

## A Vaccinia Virus-Vectored Hantaan Virus Vaccine Protects Hamsters from Challenge with Hantaan and Seoul Viruses but Not Puumala Virus

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**To investigate the ability of a vaccinia virus-vectored vaccine expressing the M and the S segments of Hantaan (HTN) virus (C. S. Schmaljohn, S. E. Hasty, and J. M. Dalrymple, *Vaccine* 10:10-13, 1992) to elicit a protective immune response against other hantaviruses, we vaccinated hamsters with the recombinant vaccine and challenged them with HTN, Seoul (SEO), or Puumala (PUU) virus. Neutralizing antibodies to HTN virus were found in all vaccinated hamsters both before and after challenge. Neutralizing antibody titers to SEO virus were present at low levels or were undetectable after two immunizations with the vaccine but were positive in all vaccinated hamsters after challenge with SEO virus and were also positive in control animals that were not challenged. Neutralizing antibodies to PUU virus were observed only in hamsters previously challenged with PUU virus. To assay for virus in the blood and tissues of the hamsters, we developed a nested reverse transcriptase (RT)-PCR with cross-reactive outer primers and serotype-specific inner primers. The RT-PCR specifically detected as little as 1 PFU of virus in serum containing high-titer neutralizing antibodies and was more sensitive than immunofluorescent antibody staining for detecting virus in lung and kidney specimens of infected hamsters. By using the RT-PCR, we found that vaccinated hamsters, challenged with HTN or SEO virus, neither were viremic nor had evidence of virus in their lungs or kidneys. In contrast, vaccinated hamsters challenged with PUU virus were viremic and had PUU virus-specific nucleic acid in their organs.**

Hemorrhagic fever with renal syndrome (HFRS) is caused by viruses in the genus *Hantavirus*, family *Bunyaviridae*. Approximately 200,000 cases of HFRS are reported annually, about half of which occur in China (12). At least three HFRS-causing hantaviruses have been described: Hantaan (HTN) virus, the cause of Korean hemorrhagic fever in Korea and epidemic hemorrhagic fever elsewhere in Asia; Puumala (PUU) virus, the cause of nephropathia epidemica, found in Europe west of the Ural mountains; and Seoul (SEO) virus, the cause of a moderately severe form of HFRS, potentially found wherever urban rat populations are abundant. Fatality rates associated with infection by HTN, SEO, and PUU viruses, respectively, are about 10, 1, and 0.1% (12, 14). Another distinct hantavirus, Dobrava virus, is also believed to cause a severe form of HFRS in the Balkans (5). Hantaviruses are maintained in nature in persistently infected rodents and are transmitted to humans as infectious aerosols of the rodents' urine, feces, or saliva, or on occasion by bite (8, 14). The principal rodent hosts for HTN, SEO, PUU, and Dobrava viruses, respectively, are *Apodemus agrarius*, *Rattus norvegicus*, *Clethrionomys glareolus*, and *Apodemus flavicollis* (5, 6, 10, 16).

Although a number of inactivated virus vaccines for HFRS have been developed (13, 25-27, 31), none have yet gained general acceptance. We developed a vaccinia virus-vectored vaccine for HFRS that expresses the M and S segments of HTN virus by inserting both genes into a plasmid transfer vector, in a head-to-head configuration, with M under control of the vaccinia virus 75,000-molecular-weight promoter (7.5K promoter) and S under control of the vaccinia virus 11K pro-

motor. Homologous recombination between the plasmid vector and the DNA of the Connaught human vaccine strain of vaccinia virus resulted in insertion of M and S into the thymidine kinase gene of vaccinia virus (22). This vaccine was tested in preclinical and phase I clinical studies and elicited neutralizing antibodies to HTN virus (18a, 20, 21). To examine the potential of this vaccine to protect against HFRS caused by other hantavirus serotypes, we vaccinated hamsters with the recombinant vaccine and compared antibody responses, the incidence of viremia, and the accumulation of virus in target organs after challenge with HTN, SEO, or PUU virus. To improve the animal model used for assaying protection (20), we developed a specific and sensitive nested reverse transcriptase (RT)-PCR to detect virus in the blood and tissues of infected hamsters.

### MATERIALS AND METHODS

**Viruses, cells, and media.** HTN virus, strain 76-118 (15); SEO virus, strain 80-39 (10); and PUU virus, strain K27 (28), were propagated in Vero E6 cells (Vero C1008; ATCC CRL 1586), which were maintained in Eagle's minimal essential medium with Earle's salts containing 10% fetal bovine serum and antibiotics as described previously (23).

**Hamster challenge model.** In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The use of outbred Syrian hamsters as a challenge model for HTN virus has been described previously (20). To prevent cross-contamination of hamsters, individual groups of hamsters were housed in filter-top cages which were contained in a HEPA-filtered laminar flow unit. Briefly, hamsters were infected intramuscularly with approximately 1,000 PFU of each virus, and the presence of viral antigens or nucleic acids was assessed, as indicated below, at various times after infection. To adapt PUU virus for use in the hamster model, cell culture-grown virus was passaged three times in suckling hamster brains. Briefly, 1-day-old suckling

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hamsters were infected intracerebrally, and brain tissue was collected approximately 2 weeks after infection. A 10% brain cell suspension of third-passage material was used as the inoculum for subsequent experiments.

