LETTER TO THE EDITOR

RESTORATION OF THYMIDINE KINASE ACTIVITY IN A POTENTIAL BOVINE HERPESVIRUS 2 VACCINE DOES NOT INCREASE ITS VIRULENCE

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Bovine herpesvirus 2 (BHV-2, bovine mammillitis virus) has a worldwide distribution. It causes an ulcerative disease of the teats and udder of cattle or may produce a generalized infection with lesions of the skin. No commercial vaccine exists for this virus, although protection has been shown to be effective after the intramuscular injection of a virulent or inactivated virus (*1*). We have decreased the thymidine kinase (TK) activity of two manipulated isolates of the BHV-2 CSIRO290 (C290) strain to less than 3% of that of the wild-type virus (*2,3*). One (C290BU5) had an associated decrease in the virulence of the virus and appeared to have potential as a vaccine (*2*), while the other (C290BU3) did not lose its virulence when tested in a guinea pig model system (*3*). Alteration of TK gene has been important for the development of commercial vaccines of some animal herpesviruses such as pseudorabies (*4*), as it has been associated with herpesvirus virulence and reactivation from latency (*2*).

This study reports on restoring of complete TK activity in C290BU5 (a potential vaccine) and testing of its ability to cause disease in a guinea pig animal model system, to confirm any association of the TK gene with the virulence of BHV-2.

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Abbreviations: BHV-2 = bovine herpesvirus 2; MDBK = Madin-Darby bovine kidney; TK = thymidine kinase

All BHV-2 isolates were grown at 36°C in the established bovine MDBK (Madin-Darby bovine kidney) cell line and a TK-deficient derivative of this cell line, MDBK (BU100) in a growth medium (EMEM with 10% of newborn calf serum) (*2*). Total of 5 x 10^5 MDBK (BU100) cells in a 35 mm tissue culture plate were infected with 1 x 10^6 PFU of the TK-deficient C290BU5 for 30 mins. The cells were then transfected with 10 μg of linearized pUC18 plasmid containing the BHV-2 TK gene inserted in *Sph*I-*Sal*I sites (*3,5*) and lipofectin (30 μg; Life Sciences) in a medium with HAT (0.27 μg/ml hypoxanthine, 0.04 μg/ml aminopterin, and 0.8 μg/ml thymidine; ICN). The virus infection continued for 30 hrs. This HAT selection process allowed for selection of TK-positive herpesviruses in TK-negative cell lines (*6*). The addition of the plasmid allowed for the TK activity to be restored by a DNA recombination event with virus DNA and subsequent selection in the HAT medium. The HAT medium would also select any spontaneous mutation of the TK gene that produced wild-type TK levels. The surviving virus after this initial selection procedure was then regrown in MDBK cells, followed by a second selection process in MDBK (BU100) cells in the HAT medium. The remaining virus isolates were plaque purified 3 times on MDBK cells in the growth medium containing 1% agarose (*2*). The virus isolates were continually monitored for acquisition of TK activity. The TK activity was determined as described earlier (*2,3*) and was based on measuring the ability of infected MDBK (BU100) cell lysates to phosphorylate [^{3}H]thymi-dine using ATP.

From 25 tissue culture plates only one virus isolate designated C290BUTK was found to have acquired any TK activity, which was the full activity of the wild-type C290 virus. The TK activity of C290BUTK was stable, as the

Initial virus inoculum	Total dose (PFU)	TK activity (%)	Initial virus response	C290 challenge response
Nil	0	0		4+
C290	5×10^5	100	3+	0
	5×10^6	100	4+	–
	1×10^7	100	4+	–
C290BU3	5×10^5	3	2+	0
	5×10^6	2	3+	0
	1×10^7	2	4+	–
C290BU5	5×10^5	3	0	0
	5×10^6	2	0	0
	1×10^7	2	0	0
	1.5×10^7	1	1+	0
C290BUTK	1×10^7	110	0	0
	1.5×10^7	110	1+	0
	3×10^7	100	1+	0

(0) = no effect.
(1+) = single small lesion lasting 2–3 days.
(2+) = two lesions lasting several days.
(3+) = skin redness, several lesions in the skin where hair would not grow back, and some virus spreading lasting a week.
(4+) = many lesions and virus spreading over the guinea pig lasting 3 or more weeks.
(–) = no virus challenge as the initial infection was severe.

activity was found not to change throughout the replaquing process and extensive passaging of the virus.

