LETTER TO THE EDITOR

THE PRESENCE OR ABSENCE OF RIBONUCLEOTIDE REDUCTASE ACTIVITY HAS NO AFFECT ON AVIRULENT BOVINE HERPESVIRUS 2

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Ribonucleotide reductase (RR) is a gene associated with virulence in herpesvirus such as herpes simplex virus 1 (HSV-1) and pseudorabies virus and is normally present in alphaherpesviruses (*1*). RR catalyses the reduction of ribonucleotides to deoxyribonucleotides, and the herpesvirus-encoded enzyme consists of two non-identical subunits coded for by neighboring genes in DNA of herpesviruses (*2*).

We have been attempting to produce a vaccine against bovine herpesvirus 2 (BHV-2) or bovine mammillitis virus), by removal of another known herpesvirus virulence-associated gene product, the thymidine kinase (TK) (*3*), BHV-2 has a world-wide distribution and causes an ulcerative disease of the teats and udder (herpes mammillitis, pseudolumpyskin disease) or a generalized infection with skin lesions. For testing potential vaccines we have been using the BHV-2 strain CSIRO 290 (C290) which causes lesions in the skin of guinea pigs (*3*). Removal of the TK activity from this virus resulted in C290BU5 strain with potential for use as a BHV-2 vaccine. But restoration of the TK activity to the C290BU5 strain (designated C290BUTK) did not alter its avirulent properties (*4*). Another strain created without TK activity (C290BU3) still retained its virulence (*4*), further suggesting that some other gene, other than TK, was involved in BHV-2 virulence. One such candidate gene investigated here is RR.

Cell extracts with RR activity were prepared and assayed at 37°C for 60 mins, essentially by the method of Dutia (*5*). The RR assay was based on the ability of the cell lysates to convert [^{3}H]CDP to [^{3}H]dCDP. Non-confluent MDBK cells were infected with 20 PFU/cell of each of the above BHV-2 strains, the wild-type BHM-1 strain with low virulence in guinea pigs (*3*) or HSV-1 for 8 hrs. C290-infected cell extracts (100 mg of protein) converted 3% of the added [^{3}H]CDP. The cells were disrupted by one freezing and thawing followed by Dounce homogenization, and no unlabeled CDP was added to the assay medium. Repeated freezing and thawing decreased the BHV-2-induced RR activity, and the RR activity was optimal at pH 8.0, characteristics previously found with equine herpesvirus 1 (EHV-1) RR (*6*). Uninfected cells were included in all experiments, but the cellular MDBK RR activity was low (5% of the virus-induced activity) when assayed in the absence of Mg^{++}. The addition of 2 mmol/l $MgCl_2$ increased the cellular RR activity four-fold while having no effect on the BHV-2 induced activities, as reported in other RR studies (*7*).

The results presented in the Table show the RR activity of the BHV-2 strains determined from 8 separate experiments using assay duplicates. Uninfected cell values were subtracted prior to the calculation of the infected cell values. The Table also indicates their virulence in guinea pigs (*3,4*), and their TK status (*4*). The RR activity of the different strains is indicated relative to the wild type C290.

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Abbreviations: BHV-2 = bovine herpesvirus 2; EHV-1 = equine herpesvirus 1; HSV-1 = herpes simplex virus 1; RR = ribonucleotide reductase; TK = thymidine kinase

Table

BHV-2 strains	Virulence	TK	RR activity (%)
C290	+	+	100
C290BU5	–	–	72±20
C290BU3	+	–	58±6
C290BUTK	–	+	0
BHM-1	±	+	91±7

A similar level of RR activity to that detected in C290-infected cells was found with another wild-type BHV-2 strain, BHM-1 (*3*), and both these activities were two-fold less than those induced by HSV-1. The TK-deficient C290BU5 and C290BU3 strains always induced RR activity, the latter strain inducing a slightly lower activity. However, the TK-positive C290BUTK never induced any detectable RR activity, even when assayed at 31°C for possible RR temperature-sensitivity (*5*). The RR activities induced by HSV-1 and all BHV-2 strains except C290BUTK, were inhibited by at least 80% by the addition of 0.2 mmol/l nonapeptide YAGAVVNDL (Sigma) to the assay medium. This peptide is known to inhibit both HSV-1 and EHV-1 RR activities by interfering with the combination of the two protein subunits of RR (*6*). All BHV-2 strains grew in MDBK cells at the same rate, and produced the same level of virus (*4*). Thus the difference in the detected RR activity was not due to the RR-negative virus growing more slowly. Furthermore, the infected cell extracts used in this RR assay were similar to those used in TK assays (*3,4*), and C290BUTK induced the same level of TK as the wild-type virus under these conditions. How the apparent RR mutant (C290BUTK) was produced is unknown. The TK-deficient C290BU5, from which it is derived (*4*), showed no loss of RR activity, and was initially developed by selecting the virus in the presence of bromodeoxyuridine, which not only allows the selection of TK mutants, but can also produce RR mutants of HSV-1 (*5*). In two HSV-1 mutants, derived from a parent strain treated with bromodeoxyuridine, alteration to either RR1 (*ts* 1207) or RR2 (*ts* 1222) occurred (*5,8*). No RR activity was induced by cell infection with *ts* 1222 at any temperature, as the loss of a single nucleotide in the RR2 gene altered the essential C-terminal end of the protein and its ability to bind to RR1 (*9*). C290BUTK was selected in a medium containing hypoxanthine, aminopterin, and thymidine (HAT) in a TK-negative MDBK cell line (*4*), a method used to select only for TK-positive viruses. It is possible that a spontaneous mutation may have occurred in the C290BUTK RR gene, as it has been estimated that spontaneous reversion within the HSV TK gene occurred at the rate of 10^{-4}–10^{-6} (*10*).

We have recently located and sequenced (GeneBank Acc. No. AF326961) both the BHV-2 RR large (RR1; 2,352 bp) and small (RR2; 939 bp) subunit gene (within the *Hind*III J DNA fragment of the BHV-2 strain BHM-1 DNA). BHV-2 RR1 (UL39)and RR2 (UL40) has 67% and 74% homology, respectively, with HSV-1 at the amino acid level, but have less homology with other herpesviruses. Both the HSV-1 and BHV-2 RR2 proteins have the identical amino acid sequence YAGAVVNDL at the C-terminal end, a sequence essential for the binding of RR1 and RR2 for enzyme activity (*6*) and catalytic cysteines in RR1 (*8,11*). The BHV-2 RR assays performed here confirm that this gene is indeed active in wild-type BHV-2-infected cells and its activity can be inhibited by the nonapeptide YAGAVVNDL. It is unknown where the change within the RR gene (RR1 or RR2) has occurred in the C290BUTK isolate, which completely lost the ability to induce RR activity. While alphaherpesviruses normally possess the R1 and RR2 genes, it has been suggested than canine herpesvirus may lack such a gene based on partial (93%) sequencing of the gene locations of the virus (*12*). It has been proposed that the lack of these RR genes may be associated with the natural lower growth temperature of this virus (*12*). BHV-2 also has a lower growth temperature; some viruses taken from herpes mammillitis-infected cattle were successfully isolated only by growing them at 32°C (*13*). Normally, BHV-2 grow at 33–36°C but not at 39°C. However, unlike the canine herpesvirus, its lower growth temperature is not due to the lack of RR. We now have developed two avirulent BHV-2 viruses, the TK-deficient BHV-2 (C290BU5) and a RR-negative BHV-2 (C290BUTK), and they both are potential vaccines against bovine herpes mammillitis infections.

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