**Immunization and tissue and RNA sample preparation.** Female outbred LAK Syrian golden hamsters (6 to 8 weeks old) were vaccinated intramuscularly with 0.2 ml of approximately  $10^8$  PFU of the recombinant vaccine (21). For challenge studies, the hamsters were infected intramuscularly with 1,000 PFU of each virus. The hamsters were bled from the retro-orbital sinus on the days indicated, and serum was separated by centrifugation and stored at  $-70^{\circ}\text{C}$  until assayed. Lung and kidney tissues were collected on the days indicated and stored at  $-70^{\circ}\text{C}$  or embedded in OCT freezing medium (Miles, Inc.). For RT-PCR, single-cell suspensions were prepared by grinding tissues in Eagle's minimal essential medium with Earle's salts with sterile glass tissue grinders. Ten volumes of Trizol (Gibco BRL) were added to 100  $\mu\text{l}$  of blood or to 100  $\mu\text{l}$  of 10% lung and kidney suspensions, and total RNA was extracted according to the manufacturer's directions. For the immunofluorescent antibody test (IFAT), 5  $\mu\text{m}$  frozen sections of lung and kidney tissues were cut with a cryostat. Tissue sections were placed on Probe-on-plus positively charged glass slides (Fisher Scientific), fixed in cold acetone for 10 min, and then air dried.

**IFAT.** Tissue sections were incubated with fluorescein isothiocyanate-conjugated convalescent HFRS patients' sera for 30 min at  $37^{\circ}\text{C}$  and then washed twice with phosphate-buffered saline for 3 min and once with distilled water for 30 s. After the samples were air dried, coverslips were mounted with buffered glycerol and observed with a fluorescence microscope.

**Plaque reduction neutralization assay.** Neutralization assays were performed essentially as described previously (11). Briefly, antibodies were diluted in growth medium (Eagle's minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum, 8 mM glutamine, 1% nonessential amino acids [Gibco BRL], penicillin [100 U/ml], and streptomycin [100  $\mu\text{g}/\text{ml}$ ]). Virus was added to each serum dilution to yield a mixture of about 100 PFU/0.2 ml. After incubation at  $4^{\circ}\text{C}$  overnight, 0.2 ml of the virus-antibody mixture was placed onto confluent monolayers of Vero E6 cells, cultured in 6-well plates. The plates were incubated at  $37^{\circ}\text{C}$  in the presence of 5%  $\text{CO}_2$  for 1 h, after which they were overlaid with growth medium containing 0.6% SeaKem agarose (FMC Corp.). To visualize plaques, a second overlay identical to the first, but with 5% fetal bovine serum and neutral red stain (Gibco BRL), was added. The HTN and SEO virus assays were stained 7 days after infection, and the PUU virus assay was stained 12 days after infection. Plaques were observed 1 to 2 days after staining. All neutralization titers are expressed as the reciprocal of the highest serum dilution resulting in at least an 80% reduction in the number of plaques observed in medium controls.

**RT-PCR.** Primers for RT-PCR will be described below. Total RNA extracted from tissue suspensions or from 100  $\mu\text{l}$  of blood was resuspended in 16  $\mu\text{l}$  of water and was mixed with 1  $\mu\text{l}$  (1 pmol) of primer MOF103, heat denatured at  $75^{\circ}\text{C}$  for 10 min, and then allowed to reanneal for 2 min at  $40^{\circ}\text{C}$ . After annealing, 1  $\mu\text{l}$  (10 U) of placental ribonuclease inhibitor, 2  $\mu\text{l}$  of 25 mM deoxynucleoside triphosphate mixture, 6  $\mu\text{l}$  of  $5\times$  SuperScript reaction buffer (Gibco BRL), 3  $\mu\text{l}$  of 0.1 M dithiothreitol, and 1  $\mu\text{l}$  (200 U) of SuperScript RT (Gibco BRL) were added. Reaction mixtures were incubated for 1 h at  $40^{\circ}\text{C}$  and heated for 3 min at  $95^{\circ}\text{C}$ , after which 30  $\mu\text{l}$  of first-strand cDNA was mixed with 7  $\mu\text{l}$  of  $10\times$  PCR buffer, 5  $\mu\text{l}$  of 25 mM  $\text{MgCl}_2$ , 1  $\mu\text{l}$  (100 pmol) each of primers MOF103 and MOR204, 55.5  $\mu\text{l}$  of distilled water, and 0.5  $\mu\text{l}$  (2.5 U) of *Taq* DNA polymerase. Thirty-five thermo-cycles, each consisting of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $50^{\circ}\text{C}$ , and 2 min at  $72^{\circ}\text{C}$ , were performed. For nested PCR, the annealing temperature was increased to  $55^{\circ}\text{C}$  in each cycle. For studies to assess the sensitivity of the RT-PCR, serial dilutions of known quantities of viruses were made in medium containing immune serum before RNA extraction and RT-PCR. The virus and antiserum mixtures were incubated overnight at  $4^{\circ}\text{C}$ , after which total RNA was extracted as described above. Amplified DNA was electrophoresed in 1.2% agarose gels, stained with ethidium bromide, and visualized on a short-wave UV light box. Images were recorded with an Eagle Eye II Still Video system (Stratagene) and saved in TIF format.

