Equine Abortion (Herpes) Virus: Evaluation of Markers in a Field Vaccination Trial

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Twelve mares were vaccinated with attenuated equine abortion virus (EAV) strain RAC-H. Two nonvaccinated mares served as controls. In at least three mares the vaccination appeared to coincide with a natural infection. This was indicated by characterization of the EAV isolated from nasal secretions of six vaccinated mares, a nonvaccinated control, and also from the lung, spleen, and liver of a fetus aborted by a vaccinated mare. The relative sensitivity of the isolated EAV to dithiothreitol was used to distinguish the RAC-H strain and wild-type virus. Of the 10 EAV isolates, four were recognized as being the vaccine strain while six were recognized as being wild-type strains. Three of the latter originated from two vaccinated mares and a nonvaccinated control, and three were recovered from the fetus. The ability of the EAV strains to form plaques in a cloned line of L cells proved to be unsuitable for use as a marker in this study.

The high-passage derivative of equine abortion virus (EAV) strain RAC-H was introduced as an attenuated, live vaccine against rhinopneumonitis and abortion in horses in West Germany (9). The importation of this vaccine to Sweden was made dependent on the fulfillment of certain requirements including stable in vitro markers (5). In search for such markers, Borgen (1, 2) described the specific inability of the vaccine strain RAC-H, as well as a few other strains, to produce plaques in a clone of L cells (L-marker). Klingeborn and Dinter (8) described the relative sensitivity of various EAV strains to inactivation by dithiothreitol (DTT), finding the vaccine strain RAC-H most resistant. The present study is an evaluation of these markers after passage of the vaccine strain RAC-H in mares.

MATERIALS AND METHODS

Vaccination. The vaccine labeled Prevaccinol and consisting of the live, attenuated RAC-H strain of EAV was kindly supplied by the manufacturer, Behringwerke AG, Marburg/Lahn. The virus in the vaccine was titrated (see below) and it was found that 1 ml contained 10^6 plaque-forming units (PFU) in PK-15 (pig kidney) cells. The vaccination trial was carried out on 14 thoroughbred mares. The horses (listed in Table 1) were kept in two stables divided by a simple door. In one of these stables were horses numbered 1 through 9, and in the other stable were horses numbered 10 through 14. There were no other horses in these stables, but there were many on the farm. Twelve mares were inoculated intramuscularly in the neck with 5.0 ml of the vaccine. At the time of the first vaccination six of the mares were in the fourth to seventh month of pregnancy. Three months after the first vaccination seven mares were revaccinated using the same vaccine dose and mode of injection. Five mares were not revaccinated because of advanced pregnancy. Prior to vaccination and at various time periods thereafter, the mares were tested for titers of serum neutralizing antibodies to EAV. The mares were repeatedly examined for clinical signs of reaction to vaccination.

Materials for virus isolation. Nasal swabs were collected from each mare on the second or third day after the first vaccination. The nasal cavity was rubbed with a sterile absorbent cotton ball soaked with 1 ml of cell culture medium containing penicillin (200 U/ml) and streptomycin (200 µg/ml). The cotton ball was then placed in a sterile test tube, and the tubes were kept at 0°C before testing. After compressing the cotton ball in the medium, 0.2 ml of each sample was used to inoculate a monolayer of PK-15 cells in a plastic petri dish. Such monolayers were also used for attempts to cultivate virus from an aborted fetus; inocula consisted of 10% suspensions of various organs prepared in phosphate-buffered saline, pH 7.4. The cells were observed daily for cytopathic effects.

Cell cultures. Three 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 a clone of mouse L cells (L-B), which was kindly supplied by H. C. Borgen, Lindholm, Denmark. Eagle minimal essential medium (MEM) with 5% calf serum (KFBL)
or MEM without serum (PK-15) was used for maintenance. The medium and methylcellulose overlay for the L-B cells were those recommended by Borgen (1).

**EAV strains.** The high-passage RAC-H strain (the vaccine strain) was obtained by courtesy of A. Mayr, Munich, Germany. This strain was used in neutralization tests and as a reference strain in tests for markers. A wild virus strain, 908, was from our own collection. This strain was isolated from an aborted fetus, and it was used as a reference strain in tests for markers. In the course of the vaccination trial, 10 EAV strains were isolated and stored. Stock virus of each strain was harvested as culture fluid from infected PK-15 cells and stored at −60°C before testing.

**Reference serum.** This was prepared in rabbits by multiple injections with a purified virus concentrate of the RAC-H strain. The serum had a neutralization titer of 1:128. In tests for identification of isolates, this serum was used at a dilution of 1:10 against two doses of virus, 100 and 1,000 PFU.

**Plaque assays.** For plaque assays, confluent monolayers of PK-15 or L-B 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. For L-B cells, the overlay and the procedures of fixation and staining were those described elsewhere (8). For PK-15 cells, after virus adsorption, 4 ml of an agar overlay was added per plate. The agar overlay was composed of Noble agar (1.5 g), Eagle MEM, 2× (48 ml), calf serum (4 ml), and distilled water (48 ml). Plaques were counted on day 3 after inoculation. The infectivity titer was expressed in terms of PFU per milliliter.

**Inactivation with DTT.** The method of inactivation with DTT was described previously (8) except that, for the present studies, infectivity was measured by a plaque assay (see above).

**Neutralization test.** Each serial twofold serum dilution was mixed with about 100 mean tissue culture infective dose (TCID₅₀) virus. The mixtures were incubated for 60 min at 37°C, and 0.2 ml was then inoculated into each of four tube cultures. The cultures were incubated in a roller apparatus at 37°C and final readings were made on day 7 after inoculation. The highest serum dilution which protected at least two of four tube cultures were considered as the neutralization titer.

