Equine Abortion (Herpes) Virus: Strain Differences in Susceptibility to Inactivation by Dithiothreitol

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The infectivity of equine abortion (herpes) virus (EAV) was inactivated by treatment with reduced dithiothreitol (DTT). According to their susceptibility to DTT, the EAV strains could be divided into three groups. The vaccine strain RAC-H (419) proved to be more resistant to DTT than all of the other 14 strains tested. The hemagglutinin of EAV was also inactivated by DTT; no strain differences were observed in this respect.

Reduced dithiothreitol (DTT) is a slow acting agent which reduces disulfide bonds. It was first described by Cleland (3). Several viruses have been shown to be inactivated by DTT. Among these viruses are members of the arbovirus groups A and B, the poxvirus vaccinia, the paramyxovirus Newcastle disease (2), and the papovavirus polyoma (7). A herpesvirus, the pseudorabies virus, has been reported to be inactivated by DTT under limiting conditions of temperature, pH, and drug concentration (6). Other viruses which have been tested, i.e., some picornaviruses, proved to be resistant to inactivation by DTT (2).

In the present report, the inactivation by DTT of another herpesvirus, the equine abortion virus (EAV), is described. However, various EAV strains showed differences in sensitivity to DTT, the vaccine strain RAC-H (8) being the most resistant one. This strain is also characterized by its inability to produce plaques in L cells (1).

MATERIALS AND METHODS

Cell cultures. Four different types of cells were used: PK-15, a continuous line of pig kidney cells; KFBL, a line of bovine lung cells transformed by simian virus 40 deoxyribonucleic acid (4); and two different clones of L cells. One clone (L-B) was supplied by H. C. Borgen, Lindholm, Denmark, and the other one (L-929) was obtained from Flow Laboratories, Irvine, Scotland. Eagle's minimal essential medium (MEM) with 5% calf serum (KFBL) or MEM without serum (PK-15, L-929) was used for maintenance. The medium for the L-B cells and the methylcellulose overlay for both L-cell clones were those recommended by Borgen (1).

EAV strains. Fifteen different EAV strains were used. Two of them were derivatives of the hamster-adapted RAC-H strain which was passaged in pig kidney cells (8). We obtained the low-passage RAC-H strain, referred to as 17, and the high-passage RAC-H strain (the vaccine strain), referred to as 419, by courtesy of A. Mayr, Munich, Germany. Strains N-3 and SL-D were kindly supplied by H.C. Borgen. The 11 other strains used were low-passage isolates derived from aborted fetuses. Stock virus of each strain was harvested as culture fluid from infected PK-15 cells and stored at −60 C. Purified virus preparations of strains 419 and 647 had also been used. The purification procedure was described elsewhere (7a).

End-point titrations. End-point titrations were carried out in tube cultures of KFBL cells. The cells were inoculated with serial 10-fold dilutions of a virus suspension, four tube cultures per dilution. The infectivity titer was calculated in log₄₀ TCD₄₀ per 0.1 ml by the method of Reed and Muench (10).

Plaque assays. For plaque assays, confluent monolayers of L-B or L-929 cells were cultivated in 60-mm plastic petri dishes at 37 C in 5% CO₂. Serial 10-fold dilutions of virus were allowed to adsorb to the cells for 45 min at 37 C. After virus adsorption, 4 ml of methylcellulose overlay, consisting of VM3 balanced salt solution, 0.2% glucose, 0.5% lactalbumin hydrolysate, 1% calf serum, and 0.75% methylcellulose (1), was added per plate. The plates were incubated for 5 days at 37 C in 5% CO₂. The overlay was then removed, and the cells were fixed with a 10% solution of Formalin and stained with undiluted Giemsa stain.