## RESULTS

**RT-PCR.** The strategy used for nested RT-PCR amplification is shown in Fig. 1A. The outer G1-specific primers, MOF103 and MOR204, were designed by comparing nucleotide sequences conserved among a number of hantaviruses (Fig. 1B). The inner primers were designed to be specific for sequences of HTN, SEO, or PUU virus. To test the specificity of the serotype-specific primers, we performed RT-PCR with RNA from homologous and heterologous viruses. We found that the HTN, SEO, and PUU virus-specific primers amplified only homologous viruses (Fig. 2A). To test the sensitivity of the RT-PCR, known quantities of HTN or PUU virus were diluted in immune human or hamster sera, total RNA was extracted, and nested RT-PCR was performed. The IFAT titers of the

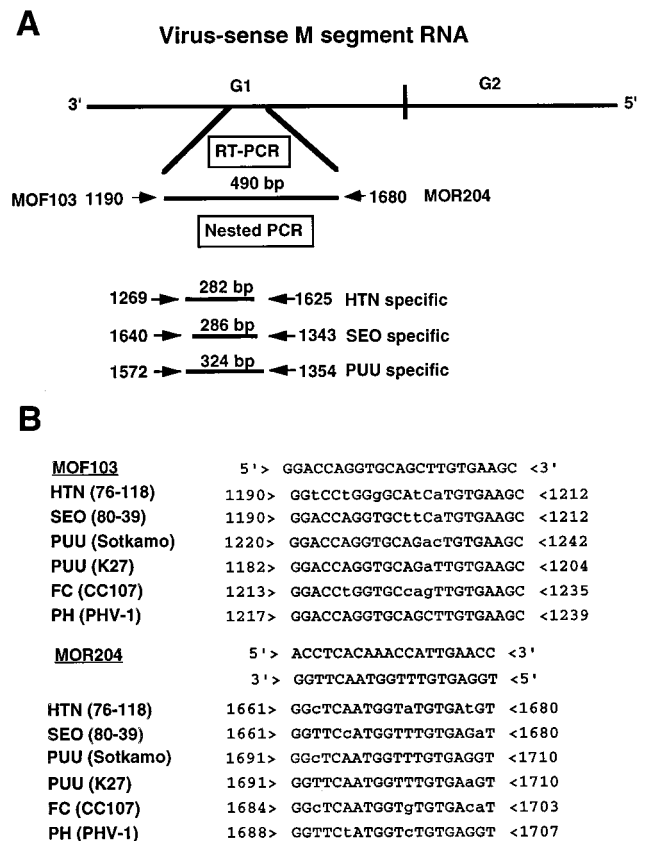


FIG. 1. Strategy for nested RT-PCR. (A) A 490-bp region of the G1 gene of each virus was reverse transcribed and amplified by using outer primers MOF103 and MOR204. Inner primers, specific for HTN, SEO, or PUU virus, were used for nested PCR. (B) The cross-reactive outer primers, MOF103 and MOR204, were designed by comparing the sequences shown for a number of different hantaviruses. The numbers shown correspond to sequences with respect to the 5' terminus of complementary-sense RNA of HTN virus, strain 76-118 (24); SEO virus, strain 80-39 (1); PUU virus, strains K27 (30) and Sotkamo (29); FC virus, strain CC107 (18); and PH virus, strain PHV-1 (17).

human and hamster sera, respectively, were 1:4,000 and 1:1,000, and the neutralizing titers were 1:320 and 1:160. In both experiments, we were able to detect as little as 1 PFU of input HTN or PUU virus by the nested RT-PCR (Fig. 2B and C). Negative controls, consisting of immune sera with no added virus, yielded no PCR products. Controls consisting of virus diluted in normal serum yielded results identical to those shown.

