Pathogenicity and immunogenicity of a mutagen-attenuated Rift Valley fever virus immunogen in pregnant ewes

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SUMMARY

A mutagenized clone of Rift Valley fever virus (RVFV; MV P12) used in inoculation of 3 pregnant ewes was immunogenic, nonpathogenic, and nonabortogenic. In contrast, inoculation of a matched group of 3 pregnant ewes with parent RVFV induced clinical disease and abortions. Ewes given MV P12 delivered healthy lambs that had RVFV antibody titers of < 1:10 at birth, increasing to ≥ 1:80 after ingestion of colostrum. Ewes inoculated with parent RVFV developed marked viremia, followed by RVFV antibody titers ≥ 1:1,280; ewes inoculated with MV P12 developed low viremia titers and RVFV antibody titers of 1:80 to 1:320. Postpartum challenge exposure of the previously MV P12-inoculated ewes with virulent Zagazig human 501 strain RVFV indicated that the ewes were protected from clinical disease. The RVFV-susceptible female Culex pipiens that fed on the MV P12-inoculated ewes failed to transmit RVFV to hamsters; mosquitoes that fed on the parent RVFV-inoculated ewes became infected and transmitted RVFV to hamsters.

Rift Valley fever (RVF) is an acute, arthropod-borne, viral disease of sheep, cattle, and goats.1,2 Infected animals develop high viremia titers, virtually all pregnant animals abort, and mortality approaches 100% in neonates. Human beings are also susceptible to RVF virus (RVFV) infection via the bite of infected mosquitoes and exposure to aerosols or infected tissues. Human infection is usually a self-limiting, acute febrile illness, sometimes complicated by hemorrhagic fever, encephalitis, and retinal lesions.3 Human beings that develop hemorrhagic syndrome have high mortalities.4

Before the Egyptian epizootic in 1977, RVF was thought to be confined to sub-Saharan Africa. The Egyptian epizootic revealed that RVFV can be spread beyond its traditional range and has the potential for global transmission.5 Because of the zoonotic potential of the virus and the severe economic impact of RVF on domestic livestock production, immunization of domestic animals in enzootic areas, as well as in adjacent areas, is desirable to limit spread of the disease.6 The existing Smithburn mouse-adapted vaccine strain is efficacious, but may cause fetal anomalies and has shown changes in mouse virulence under certain circumstances.7,8

Serial mutagenesis may be an alternative to the conventional approach of serial passage in cell culture or with parent RVFV strain Zagazig human (ZH)-548 resulted in progressive attenuation for mice.9 Furthermore, serial mutagenesis of RVFV strain Zagazig human (ZH)-548 resulted in productive infection in mice.10 The virus had been passaged twice in suckling mouse brain. Virus was propagated once in DBS-103 fetal rhesus lung cells and then in MRC-5 human diploid fibroblast cells grown in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (heated at 56°C for 30 minutes), 1% L-glutamine, and 0.1% gentamicin. For attenuation, RVFV virus was grown in the presence of 5-fluorouracil (5-FU). The method of virus cloning and serial mutageneses has been reported.11

Materials and Methods

Ewes—Six healthy, crossbred ewes in the second third of pregnancy were obtained from a local flock. Ewes were seronegative to RVFV by plaque-reduction neutralization assay.

Viruses—The Egyptian ZH-548 strain of RVFV was isolated from the serum of a human being with nonfatal RVF in 1977. The virus had been passaged twice in suckling mouse brain. Virus was propagated once in DBS-103 fetal rhesus lung cells and then in MRC-5 human diploid fibroblast cells grown in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (heated at 56°C for 30 minutes), 1% L-glutamine, and 0.1% gentamicin. For attenuation, RVFV was grown in the presence of 5-fluorouracil (5-FU). The method of virus cloning and serial mutageneses has been reported.11