**RESULTS**

**Clinical signs.** None of the mares showed any apparent reaction after vaccination, except that mare no. 8 aborted on day 2 after vaccination; she was in the beginning of the seventh month of pregnancy. This mare showed no signs of illness prior to, or after, vaccination. Because of advanced decay the pathological examination of the aborted fetus revealed no macroscopic or microscopic changes (inclusion bodies).

**Virus recovery.** Cultivation of virus was attempted from 14 nasal swabs collected from 12 vaccinated mares and 2 control mares 2 or 3 days after vaccination. Virus was recovered from 7 swabs, 6 collected from vaccinated mares on day 2 and 1 from a control mare on day 3. Virus was also isolated from suspensions of the lung, liver, and spleen of the aborted fetus.

**Evaluation of the DTT marker.** A total of 10 isolates, seven from nasal swabs and three from organs of the aborted fetus, were first examined for identity as EAV and then for sensitivity to DTT. A reference rabbit antiserum was used for identification. Each of the 10 isolates induced cytopathic changes typical of EAV in PK-15 cells and each of them was neutralized by the reference serum. The results of tests for the DTT marker are presented in Table 1. It is seen that 4 of the nasal isolates showed the same resistance to inactivation by DTT as the vaccine strain RAC-H. Three nasal isolates, as well as three isolates from the fetus, were as sensitive to DTT as the wild strain 908, suggesting that in these mares the vaccination coincided with a natural infection.

**Unsuitability of the L marker.** In the course of the vaccination trial the L marker was found unsuitable because of variability of the L-B cells used. During propagation of these cells in our laboratory, their susceptibility for EAV changed. Plaques were produced in cells of this new clone by isolates and reference strains. It has been previously reported (8) that plaques

| Table 1. Differentiation of equine abortion virus (EAV) isolates by the DTT marker |
|-------------------------------------|----|----|
| Origin isolate or strain | Label | DTT marker |
| NS³ | 3 | 2.25 |
| NS | 5 | 3.00 |
| NS | 6 | 0.75 |
| NS | 7 | 0.75 |
| NS | 8 | 0.75 |
| NS | 9 | 0.75 |
| NS | 10 | 2.25 |
| Lung, AF³⁴ | 8 | 2.25 |
| Spleen, AF | 8 | 2.50 |
| Liver, AF | 8 | 2.50 |
| Strain | RAC-H | 0.75 |
| Strain | 908 | 2.50 |

- Reduction in virus titer (logₐ PFU) by exposure to DTT for 30 min at 30°C, pH 7.8.
- Number of the mare (cf. Table 2) or the name of the strain.
- NS, Nasal secretion.
- AF, Aborted fetus.
were produced by all EAV strains tested when using another clone of L cells, L-929.

Antibody response to EAV in mares. The antibody response to EAV in mares is shown in Table 2. All the mares of the vaccination trial, i.e. 12 vaccinees and 2 nonvaccinated controls, had neutralizing antibodies to EAV on the day of vaccination. This means that they all had been infected with this virus prior to vaccination. Three weeks after the first vaccination 10 of the 12 mares, and also 1 nonvaccinated control (no. 3), showed a significant rise of neutralizing antibodies to EAV, whereas only 3 of 7 revaccinated mares showed a subsequent significant rise of neutralization titer. However, no titer rise could be evaluated with respect to antigenicity of the RAC-H strain in the vaccine Prevaccinol since the vaccination coincided with a natural EAV infection (Table 1) and the failure to isolate EAV does not preclude infection.

DISCUSSION

The original purpose of the vaccination trial described was to test the safety of vaccination and to measure the antibody response to the vaccine strain. However, since in some mares the vaccination coincided with a natural EAV infection, the trial turned out to be an excellent opportunity to evaluate the DTT marker for differentiation of the vaccine strain from wild isolates.

When a mare (no. 8) aborted on day 2 after vaccination, and EAV was isolated from lungs, spleen, and liver of the fetus, the vaccine strain was initially suspected of causing the abortion. However, when the isolates from the fetus were tested for the DTT marker, they behaved like a wild strain. On the other hand the isolate from the nasal swab of the dam behaved like the vaccine strain (Table 1). It seems most likely that a natural infection coincided with vaccination. An isolate from the nasal swab of one of the nonvaccinated mares was also found to behave like a wild strain, whereas the three additional isolates from vaccinated mares, behaved like the vaccine strain.

Furthermore, when serum samples from 129 horses were tested for neutralizing antibodies to EAV, 102 samples (79%) were found positive (unpublished data). Also, in 1969/1970 20% of equine abortions in Sweden were diagnosed as due to infection with EAV (G. Rockborn, personal communication). Thus, EAV is circulating in the horse population to the extent that a coincidence of natural infection with vaccination is rather likely. However, in order to determine whether the DTT marker remains stable on serial passages in vivo, more experiments on horses are required.

At the time of abortion mare no. 8 had a high titer of neutralizing antibodies (Table 2). However, the infection of this mare might have happened long before abortion, and also long before the fetus became infected. It was found that the time interval between the infection of a mare and abortion can vary from 1 to 4 months, and between the infection of a fetus and abortion from 3 to 9 days (7). Since leukocytes are carriers of EAV, the fetus can be infected in spite of circulating antibodies in the mare (3). Doll (6) described several cases of abortion in dams with high titers of neutralizing antibody in their sera. When EAV has once reached the fetus, the infection proceeds quickly because there is no antibody transfer through the equine placenta.

Summarizing, the results of the present study point out the value of markers for live attenuated vaccine strains.

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