**Evaluation of the hamster infection model for SEO and PUU viruses.** We previously reported that HTN viral antigen could be detected in infected hamsters' lungs and kidneys at 28 days after infection with 1,000 PFU of virus (20). To determine if a similar model of infection could be used with SEO and PUU viruses, we infected hamsters with 1,000 PFU of each virus and then bled the animals and harvested tissues at various times after infection. Because initial studies with PUU virus (K27) revealed no viral antigen in the lungs or kidneys of hamsters infected with serial 10-fold dilutions of virus from 0.05 to  $5 \times 10^5$  PFU (data not shown), we attempted to select for virus exhibiting more vigorous growth in hamsters by passaging the cell culture-derived PUU virus three times in suckling hamster brains. The hamster-adapted PUU virus and SEO virus were then assayed in hamsters to determine the optimal time for detecting virus in the lungs and kidneys. Sera from all

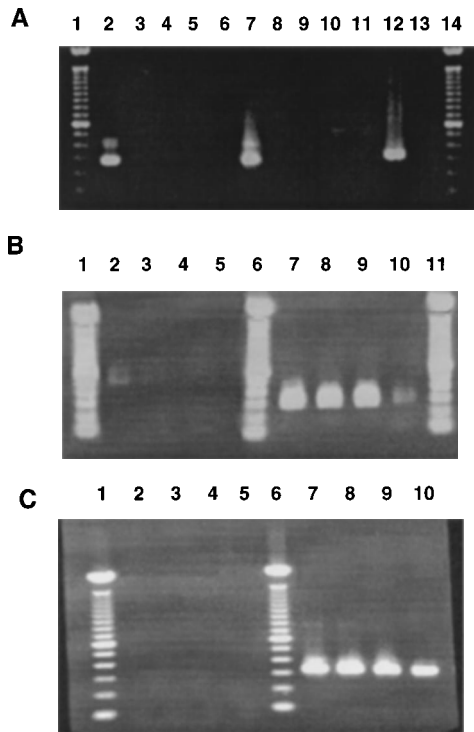


FIG. 2. Specificity and sensitivity of the nested RT-PCR. Agarose gel electrophoresis of PCR products was performed in 1.2% agarose. (A) PCR products are shown in comparison to 100-bp size markers (lanes 1 and 14). Shown are nested RT-PCR products obtained from HTN virus RNA amplified by using HTN (lane 2), SEO (lane 3), or PUU (lane 4) virus-specific primers; nested RT-PCR products obtained from SEO virus RNA and HTN (lane 6), SEO (lane 7), or PUU (lane 8) virus-specific primers; and nested RT-PCR products obtained from PUU virus RNA and HTN (lane 10), SEO (lane 11), or PUU (lane 12) virus-specific primers. Controls were performed with no added templates and with HTN (lane 5), SEO (lane 9), or PUU (lane 13) virus-specific primers. (B and C) PCR products are shown in comparison to 100-bp size markers (lanes 1, 6, and 11). HTN virus (B) or PUU virus (C) was incubated with immune serum, and RNA was extracted and used for RT-PCR. Products shown are from RT-PCR performed with RNA extracted from 1,000 PFU (lane 2), 100 PFU (lane 3), 10 PFU (lane 4), or 1 PFU (lane 5) of input HTN or PUU virus and outer generic primers MOF103 and MOR204. Lanes 7 to 10 display nested PCR products generated with HTN or PUU virus-specific primers amplified from the first-round products.

hamsters infected with 1,000 PFU of PUU or SEO virus were found to react strongly with homologous antigens in an IFAT, indicating that the animals were infected (data not shown). Only one animal in each of the three groups infected with PUU virus had antigen detectable by IFAT in their lungs and/or kidneys at days 14, 21, and 28 after infection (Table 1). However, RT-PCR, which was used only to examine tissues collected at 21 and 28 days after infection, demonstrated the presence of PUU virus nucleic acid in all of the tissues (Table 1). Hamsters infected with SEO virus did not have IFAT-detectable antigen in their lungs or kidneys until 35 days after infection, and even then, fluorescence was minimal (Table 1). All tissues, however, were positive for SEO virus nucleic acid at 35 days after infection (Table 1). Although tissues of these hamsters were not examined for SEO virus nucleic acid at earlier time points, in other experiments (e.g., the results discussed below) we found that SEO virus nucleic acid could also be detected by the nested RT-PCR at 28 days after infection.

**Viremia in vaccinated and control hamsters infected with HTN, SEO, or PUU virus.** We used RT-PCR to detect virus in the blood of experimentally infected hamsters over 60 days.

Viremia was not observed with any of the three viruses at 4 days after infection; however, by 8 days some hamsters in each group were viremic (Table 2). In other, similar experiments, viremia was also detected 7 days after infection (e.g., see Table 3). For some hamsters in each group (e.g., hamsters H-1, S-3, and P-5 [Table 2]), viremia could not be detected at either 14 or 21 days after infection but was observed again at later times (Table 2). These results suggest that the viremia detected by RT-PCR was near its lower limits of sensitivity, i.e., less than 1 PFU/100  $\mu$ l of blood, and/or that viremia was increased at later times because of additional virus replication in target sites.

To assess the ability of the vaccinia virus-vectored vaccine to prevent viremia, vaccinated hamsters were challenged with HTN, SEO, or PUU virus; the RT-PCR was used to assay virus in the blood at 1, 2, 3, and 4 weeks after challenge. None of the hamsters vaccinated and subsequently challenged with HTN or SEO virus had detectable viremia at any time (Table 3). In contrast, 7 of the 13 hamsters challenged with PUU virus were viremic at one or more of the time points tested (Tables 3 and 4). As with the nonvaccinated hamsters, at least one of these hamsters (P-13) had detectable viremia at early and late time points but not at an intermediate time (Table 4).

