SHORT COMMUNICATION

Immunization with VP2 Is Sufficient for Protection against Lethal Challenge with African Horsesickness Virus Type 4

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Horses were immunized by inoculation with a vaccinia construct containing a full-length cDNA corresponding to the L2 gene segment of African horsesickness virus type 4 (AHSV-4). All immunized horses developed serum neutralizing antibodies prior to challenge with virulent AHSV-4. No ELISA-reactive antibodies were present prior to challenge. A group of four seronegative control horses died after developing clinical signs and lesions typical of the pulmonary form of African horsesickness while the immunized horses were clinically normal. Increases in serum neutralizing and ELISA-reactive antibody titers following challenge indicate that at least some replication of challenge virus occurred in immunized horses. These results demonstrate that AHSV VP2 alone is sufficient to induce a protective immune response in horses and indicate the usefulness of ELISA-reactive antibodies for differentiation of vaccinated and naturally exposed horses.

African horsesickness (AHS) is a noncontagious disease of horses and other solipeds caused by an arthropod-borne orbivirus of the family Reoviridae. There are currently nine known serotypes, with the most recent outbreaks caused by African horsesickness virus (AHSV) types 4 and 9 (1, 2). The disease is enzootic in sub-Saharan Africa and severe epizootics have occurred in the Iberian peninsula, North Africa and the Middle East (3–5). In 1987 an outbreak was reported in Spain, with extension to Portugal and Morocco in 1988 (5, 6). This outbreak continued until 1990, resulted in the death of numerous horses, and required the vaccination of thousands of other horses. An outbreak of AHS in the United States or Europe, with the native Culicoides vectors competent for AHSV in both areas, would be devastating to the horse industry (7, 8).

AHSV is a typical orbivirus with a double-layered capsid containing the genomic material consisting of 10 segments of double-stranded (ds) RNA, each encoding one to two proteins (9). The major outer capsid and core structural viral proteins are VP2, VP5 and VP3, VP7, respectively. In addition, there are three minor structural proteins, VP1, VP4, and VP6, associated with the core and four nonstructural (NS) proteins, NS1, 2, 3, and 3a (10–12).

Current vaccines for AHS include modified live virus (MLV) and inactivated whole virus vaccines (13–17). Although these vaccines are generally effective, there are some concerns about their use, including possible reversion to virulence by back mutation or reassortment with wild-type virus, incomplete attenuation or inactivation, and owner concerns about effects of modified live vaccines on performance of their horses. There are two significant problems with both MLV and inactivated AHS vaccines. First, both involve growth of large amounts of live virus which may require a biocontainment facility outside enzootic areas. Second, it is difficult to serologically differentiate vaccinated from naturally infected horses. These problems would be eliminated by the use of subunit vaccines.

A critical step in developing a subunit vaccine is to determine which viral protein(s) are responsible for inducing a protective immune response. It has been shown that VP2, which is encoded by gene segment L2, bears the major neutralizing epitopes and thus is responsible for inducing serotype-specific responses (18–20). AHSV type 4 (AHSV-4) VP2 bears at least three distinct, highly conformational neutralizing epitopes (T. Burrage, unpublished observation). Of particular relevance to vaccine development are experiments performed demonstrating that neutralizing monoclonal antibodies to VP2 passively protect mice against a lethal challenge of virus, suggesting that neutralizing antibodies are sufficient for protection (18).

To determine if VP2, presumably by induction of neutralizing antibodies, is sufficient for protection of horses, we used a vaccinia construct expressing a full-length L2 gene segment (AHSV-4 VP2) to immunize horses which
were then challenged with a lethal dose of AHSV-4, a 1990 isolate from a horse in Spain (AHSV/4SP). The data described in this paper show that AHSV VP2, expressed in vivo, induces neutralizing antibodies and confers complete protection from disease.