Inactivation with reduced DTT. DTT was obtained from Calbiochem Corp., Los Angeles, Calif. A fresh DTT solution in 0.01 M tris(hydroxymethyl)- aminomethane-hydrochloride buffer (pH 8.1) was prepared on the day of the experiment. The final DTT concentrations were 0.001 M or 0.002 M at pH 1121.
Drug-virus mixtures were incubated at 30 or 37 C. Samples were taken at different times of incubation and immediately placed in an ice bath to stop further inactivation. The infectivity of the samples was measured by end-point titrations (see above).

**HA tests.** The hemagglutinin of EAV for horse red blood cells (9) was titrated by the microtiter method. Serial twofold dilutions of a virus suspension in 0.05 ml of phosphate-buffered saline were made, and 0.05 ml of a 0.5% suspension of horse blood cells was added to each dilution. The microtiter plates were kept at room temperature. The highest virus dilution showing complete hemagglutination (HA) was taken as the HA titer.

**RESULTS**

**Kinetics of inactivation by DTT.** Gainer et al. (6) showed that the rate of inactivation of pseudorabies virus by DTT was temperature dependent under mild alkaline conditions, being more rapid as the temperature was raised from 30 to 41 C.

The conditions of exposure of two different strains (419 and 647) of EAV to DTT were studied at two different concentrations of DTT (0.001 or 0.002 M) and at two different temperatures (30 and 37 C), at pH 7.8. At 37 C, both strains were inactivated within 60 to 75 min of exposure; the inactivation was rapid at both DTT concentrations used. At 30 C, the inactivation was slower than at 37 C so that inactivation curves could be obtained for both concentrations of DTT (Fig. 1 and 2). The inactivation was more rapid with 0.002 M DTT than with 0.001 M DTT. At both concentrations, the strains showed differences in sensitivity to DTT, the vaccine strain 419 being more resistant than the wild-type strain 647. Similar results were obtained with purified preparations of strains 419 and 647. These were used to ascertain that the inactivation by DTT was not influenced by nonviral constituents of the reaction mixture. Suspensions of untreated virus kept at 30 or 37 C and pH 7.8 had about the same titers throughout the test period.

**Strain differences in the sensitivity to DTT.** Since the vaccine strain 419 was more resistant to DTT than the wild-type strain 647, 13 additional wild-type strains were tested. The conditions of exposure were 0.002 M DTT for 30 min at 30 C and pH 7.8. The data in Table 1 show that, according to their sensitivity to DTT, the strains could be divided into three groups. Of all strains tested, strain 419 was the most resistant one. Strains SL-D and N-3 were placed in an intermediate group, whereas the remaining strains belong in the most sensitive group.

**"L character" of EAV strains.** Differences among EAV strains were described earlier by Borgen (1). The inability to produce plaques in L cells was considered as a marker for a few strains, including the vaccine strain RAC-H. We studied the ability of our strains to produce plaques in two different clones of L cells, L-B and L-929, respectively. In L-B cells the strains behaved as described by Borgen (1, 7a)
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Table 1. Grouping of equine abortion virus (EAV) strains by sensitivity to dithiothreitol (DTT)

| EAV strain | Reduction in virus titer by DTT (log_{10} units TCD_{50}) | L character* |
|------------|----------------------------------------------------------|---------------|
|            | 0-1| 1-2| 2-4                  |               |
| RAC-H 419  | 0.75| 1.25|                      | L-            |
| SL-D       | 1.5 |      |                      | L-            |
| N-3        |     | 3.0 |                      | L-            |
| RAC-H 17   | 2.25| 2.25|                      | L-            |
| 647        | 2.5 | 2.5 |                      | L+            |
| 511        | 2.5 | 2.5 |                      | L+            |
| 465        | 2.5 | 2.5 |                      | L+            |
| 908        | 2.75| 2.75|                      | L+            |
| 1080       | 3.0 | 3.0 |                      | L+            |
| 510        | 3.25| 3.25|                      | L+            |
| 632        | 3.25| 3.25|                      | L+            |
| 825        | 3.5 | 3.5 |                      | L+            |
| 302        |     |     |                      | L+            |
| 800        |     |     |                      | L+            |

*Ability or inability to produce plaques in L-B cells (1).

whereas in L-929 cells all strains produced plaques with a plaque size of about 1 to 3 mm diameter. Strain 419 was characterized by two markers (Table 1), i.e., the inability to produce plaques in L-B cell monolayers, and relative resistance to DTT. Among the other strains, SL-D, N-3, and 17 were also unable to produce plaques in L-B cell monolayers but proved to be less resistant to DTT than strain 419.