**Neutralizing antibody responses of vaccinated hamsters challenged with HTN, SEO, or PUU virus.** Hamsters were immunized two times, 30 days apart, with the vaccinia virus-vectored HTN vaccine and were challenged 30 days after the second immunization with HTN, SEO, or PUU virus or were mock challenged. The hamsters were individually tagged, and changes in antibody titers were assessed. All of the hamsters had neutralizing antibodies to HTN virus before challenge (D-0, Fig. 3). Four weeks after challenge with HTN virus, 9 of 13 hamsters had neutralizing titers which were the same as or lower than those before challenge, and titers of the remaining

TABLE 1. Detection of virus in the lungs and kidneys of hamsters infected with SEO or PUU virus

| Virus and hamster | Day killed | Result for <sup>a</sup> : |     |                |     |
|-------------------|------------|---------------------------|-----|----------------|-----|
|                   |            | Lungs                     |     | Kidneys        |     |
|                   |            | IFAT                      | PCR | IFAT           | PCR |
| <b>PUU</b>        |            |                           |     |                |     |
| P-1               | 14         | + <sup>b</sup>            | NT  | + <sup>b</sup> | NT  |
| P-2               | 14         | -                         | NT  | -              | NT  |
| P-3               | 14         | -                         | NT  | -              | NT  |
| P-4               | 21         | + <sup>b</sup>            | +   | + <sup>b</sup> | +   |
| P-5               | 21         | + <sup>b</sup>            | +   | + <sup>b</sup> | +   |
| P-6               | 21         | + <sup>b</sup>            | +   | + <sup>b</sup> | +   |
| P-7               | 28         | -                         | +   | + <sup>b</sup> | +   |
| P-8               | 28         | -                         | +   | -              | +   |
| P-9               | 28         | -                         | +   | + <sup>b</sup> | +   |
| P-10              | 28         | -                         | +   | -              | +   |
| <b>SEO</b>        |            |                           |     |                |     |
| S-1               | 28         | -                         | NT  | -              | NT  |
| S-2               | 28         | -                         | NT  | -              | NT  |
| S-3               | 28         | -                         | NT  | -              | NT  |
| S-4               | 28         | -                         | NT  | -              | NT  |
| S-5               | 28         | -                         | NT  | -              | NT  |
| S-6               | 35         | -                         | +   | -              | +   |
| S-7               | 35         | -                         | +   | +w             | +   |
| S-8               | 35         | -                         | +   | +w             | +   |
| S-9               | 35         | +w                        | +   | +              | +   |
| S-10              | 35         | +w                        | +   | +w             | +   |

<sup>a</sup> NT, not tested; +w, weakly positive.

<sup>b</sup> One to three percent of cells were positive.

TABLE 2. Viremia in unvaccinated hamsters infected with HTN, SEO, or PUU virus

| Virus and hamster | Result at day after infection <sup>a</sup> : |   |    |    |    |    |    |
|-------------------|--|---|----|----|----|----|----|
|                   | 4  | 8 | 14 | 21 | 28 | 45 | 60 |
| <b>HTN</b>        |  |   |    |    |    |    |    |
| H-1               | –  | – | –  | +  |    | k  | k  |
| H-2               | –  | + | –  | +  | +  | k  | k  |
| H-3               | –  | + | –  | +  | –  | k  | k  |
| H-4               | –  | + | +  | +  | +  | –  | k  |
| H-5               | –  | + | +  | –  | –  | +  | k  |
| H-6               | –  | + | +  | +  | +  | +  | k  |
| H-7               | –  | + | –  | –  | –  | –  | –  |
| H-8               | –  | + | –  | +  | –  | –  | –  |
| H-9               | –  | + | +  | +  | –  | –  | –  |
| H-10              | –  | – | –  | +  | +  | +  | +  |
| <b>SEO</b>        |  |   |    |    |    |    |    |
| S-1               | –  | + | +  | +  | –  | k  | k  |
| S-2               | –  | + | +  | +  | +  | k  | k  |
| S-3               | –  | + | +  | –  | +  | k  | k  |
| S-4               | –  | + | +  | +  | –  | –  | k  |
| S-5               | –  | + | –  | –  | –  | –  | k  |
| S-6               | –  | + | +  | –  | –  | –  | k  |
| S-7               | –  | + | NT | +  | –  | –  | –  |
| S-8               | –  | – | NT | –  | +  | –  | –  |
| S-9               | –  | + | NT | –  | +  | –  | –  |
| S-10              | –  | + | NT | –  | –  | –  | –  |
| <b>PUU</b>        |  |   |    |    |    |    |    |
| P-1               | –  | + | –  | +  | –  | k  | k  |
| P-2               | –  | – | –  | –  | –  | k  | k  |
| P-3               | –  | – | +  | –  | –  | k  | k  |
| P-4               | –  | + | –  | –  | –  | –  | k  |
| P-5               | –  | – | –  | –  | +  | –  | k  |
| P-6               | –  | – | –  | –  | –  | –  | k  |
| P-7               | –  | + | –  | +  | –  | –  | –  |
| P-8               | –  | – | –  | –  | –  | –  | –  |
| P-9               | –  | + | +  | +  | –  | –  | –  |
| P-10              | –  | + | –  | –  | –  | –  | –  |

<sup>a</sup> k, hamsters were killed to assay for virus in lungs and in kidneys; NT, not tested.