A recombinant vaccinia virus expressing VP2 of AHSV-4 was constructed by cloning the full-length L2 cDNA (encoding the VP2 protein) into the BglII site of the transfer vector pSC11 (21). Correct orientation of the insert was confirmed by restriction enzyme digest and sequence analysis. Transfection was carried out with the WR strain of vaccinia virus. Recombinants were selected and plaque purified three times.

All virus stocks used were propagated and assayed on Vero cell monolayers in DMEM media with 10% fetal bovine serum and antibiotics. Vaccinia viruses, parental WR, and the construct containing AHSV type 4 L2 gene were subjected to two freeze/thaw cycles, sonicated for 1 min, and stored at −70° until being used for vaccination. The challenge virus, AHSV/4SP, was isolated in 1990 from an infected horse in Spain (5). This plaque purified isolate propagated in Vero cells was previously shown to cause 100% mortality in horses, consistently inducing the pulmonary form of AHS (22, 23). Titers of parental vaccinia virus and the vaccinia-L2 construct were determined by plaque titration while AHSV titers were determined by microtitration, both on Vero cells.

The horses used in this experiment were clinically normal and seronegative for AHSV neutralizing and ELISA antibodies (18, 24). Horses were allowed to acclimatize to the biocontainment facility for 2 weeks prior to primary inoculation. Two horses received parental vaccinia WR strain while four horses received the vaccinia-L2 construct via three intradermal inoculations of 10⁹ PFU at 3-week intervals. A negative control horse received Vero lysate prepared in the same manner as the vaccinia-L2 Vero lysate. All inoculations were given on the lateral aspect of the neck. The immunized and control horses were challenged 3 weeks after the last inoculation by intravenous injection of 5 × 10⁴ TCID₅₀ of AHSV/4SP. Three naive control horses from a parallel experiment were included for statistical purposes in analysis of this experiment. After challenge, the horses were observed each day for clinical manifestations of AHS. Blood samples for virus titrations, serum neutralization, and ELISA were taken daily. To determine if VP2 was expressed in vivo prior to challenge, radioactive labeling with [³⁵S]methionine-labeled AHSV/4SP-infected Vero cell lysate as antigen (18). RIPs utilizing preinoculation, prechallenge, and postchallenge horse sera were analyzed by electrophoresis on 10% SDS – PAGE gels with autoradiographic detection.

Horses that received the vaccinia-L2 construct developed small swellings at inoculation sites, suggesting local replication of the construct. Control horses and those receiving parental vaccinia WR strain developed fevers starting on day postchallenge (dpc) 2 or 3 (Fig. 1). On dpc 4 all control and vaccinia WR strain horses were febrile and showing clinical signs typical of the pulmonary form of AHS, including anorexia, depression, and dyspnea (Fig. 1). Fevers averaging 104°F peaked approximately 24 hr prior to death when a sudden drop in temperature was recorded in all control horses. Immediately prior to death, serous exudation from the nares was observed in some horses. Horses either died suddenly or were euthanized in extremis by injection of pentobarbital (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI). On postmortem examination the predominant lesion was severe pulmonary edema, sometimes accompanied by mild pleural effusion. Horses that received the vaccinia-L2 construct did not show any clinical signs associated with AHS during the 21-day observation period (Fig. 1). Titers of AHSV in blood correlated well with fevers in control horses (Fig. 2). All horses that did not receive the vaccinia-L2 construct were viremic starting on dpc 3, with titers of at least 10⁸ TCID₅₀/ml recorded prior to death. Viremia above a limit of detection of 10⁰.₅ TCID₅₀/ml was not present in any of the animals that received the AHSV/4SP isolate. Virology
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FIG. 2. Viremia: Heparinized whole-blood samples were collected from all horses each day after challenge for 14 days. Presented are the viremic titer of each horse. Viremia was quantified by microtitration as previously described (25). Endpoints were determined by the method of Spearman and Karber (26). Control horses received Vero cell lysates, vaccinia-control horses received the construct parent virus (WR), and vaccinia-L2 received a vaccinia construct which expressed the VP2 protein of AHSV-4. Limit of detection in this experiment because of the manipulations required was 0.5 (--)log10/ml.

