virulence patterns. In fact, previous studies demonstrated that the BR-VACVs do not consist of a genetic or biologic homogeneous group[18-20], but whether BR-VACVs exhibit different virulence patterns still remains to be addressed.

Although natural infections caused by *Orthopoxvirus* can occur through the respiratory tract[21] and the infection by intranasal route is an efficient model to differentiate virulence among *Orthopoxviruses*, the intradermal model could better mimic the infection course of BR-VACVs in nature. Thus, the aim of this work was to study the intradermal infection course in a Balb/c mice model of different BR-VACVs isolated from rodent, cow and human.

MATERIALS AND METHODS

Viruses and cells

Three BR-VACVs were used in this study: BeAn58058 virus (BAV), isolated from a rodent *Oryzomis* sp in 1963, Pará (PA); Araçatuba virus (ARAV), isolated from a dairy cow in 2000, São Paulo (SP); and Muriaé virus (MURV), isolated from a milker in 2000, Minas Gerais (MG). *Vaccinia virus* strain Western Reserve (VACV-WR), which was provided by Dr. C. Jungwirth (Universität Wurzburg, Germany), was used as a reference strain. Vero cells were used for viral multiplication and neutralization assays[22, 23]. All viral stocks were grown in Vero cells (ATCC CCL-81) cultivated in Eagle's minimum essential medium supplemented with 5% of fetal calf serum (Cultilab), glutamine and antibiotics at 37℃ in a 5% CO_2 atmosphere. Viruses were purified on sucrose gradients as described elsewhere[24].

Experimental animals

Four-week-old male Balb/c mice, which were housed in filter top micro isolator cages, were used for virus infection and cross-protection assays. All mouse experimentation was carried out in accordance with regulations and guidelines of the Ethical and Animal Use Committee of the Universidade Federal de Minas Gerais/Brazil.

Intradermal inoculation

In order to compare the infection course of VACVs, groups of 6 four-week-old male Balb/c mice were used for virus inoculation in the footpad and in the back. Mice were anesthetized by intraperitoneal injection of anesthetic cocktails containing ketamine and xilazine (3.2 mg and 0.16 mg/mice, respectively, in 0.9% phosphate-buffered saline (PBS)[25]. Following anesthesia, mice were inoculated with 10^6 plaque forming units (PFU) of purified virus in a volume of 10 μL by either footpad scarification or back scarification. For inoculation on the back, fur was previously removed using Nair hair remover. For both inoculations routes, 10 needle scratches were made horizontally across the right footpad or back and the solution containing the virus was placed onto the abraded skin and rubbed in with the side of the pipette tip[26]. Animals from the control group were injected with PBS.

Mice infected by footpad or back scarification were weighed daily and the effects of injections were monitored by measuring the footpad swelling response using a micrometer, before and after injection during 20 days post infection (dpi) and comparing with control mice[27]. Significant weight loss variations were calculated using the Student's *t*-test ($P \leqslant 0.05$: statistical significance) and Graph Prism Excel software.

Cross-protection test

In order to test if the intradermal VACV inoculation could confer cross-protection against VACV, four groups containing nine mice each were infected by footpad scarification with 10^6 PFU/10 μL of each virus: BAV, MURV, ARAV and WR. After 30 dpi, three mice of each group were reinfected by the same method and viral doses with different viruses (Figure 1). Animals were observed daily to check lesion development.

Blood samples were collected from Balb/c mice and sera aliquots were used in the neutralization assay to check for the presence of antibodies against *Orthopoxvirus* in non-infected and infected mice after forty days. The antibody neutralization titers were determined as previously described[28]. Dilutions of heat-inactivated sera were added to an equal volume

of VACV suspension, and then inoculated onto Vero cell monolayer´s. The antibody neutralization titer was estimated by determining the reciprocal dilution of sera that caused a 50% reduction in the VACV plaque count as compared to the negative control.