4 animals included 2 that were twofold higher and 2 that rose from 1:10 to 1:40. Plaque reduction neutralization test (PRNT) titers of the HTN virus-challenged hamsters to SEO virus were the same or twofold different before and after challenge, except for five animals, which had titers of <10 before challenge and 1:16 or 1:32 after challenge. Similar results were seen for the animals that were mock challenged; thus, these titer rises were probably unrelated to infection with HTN virus (Fig. 3). None of the HTN virus-challenged hamsters had PRNT titers to PUU virus greater than 1:10 either before or after challenge. Titers of the mock-infected animals to HTN virus before and after challenge were the same, were twofold higher or lower, or, in one case, were fourfold lower.

Approximately half of the animals (24 of 50) had no detectable titers to SEO virus before challenge, and of those that did, the highest titer observed was 1:40 (Fig. 3). In hamsters challenged with SEO virus, titers to HTN virus were the same or lower for all animals, except for four animals that had twofold rises in titer. None of the PRNT titers of the SEO virus-challenged hamsters to SEO virus were greater than 1:40 before challenge or 1:80 after challenge (Fig. 3). Of these hamsters, three had the same or twofold-different titers pre- and postchallenge, five had undetectable titers before challenge but after challenge had titers of 1:16 or 1:32, and the rest had

TABLE 3. Viremia in vaccinated and control hamsters challenged with HTN, SEO, or PUU virus

| Challenge virus | Vaccine <sup>a</sup> | No. of viremic hamsters/total no. at week after challenge: |                  |      |      |
|-----------------|----------------------|--|------------------|------|------|
|                 |                      | 1 <sup>b</sup>   | 2 <sup>c</sup>   | 3    | 4    |
| HTN             | +                    | 0/13   | 0/8              | 0/13 | 0/13 |
|                 | –                    | 8/10   | 4/10             | 8/10 | 4/10 |
| SEO             | +                    | 0/13   | 0/7 <sup>d</sup> | 0/12 | 0/12 |
|                 | –                    | 9/10   | 5/6 <sup>e</sup> | 4/10 | 5/10 |
| PUU             | +                    | 2/13   | 3/8              | 4/13 | 3/13 |
|                 | –                    | 5/10   | 2/10             | 3/10 | 1/10 |

<sup>a</sup> Results of controls include those shown in Table 1.

<sup>b</sup> Vaccinated and control hamsters were bled 7 or 8 days after infection, respectively.

<sup>c</sup> Five hamsters from each vaccinated group were not bled 2 weeks after challenge.

<sup>d</sup> One hamster died of unknown causes.

<sup>e</sup> Four hamsters were not tested.

fourfold titer rises: i.e., from 1:10 up to 1:40 or from 1:20 up to 1:80. As indicated above, these results are similar to those observed for the mock-infected hamsters and probably indicate that SEO virus infection did not occur. It is possible, however, that limited or abortive SEO infections which we were unable to detect by RT-PCR did transpire. No rise in titers to PUU virus was observed for the hamsters challenged with SEO virus.

Only one hamster had a neutralizing antibody titer greater than 1:10 to PUU virus before challenge (Fig. 3, PUU challenge group). This animal, which had a 1:40 titer before challenge, also had a 1:40 titer after challenge. All of the other hamsters had fourfold or greater increases in antibody titer after challenge, which suggested that they were not protected from infection with PUU virus.

**Detection of viral antigen or RNA in the tissues of vaccinated and control hamsters challenged with HTN, SEO, or PUU virus.** Hamsters were vaccinated with the recombinant vaccinia virus-vectored vaccine and challenged with HTN, SEO, or PUU virus, as described above. None of the vaccinated hamsters challenged with HTN virus had evidence of viral antigen or RNA in their organs, while 9 of 10 nonvaccinated hamsters had HTN virus in their lungs and kidneys,

TABLE 4. Viremia in vaccinated hamsters challenged with PUU virus

| Hamster | Result at day after infection: |                 |    |    |
|---------|--------------------------------|-----------------|----|----|
|         | 7                              | 14              | 21 | 28 |
| P-1     | –                              | NT <sup>a</sup> | –  | –  |
| P-2     | +                              | NT              | –  | –  |
| P-3     | –                              | NT              | +  | +  |
| P-4     | –                              | NT              | +  | +  |
| P-5     | –                              | NT              | +  | –  |
| P-6     | –                              | –               | –  | –  |
| P-7     | –                              | –               | –  | –  |
| P-8     | –                              | –               | –  | –  |
| P-9     | –                              | –               | –  | –  |
| P-10    | –                              | +               | –  | –  |
| P-11    | –                              | +               | +  | –  |
| P-12    | –                              | –               | –  | –  |
| P-13    | +                              | +               | –  | +  |

<sup>a</sup> NT, not tested.