Monolayers of MRC-5 cells were inoculated with the uncloned ZH-548 strain of RVFV, adsorbed for 1 hour, and washed repeatedly with growth medium. New growth medium containing 5-FU (200 µg/ml) was added, and cells were inoculated at 37.5°C in a 5% CO2 atmosphere. Nonadsorbed virus was removed 3 to 4 hours after inoculation by replacing the medium with fresh growth medium containing the same concentration of 5-FU. After incubation for an additional 45 to 65 hours, culture supernatants were harvested and titrated by plaque assay in MRC-5 cells. The 2 largest well-separated plaques from each titration were picked, and each was suspended in 0.5 ml of growth medium. Virus from each suspension was grown in MRC-5 cells, using growth medium that lacked 5-FU. Infected cell supernatants were harvested at 48 to 72 hours after inoculation, clar-
TABLE 1—Experimental design: group allocation, treatment, and pregnancy results

<table>
<thead>
<tr>
<th>Sheep No.</th>
<th>Pregnancy results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>× 10⁴ PFU of ZH-548 parent RVFV MV P1</td>
</tr>
<tr>
<td>8</td>
<td>× 10⁴ PFU of ZH-548 parent RVFV MV P1</td>
</tr>
<tr>
<td>11</td>
<td>Aborted term fetus, PID 5</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Delivered viable twin lambs, PID 39</td>
</tr>
<tr>
<td>9</td>
<td>Delivered viable lamb, PID 48</td>
</tr>
<tr>
<td>10</td>
<td>Delivered viable lamb, PID 59</td>
</tr>
</tbody>
</table>

* Challenge inoculated SCC with 1 × 10⁴ PFU of ZH-501 on PID 63.

** tried by low-speed centrifugation, and stored frozen at -70 C. On the basis of its higher titer and/or larger plaque size, 1 of the 2 virus clones was used to initiate another cycle of mutagenesis. The 1st MRC-5 mutagenesis passage of the parent RVFV (MV P1) and a 12th mutagenesis passage of RVFV (MV P12) were used. Because different clones of RVFV are known to vary in their mouse virulence,12 we used the 1st mutagenesis passage as the virulent partner for comparison in sheep, thereby emphasizing the role of subsequent mutagenization on attenuation. The virulence of these viruses for 4- to 6-week-old ICR for histopathologic examination. Tissue specimens also were embedded in a compound,4 frozen at -70 C, sectioned at 5 μm in a freezing microtome, fixed in acetone, and stained by standard direct fluorescent antibody (DFA) staining techniques. Suspensions (10%, v/v) of selected tissues were prepared in HBSS containing antibiotics, homogenized with tissue grinders, clarified by centrifugation at 800 × g for 30 minutes, and stored frozen at -70 C until assayed for RVFV and neutralizing antibody.

** Necropsies were performed on all ewes and lambs. Tissues were fixed in neutral-buffered 10% formalin and were processed for histopathologic examination. Tissue specimens also were fixed in neutral-buffered 10% formalin and were processed for histopathologic examination.

** Viral plaque assay—Viral isolation from specimens was conducted in monolayers of Vero cells established in 24-well, plastic tissue culture plates.5 Serial 10-fold dilutions of each specimen were prepared in HBSS, and 50 μl of each dilution was adsorbed on duplicate cell monolayers for 1 hour at 37 C and was overlaid with 0.5 ml of a mixture of 1 part 1% agarose4 and 1 part 2× Eagle’s basal medium with Earle’s salts, 17 mM HEPES, 8% heat-inactivated bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin sulfate/ml. After incubation for 72 hours at 37 C in a 5% CO₂ atmosphere, each cell monolayer was stained by adding 0.5 ml of a second overlay, identical to the first, but containing 1:9,000 neutral red stain. After an additional 24 hours’ incubation, plaques were counted, and viral titers were calculated.

** Plaque-reduction neutralization test (PRNT)—Serial 4-fold dilutions of sera and CSF were prepared in HBSS. An equal volume of ZH-501 strain of RVFV suspension containing 80 to 100 PFU/ml was added to each specimen dilution. After incubation at 37 C for 1 hour, 50 μl of each dilution was inoculated into each of 2 Vero cell monolayers. The virus-serum mixture was adsorbed for 1 hour at 37 C and was overlaid with 0.5 ml of the agarose-medium mixture used in the viral assay. After 72 hours’ incubation at 37 C in a 5% CO₂ atmosphere, each cell monolayer was inoculated with 0.5 ml of a 2nd agarose overlay containing neutral red dye. Plaques were counted, as described. An 80% reduction in the number of plaques was used as the end point for virus-neutralization titer (PRNT80).