RESULTS

Infection course after intradermal inoculation by back scarification

Mice inoculated with WR, BAV, MURV and ARAV in the back did not show significant weight loss (Student´s t-test; $P<0.05$) until 20 dpi when compared to control mice (Figure 2A). Infected mice presented the same lesion development pattern. At fourth dpi, irregular yellow lesions were observed characterized as pustules (Figure 3A), and evolving to scabs nine dpi (Figure 3B); the cicatrisation occurred thirteen dpi. Mice injected with PBS did not show any lesions and had a normal cicatrisation reaction ending at third dpi (Figure 3C).

Infection course after intradermal inoculation by footpad scarification

Statistically weight loss was not observed in mice inoculated via intradermal injection with WR, BAV, MURV and ARAV, when compared to control mice, from infection to 20 dpi (Figure 2B).

The infection course of animals inoculated with the four VACV strains was similar and typical poxvirus lesions in different stages were observed. The lesion thickness of infected right footpads was compared with the left control footpads that measured around 0.2 cm. On day one pi, no lesions were observed, however, on day two pi, the infected footpads presented edema and swelling compared with control one (Figure 4A) that persisted for two more days. On day four vesicles with 0.3-0.7 cm vesicles on the plantar faces of footpads were observed. On days 10-12 pi, the swelling decreased and the vesicles persisted (Figure 4B), and from day 14 pi to 17 pi, the vesicles evolved to pustules measuring from 0.25 cm to 0.61 cm, as shown in Figure 4C. Scabs measuring from 0.24 cm to 0.45 cm appeared on day 18 pi (Figure 4D) and there was complete cicatrisation on day 20 pi. Control mice inoculated with PBS showed a normal cicatrisation reaction, detected in dorsal and plantar regions of their footpads, that ended at three dpi (Figure 4E, F).

First infection 9 mice/group		Group 1 VACV-WR (L+)	Group 2 BAV (L+)	Group 3 MURV (L+)	Group 4 ARAV (L+)	MOCK (PBS)
⇩		⇩	⇩	⇩	⇩	⇩
Reinoculation	ARAV	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L+)
	MURV	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L+)
	BAV	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L+)
	VACV-WR	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L+)
	MOCK (PBS)	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L-)	3 mice (L+)
Total		15 mice	15 mice	15 mice	15 mice	15 mice

Figure 1 Strategy of virus infections used in the cross-reactivity assay of *Vaccinia virus* strains. Balb/c mice were infected by footpad scarification route.

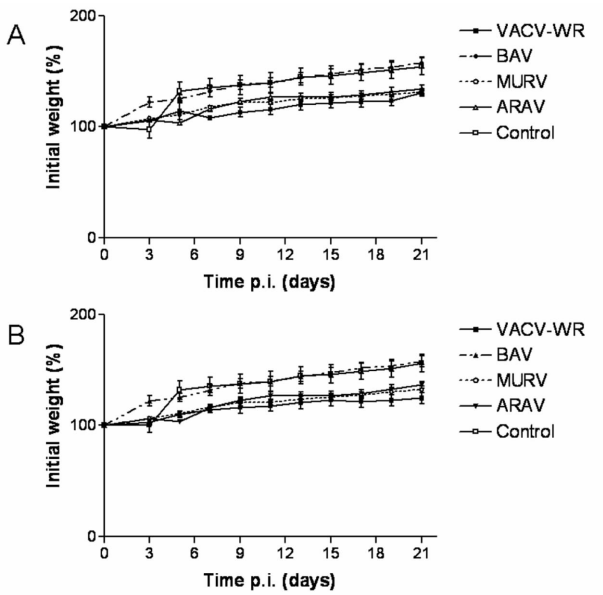

Figure 2 Body weight of mice infected with *Vaccinia virus* strains by intradermal route. Groups of six mice were infected with 10^6 PFU/10μL of VACV-WR, BAV, MURV and ARAV by back scarification (A) or (B) footpad scarification. Mean body weight as a percentage was obtained in comparison with initial body weight. The control group was injected with PBS. The weight loss showed no significant difference in relation to body weight of PBS inoculated mice ($P\leqslant 0.05$).