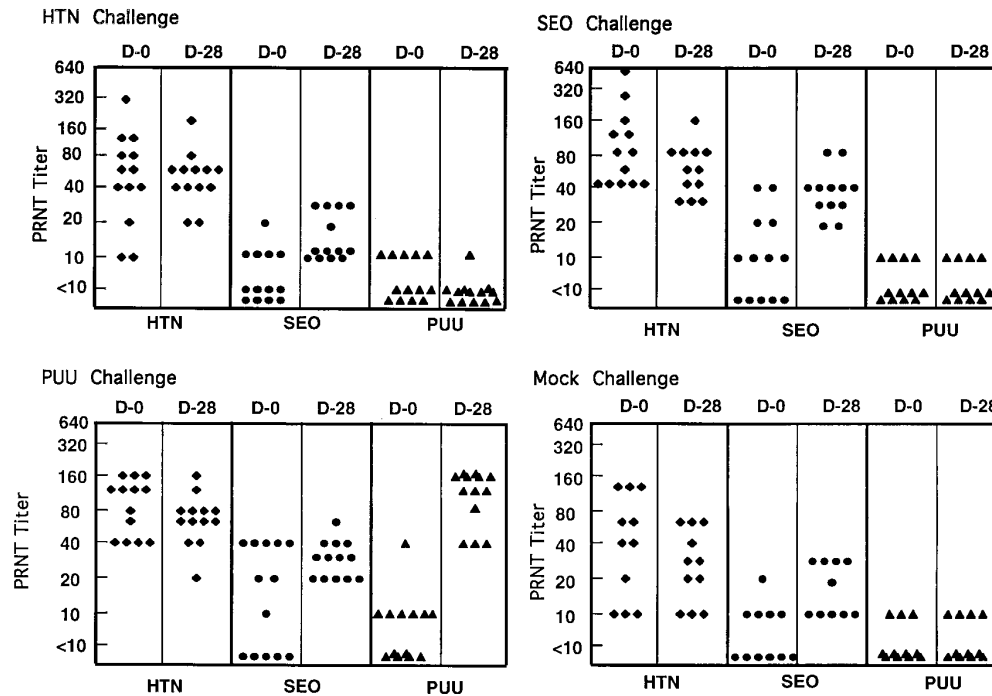


FIG. 3. Plaque-reduction neutralization titers (80%) of individual hamsters vaccinated with the vaccinia virus-vectored HTN vaccine and challenged with HTN, SEO, or PUU virus or mock challenged. Hamsters received two immunizations, 30 days apart, with the recombinant vaccine. Hamsters were bled 30 days after the second vaccination, just before challenge (D-0) and 28 days after challenge. Titers are expressed as the reciprocal of the highest dilution causing >80% reduction in the number of plaques compared with that for controls not incubated with immune serum.

according to both assays. Likewise, none of the vaccinated hamsters that were challenged with SEO virus had detectable antigen or RNA in their tissues, while all nonvaccinated controls displayed SEO-specific nucleic acid in both their lungs and kidneys (Table 5). In contrast, most of the vaccinated and control animals that were challenged with PUU virus had virus-specific RNA in their lungs and/or kidneys (Table 5). These data correlate with the PRNT results and indicate that

the vaccinia virus-vectored HTN virus vaccine did not protect the hamsters from infection with PUU virus.

DISCUSSION

HFRS is a serious and sometimes fatal disease which is characterized by abrupt onset of fever accompanied by various degrees of kidney involvement and hemorrhagic manifestations, potentially including shock, and which may end in death (9). Although a number of traditional, inactivated vaccines, including a rodent brain-derived vaccine (13) and cell culture-derived vaccines (25, 26), have been developed and tested in humans, none have yet been proven to be effective against HFRS in controlled clinical trials. We have developed a recombinant vaccinia virus vaccine that expresses the M and S segments of HTN virus (22). This vaccine induces neutralizing antibodies to HTN virus in both preclinical and phase I clinical studies (18a, 20, 21) and prevented antigen accumulation in the lungs of hamsters challenged with virulent HTN virus (20). In this study, we sought to determine if our vaccine would induce protective immunity in hamsters to hantaviruses serologically distinct from HTN virus and known to cause HFRS. For these experiments, we used a hamster challenge model. This model is based on our finding that animals infected by a peripheral route with HTN virus will display viral antigen in their organs, particularly the lungs and kidneys (20). While we were able to use IFAT with HTN virus to detect viral antigens in the target organs, because SEO and PUU virus antigens were more difficult to detect, we developed and used a more sensitive RT-PCR method for measuring protection.