able ELISA-reactive antibodies in sera from control or immunized horses. However, ELISA-reactive antibody titers increased in immunized horses starting on dpc 7 (Fig. 4). Control and vaccinia WR strain horses died before developing detectable levels of either neutralizing or ELISA antibodies. Protein specificity of the antibody response to immunization and challenge was determined by RIP. A single band was precipitated by sera from immunized horses prior to challenge which comigrates with VP2 at 116 kDa (Fig. 5). Sera from vaccinia-

L2-immunized horses recognized all structural and non-structural AHSV proteins by dpc 21 (Fig. 5).

The results of this study, utilizing a vaccinia construct containing the L2 gene from AHSV-4 and a well-characterized homologous challenge system, indicate that AHSV VP2 is sufficient to induce a protective immune

FIG. 3. Serum neutralization: Assays were performed as previously described (8). Twofold dilutions of sera were incubated with 100 TCID50 of challenge virus (AHSV/4SP) for 1 hr at 37°C, 5% CO2 in 96-well plates after which, Vero cells were added and incubated for 5–6 days. Endpoints were defined by those wells in which 75% of the monolayer remained intact. The neutralizing titers of Horses 1, 9, 14, and 23 (vaccinia-L2 immunized) in this study are compared to titers from horses vaccinated with a MLV vaccine and challenged with the same isolate used in this study. The values presented for the MLV vaccinates are geometric mean titers (n = 4).

FIG. 4. ELISA. A single dilution ELISA was performed as previously described, utilizing plates coated with the homologous challenge virus (AHSV/4SP) (24). Binding of anti-AHSV antibodies was detected using biotin-labeled goat anti-horse IgG (Kirkegaard and Perry Laboratories, Gaithersburg MD) and avidin-alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IN). The absorbance at 495 nm was determined and compared to that derived from hyperimmune horse sera to obtain titers. No ELISA-reactive antibodies were detected in control horses (data not shown).

FIG. 5. Radioimmunoprecipitation of [35S]methionine-labeled AHSV proteins by serum from a horse immunized with vaccinia-L2 and challenged with AHSV/4SP. On the day of challenge (dpc 0) serum from this horse recognizes only VP2 while 3 weeks postchallenge most AHSV proteins are precipitated. Immunoprecipitates by serum from naive (control) and hyperimmunized horses are included in the two right-hand lanes.
response in horses (22). Horses that received the vaccinia-L2 construct were fully protected against disease, showing no clinical signs or viremia (Figs. 1 and 2). In contrast, all control horses succumbed to challenge with clinical signs, lesions, and viremia characteristic of the acute pulmonary form of AHS. Increasing neutralizing and ELISA antibody titers, along with recognition of the full complement of AHSV proteins by RIP, suggest that at least limited replication of the virus occurred in immunized horses after challenge (Figs. 3 and 4). This is, however, similar to the response observed in horses which received an AHSV-4 MLV vaccine and suggests that the level of protection conferred by the vaccinia-L2 construct is similar to that of existing vaccines (Fig. 3 and W. Laegreid, unpublished data). Horses that received the vaccinia-L2 construct did not produce ELISA-reactive antibodies to AHSV prior to challenge (Fig. 4). Thus, it is likely that existing ELISA techniques may be used for serologic distinction between vaccinated and naturally infected horses, a feature important to facilitate international movement of horses.

These results clearly indicate AHSV VP2 is sufficient to induce protection against homologous challenge with highly virulent AHSV. Protection is presumably mediated by neutralizing antibody although other mechanisms such as ADCC or other cell-mediated responses cannot be ruled out by this experiment. The potential efficacy of subunit vaccines based on AHSV VP2 alone is established by these studies.

REFERENCES