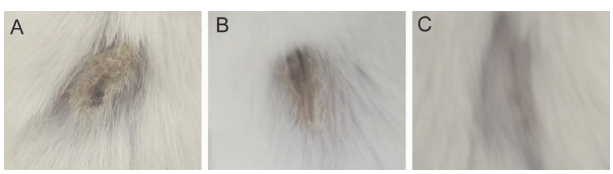

Figure 3 Back scarification. Development of lesions on back of Balb/c mice infected with 10^6 PFU/10 μL of virus. (A) Pustule on day 4 pi (B) Scabs on day 9 pi. (C) Control mice injected with PBS. All VACV strains (WR, BAV, ARAV and MURV) show the same lesions.

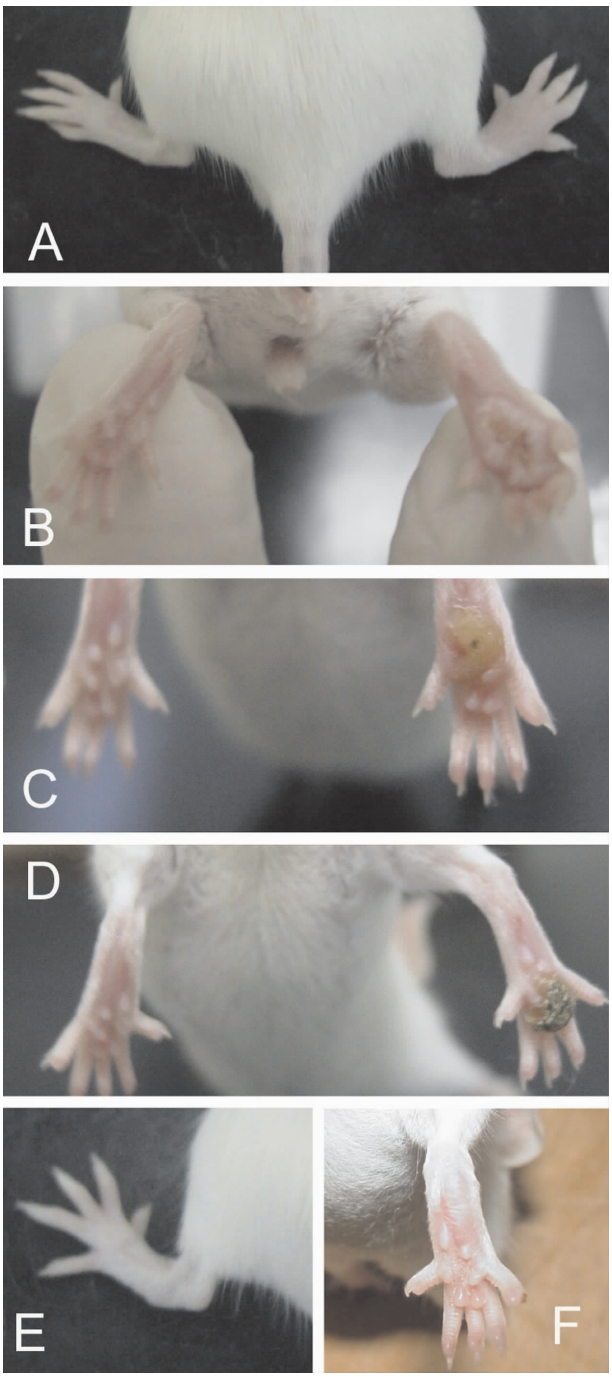

Figure 4 Footpad scarification. Lesions evolution in Balb/c mice infected with 10^6 PFU/10μL of virus. (A) Edema of right footpad on day 2 pi (B) Vesicles on day 6 pi (C) Pustle on day 7 pi (D) Dark scabs on day 11 pi (E, F) Dorsal and plantar place of control mice, injected with PBS. All VACV strains (WR, BAV, ARAV and MURV) show the same lesions.

Cross-protection between VACV strains after challenge

In the first infection, all VACVs induced skin lesions in the footpads of mice, beginning with swelling and followed by vesicles evolving to pustules and scabs. However, at 30th dpi, no mice showed pox infection clinical signs when challenged with different viral strains. The footpads maintained a thickness of 0.2 cm as the control.