Inactivation of EAV hemagglutinin by DTT. It was proposed that, in viruses sensitive to DTT, the protein coat of the virion became disrupted (6). The EAV hemagglutinin is supposedly located in the viral envelope. Therefore, we investigated whether the hemagglutinin is inactivated by DTT. Virus suspensions of strains 419 and 647, with a HA titer of 1:64 each, were incubated with 0.002 M DTT for 60 min at 30 C and pH 7.8. Samples of the virus suspensions were titrated for HA activity after different times of exposure to DTT. The results in Table 2 show that the hemagglutinins of both strains are inactivated at a similar rate. Untreated virus suspensions kept at 30 C and pH 7.8 have constant HA titers throughout the test period.

**DISCUSSION**

The high-passage derivative of the RAC-H strain was introduced as an attenuated, live vaccine against rhinopneumonitis and abortion in horses in West Germany (8). The importation of this vaccine strain to Sweden was made dependent on the fulfillment of certain requirements as, for instance, that for stable in vitro markers (5). In search for such markers, Borgen (1, 1a) described the inability of the vaccine RAC-H strain as well as of a few other strains (low-passage RAC-H, SL-D, N-3) to produce plaques in a clone of L cells, here called L-B. The majority of the strains tested were plaque producers in this cell line. In the present study, the findings of Borgen were confirmed as far as L-B cells were concerned. When another clone of L cells (L-929) was used, all strains did produce plaques. The difference between L-cell clones could not be explained.

In our search for markers, we found that the vaccine strain RAC-H is more resistant to inactivation by DTT than all other strains tested (Table 1). At present, the strain differences in sensitivity to DTT are difficult to explain. In studies on another herpesvirus, the pseudorabies virus, Gainer et al. (6) tried to explain the mechanism of inactivation by this drug. The main effect of DTT as determined by electron microscopy is disruption of the coat of the virion, probably due to cleavage of S-S linkages in cystine. Under similar conditions of exposure to DTT, the inactivation rate of the vaccine RAC-H strain is similar to that of the pseudorabies virus, whereas some of our other strains have proved to be much more sensitive to DTT than the pseudorabies virus.

The inactivation of hemagglutinin does support the notion that DTT interacts with and alters the envelope. However, the inactivation rates of the vaccine RAC-H strain and a wild-type strain are identical.

The vaccine RAC-H strain is characterized by two markers: a relative resistance to DTT (DTT marker) and the inability to produce plaques in L-B cells (L- marker). The L- marker is also found associated with the low-passage RAC-H strain, indicating that this marker is an intrinsic property of the RAC-H

Table 2. Inactivation by dithiothreitol (DTT) of equine abortion virus (EAV) hemagglutinin

| EAV strain | HA titer* at time (min) of exposure to DTT |
|------------|------------------------------------------|
|            | 0   | 15  | 30  | 45  | 60  |
| RAC-H 419  | 64  | 16  | 8   | 2   | 2   |
| 647        | 64  | 16  | 8   | 2   | 2   |

* Reciprocals of initial virus dilutions. HA, hemagglutinin.
strain (1). Since the vaccine RAC-H strain proves to be much more resistant to DTT than the low-passage RAC-H strain, it appears that this property may have been acquired during the course of numerous (419) passages in pig kidney cells. Thus, the $L^-$ and DTT markers are not coupled properties, and the DTT marker does not necessarily indicate attenuation.

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