Our results indicate that the vaccine can prevent viremia and accumulation of virus in target organs of animals challenged with SEO virus but cannot uniformly prevent these manifesta-

TABLE 5. Detection of viral antigen or nucleic acid in the lungs and kidneys of hamsters after challenge with HTN, SEO, or PUU virus

| Challenge virus <sup>a</sup> | Vaccine | Result for <sup>b</sup> : |       |         |       |
|------------------------------|---------|---------------------------|-------|---------|-------|
|                              |         | Lungs                     |       | Kidneys |       |
|                              |         | IFAT                      | PCR   | IFAT    | PCR   |
| HTN                          | +       | 0/13                      | 0/13  | 0/13    | 0/13  |
|                              | -       | 9/10 <sup>c</sup>         | 9/10  | 9/10    | 9/10  |
| SEO <sup>d</sup>             | +       | 0/13                      | 0/13  | 0/13    | 0/13  |
|                              | -       | 0/10                      | 10/10 | 0/10    | 10/10 |
| PUU                          | +       | 0/13                      | 11/13 | 0/13    | 5/13  |
|                              | -       | 0/10                      | 7/10  | 0/10    | 7/10  |
| None (mock infected)         | +       | 0/13                      | 0/13  | 0/13    | 0/13  |

<sup>a</sup> All vaccinated, challenged hamsters were killed 4 weeks after challenge. Of the 10 nonvaccinated controls, 3 were killed on day 29, 3 were killed on day 45, and four were killed on day 60 after infection.

<sup>b</sup> Data are number of hamsters with detectable viral antigen or RNA/total number of hamsters.

<sup>c</sup> The hamster testing negative belonged to the group killed 60 days after infection.

<sup>d</sup> Results shown include those of one hamster that died at day 14.

tions of infection in animals challenged with PUU virus. There was a hint of cross-protection in a single hamster, which displayed a neutralizing titer to PUU virus before challenge and did not have a rise in titer after challenge with PUU virus. These observations probably correlate with earlier findings indicating that HTN virus is genetically more similar to SEO virus than it is to PUU virus. That is, gene sequence homologies of HTN to SEO viruses are approximately 71% for both the M and S segments, and the amino acid sequence homologies are approximately 75% and 82% for the gene products encoded by the M (G1 and G2) and S (nucleocapsid) segments, respectively. Gene sequence homologies of the M and S segments of HTN and PUU viruses are 58 and 56%, respectively, and the amino acid sequence homologies are 53 and 61%, respectively, for the M and S segment gene products.

The genetic characteristics of these viruses are reflected in their antigenic properties, in that HTN and SEO viruses are more cross-reactive than are HTN and PUU viruses in a variety of serological tests. For example, we found that sera from animals experimentally infected with a number of strains of HTN or SEO virus were highly cross-reactive with HTN and SEO antigens by enzyme-linked immunosorbent assay, IFAT, and hemagglutination inhibition assays, although the two viruses could be clearly differentiated by PRNT (7). In contrast, minimal cross-reactivity was observed with antisera raised to HTN and PUU viruses (7). Despite these findings with polyclonal sera, conserved epitopes on the G2 proteins of HTN and PUU viruses have been identified by cross-reactive neutralizing and nonneutralizing monoclonal antibodies raised to HTN virus (2, 7). At least one of the monoclonal antibodies (11E10) was previously shown to passively protect animals from challenge with HTN virus (20), and although not tested against PUU virus challenge, the ability of the monoclonal antibody to neutralize PUU virus suggests that at least some level of cross-protective humoral immunity could be induced by HTN virus antigens.

In addition to such cross-reactive humoral immunity, earlier studies pointed to cross-protective cellular immune responses to hantaviruses in mice. In the first of these studies, cytotoxic T lymphocytes induced by infection of mice with HTN virus or with SEO virus cross-reacted with target cells infected with the heterologous virus (4). In a follow-up study, spleen cells primed by Prospect Hill (PH) or PUU virus were found to cross-react with HTN virus-infected target cells, even though no cross-reactive neutralizing antibodies to HTN virus were observed (3). In this same study, transfer of either T lymphocytes primed by PUU or PH virus or immune serum to PUU or PH virus to nude mice resulted in reduced titers of HTN virus after a subsequent challenge (3). The importance of cellular immune responses to hantaviruses for protection and/or recovery from disease in humans is not known; however, limited T-cell cross-reactivity between HTN and FC viruses was demonstrated with lymphocytes collected from three HTN virus-seropositive individuals (19).

In conclusion, it is clear from the data presented here that hamsters vaccinated with the recombinant vaccinia virus-vectored Hantaan virus vaccine cannot be protected from challenge with PUU virus. However, the hamster is not a disease model but rather is an infectivity model, and no animal is known to develop a disease similar to HFRS after infection with a hantavirus. Thus, despite our findings, cross-reactive cellular or humoral immune responses in humans cannot be ruled out.

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