Neutralizing antibodies against OPXV at a titer of 1:160 were detected in sera from mice infected with all VACV strains. Neutralizing antibodies were also not detected in mice inoculated with PBS.

DISCUSSION

It is known that *Orthopoxvirus* species can induce cross-protection[29] in this study it was demonstrated that mice infected with one of the VACV strains, MURV, BAV, ARAV or VACV-WR, were protected from infection with diverse different strains and did not develop any lesions or clinical signs. This demonstrates that primary infection by the intradermal route induced specific humoral and/or cellular responses that can neutralize the viral infectivity and clear the virus. One could expect that during the bovine vaccinia outbreaks, once a cow, calf or a human gets infected by one VACV strain, they will be protected from other VACVs or *Orthopoxvirus* infections.

Several models have been used to study poxvirus pathogenesis, such as *Myxoma virus* in the European rabbit and *Ectromelia virus* (ECTV), CPXV and VACV in mice[29]. In the case of ECTV and CPXV, the use of that model is appropriate since these viruses are natural pathogens of rodents[30, 31]. In contrast to those viruses, the natural host of VACV is still unknown and consequently, the most appropriate animal model in which to study VACV pathogenesis is uncertain[32]. In this study, we have inoculated Balb/c mice by scarification on the footpad and back with VACVs isolated from different species to study the virus infection course, since these inoculation routes probably mimic the natural infection caused by VACVs in humans and animals in nature.

Virus inoculation by footpad and back scarification in mice resulted in the appearance and development of lesions that were restricted to the inoculation site, but no clinical signs were observed using high virus doses, such as 10^6 PFU. Differences in the lesion evolution pattern were observed as the time

course of infection in mice infected by back scarification was shorter (13 days) than the ones infected in the footpad (20 days). Moreover, while animals infected on the back developed pustules and scabs, mice infected on the footpad showed edema/swelling, vesicles, pustules and scabs.

Comparing those results with the data from natural infections of cows, calves and humans during bovine vaccinia outbreaks, a similar pattern of lesion evolution is observed after footpad injection. Dairy cows usually exhibit lesions on teats and udders, resembling typical poxvirus lesions. After contact with cow lesions, milkers can get infected and develop similar lesions on their hands and calves similarly become infected, exhibiting the same kind of lesions on oral mucosa and muzzles. At first, cows present a roseolar erythema and localized edema that evolve to form vesicles. The vesicles progress to papules and pustules and subsequently to thick dark scabs[12, 14, 15, 33, 34]. In addition, the time course of natural infections in cows and humans takes from 20 to 30 days[12], which was similar to the time course infection observed for Balb/c mice infected by footpad scarification (20 days). Therefore, footpad scarification has shown to be an appropriate method to mimic the infection course of different VACV strains in nature. Another advantage of the use of this inoculation route is that infection elicits inflammatory responses resulting in footpad swelling, which can be conveniently measured with a micrometer[27].

Animals infected with different strains by back scarification showed the same lesion evolution pattern as the animals infected by footpad scarification. By using the intradermal infection route, it was not possible to detect differences in the virulence patterns of different VACVs strains that were isolated from different hosts, at different places and times, and actually exhibit genetic and biologic differences[18-20]. For example, since BAV was isolated from a rodent, maybe it could be more adapted to the mice model. VACV infections by intracerebral and intranasal routes cause systemic infections in mice[35, 36] and moreover, the intranasal infection route has shown to be good to differentiate virulence between poxviruses[37, 38]. As discussed above, the most appropriate animal model to study VACV pathogenesis is still uncertain[32], but the inoculation of mice with VACVs by different infection routes could yield important information about their pathogenesis. The use of VACV infection in mice by intradermal (footpad scarification) and by intranasal infection routes is recommended to study the infection course and the virulence patterns of BR-VACVs in order to get information about the biology of those naturally occurring viruses, which have significant impacts on public and animal health and also on local economies.

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