The reverent TK-positive C290BUTK was tested for its ability to cause skin lesions in outbred Hartley guinea pigs along with the other BHV-2 C290 strains as described earlier (*2,3*). A summary of the results of 28 inoculations of guinea pigs with various BHV-2 C290 strains is shown in the table; each strain was tested in at least four guinea pigs in at least two separate experiments. The guinea pigs were inoculated subdermally with 0.5 ml of the BHV-2 strains (total dose is indicated in the table). Two weeks after the initial disease symptoms disappeared, the guinea pigs were challenged by subdermal injection of a high dose of wild type C290 strain (3×10^7 PFU in 0.5 ml). Initial virus inocula were prepared from the viruses grown in MDBK cells by a 3-fold freezing and thawing of the infected cultures, centrifugation at 12, 000 x g for 10 mins, and diluting of the obtained supernatants with the growth medium (*2*). The inoculation of guinea pigs with the virulent C290 and C290BU3 (TK-deficient) viruses normally took 2–3 days for the symptoms to appear at high virus inoculum dose ($=1 \times 10^7$ PFU) or 5–7 days at low dose ($= 0.5 \times 10^6$ PFU). Lesions appeared initially at the site of injection before spreading to other sites on the guinea pig (side of the head, nose, ear or stomach region). BHV-2 is naturally temperature-sensitive and therefore tends to cause disease symptoms on the cooler parts of the animal body (*7*). The table shows that inoculation with a high dose of the avirulent C290BU5 (TK-deficient) produced a small single lesion but not at the site of injection, within 7–10 days. In contrast, inoculation with the same dose of C290BUTK (TK-positive) produced a small single lesion but at the site of infection within 3 days. Differences in the time and site of appearance of symptoms were the only ones observed in the disease development with these two viruses. Clearly, the reverent TK virus, C290BUTK, never reacquired the virulence of the wild-type C290. The guinea pigs that were previously inoculated with any of the viruses were found to be protected from the disease symptoms when rechallenged with the virulent C290. Some guniea pigs inoculated with C290 or C290BU3 could not be rechallenged as their symptoms continued to be present even 8 weeks after the initial infection. Therefore,TK-positive, revertant C290BUTK virus was essentially identical to the potential vaccine C290BU5 virus in its ability to cause disease and to protect guinea pigs from the wild-type virus.

The TK-deficient strain of BHV-2, C290BU5 is potentially a better vaccine than wild-type unattenuated BHV-2 viruses (*1*) as it did not reactivate from latency (*2*) and it is a selectable marker. Further support for C290BU5 as a potential vaccine was the fact that the reacquisition of TK activity did not significantly increase the virulence of C290BU5 and alter its ability to protect against high doses of wild-type virus. Therefore, if C290BU5 did spontaneously revert to TK-positive phenotype, it appears that the virus would still remain avirulent and suitable for use as a vaccine. Nevertheless, this restoration of TK activity has been found to be a rare event. This was indicated by isolation of only a single C290BUTK revertant from C290BU5 despite 25 separate experiments that used the strong selection pressure of the HAT selection method. Furthermore, as this TK reverent did not reacquire any significant virulence, it suggests that the TK may not be the major determinant of virulence in BHV-2. This was further supported by the finding that the TK-deficient C290BU3 had no significant reduction in its virulence from the wild-type C290 (table) (*3*). Differences in the TK gene nucleotide sequences of the two TK-deficient variants, C290BU5 and C290BU3, could not even account for the differences in their virulence (*8*). No additional change in C290BU5 besides the alteration of the TK activity is known. Nevertheless, different animal systems can occasionally produce variable virulence data as those found with a TK-deficient pseudorabies isolate which had no virulence for pigs and protected against virulent pseudorabies virus, yet still maintained its virulence for rabbits and mice (*6*). Other animal herpesvirus vaccines used to protect against pseudorabies virus and bovine herpesvirus 1 contain viruses lacking glycoprotein E gene (*9*). The original C290BU5 was isolated using a bromodeoxyuridine selection process (*2*). Bromodeoxyuridine is now known to affect the glycoprotein E gene product of

herpesviruses (*10*). Thus the possibility exists that some other gene(s) besides the TK gene, such as glycoprotein E gene, which is related to BHV-2 virulence, has been affected in C290BU5. This remains to be determined at the level of the virus genome where very few genes have been sequenced or their products investigated.

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