** RVFV enzyme-linked immunosorbent assay (ELISA)—A double-antibody ELISA14 was used to detect RVFV antigen in 10%
tissue homogenates from aborted fetuses. Microtitration plates were read spectrophotometrically at 405 nm, and samples were considered positive if their optical density was greater than the mean background value plus 3 SD.

Mosquito infection studies—Female Culex pipiens from a colony derived from specimens collected in the Sharqiya Governorate of Egypt in 1981 were used. These mosquitoes were susceptible to infection with RVFV. Approximately 50 mosquitoes in a 0.5-L cardboard container, with netting over one end, were applied to the shaved flank area of each sheep at about 24, 48, and 72 hours after the sheep had been inoculated with virus. After the feeding period, engorged mosquitoes were removed, placed in another 0.5-L container, and stored in an incubator maintained at 26 ± 1 C with a 16-hour photophase. An oviposition dish was provided 3 days later. Each group of mosquitoes that fed on the sheep was allowed to refed on anesthetized golden Syrian hamsters 7 to 8 days after the initial blood meal. After this meal, each mosquito was individually triturated in 1 ml of diluent (10% calf serum in medium 199 with Hanks' salts and 0.075% NaHCO3) and was frozen at −70 C until assayed for virus by plaque assay on Vero cell monolayers. A portion of liver from each hamster that died after mosquito feeding was assayed for RVFV. The correlation of mosquito infection with sheep viremia was determined by Spearman's rank correlation.

Results

Clinical response—After inoculation with RVFV, group-A ewes experienced viremia and mild clinical disease with pyrexia (Fig 1), hyperemia of mucous membranes, lethargy, and abortion. Virus titers of 2.4 to 5.6 log10 PFU/ml of serum were detected in all 3 ewes (Fig 2). Neutralizing antibody to RVFV was first detected on PID 4 or 5, and titers increased to ≥ 1:1,280 by PID 14 (Fig 2). By PID 17, all 3 ewes aborted fetuses in varying stages of postmortem decomposition (Table 1).

Group-B ewes inoculated with MV P12 did not become clinically ill or abort, but did experience low and transient viremias. Rift Valley fever virus was not detected in serum samples, but low viremia titers of 0.7 to 1.4 log10 PFU/ml were detected in the plasma fraction of group-B ewes on PID 1. The RVFV-neutralizing antibody was detected at PID 4 to 6 and increased to titers of 1:320 by PID 14 (Fig 3). Group-B ewes delivered healthy, viable lambs on PID 39, 48, and 59. At birth, these lambs had no detectable antibody to RVFV, and virus was not recovered from serum or placental membranes and fluids. All lambs nursed after birth, and by day 5, each lamb had a serum PRNT80 titer of ≥ 1:80 to RVFV. The colostrum of all group-B ewes had PRNT80 titers of ≥ 1:320 at parturition (Table 2). Lambs were euthanatized at 5 to 24 days of age.

The RVFV-neutralizing antibody was detected in CSF collected at necropsy from 3 ewes infected with parent virus (Table 3). Rift Valley fever virus neutralizing an-

![Graph 1](image1.png)

**Fig 1**—Mean rectal temperatures of parent RVFV (MV P1) and mutagenized RVFV (MV P12)-inoculated ewes.

![Graph 2](image2.png)

**Fig 2**—Viremia and antibody titers of parent RVFV (MV P1)-inoculated ewes.

- ewe 3:
- ewe 8; and • = ewe 11.

![Graph 3](image3.png)

**Fig 3**—Viremia and antibody titers of mutagenized RVFV (MV P12)-inoculated ewes. • = ewe 4; — = ewe 9; and △ = ewe 10.

| TABLE 2—Ewe colostral and lamb serum antibody titers |
|--------------|---|---|---|---|
| Specimen     | 4A | 4B | 9A | 10A |
| Colostrum    | 320 | 320 | 1,280 | 320 |
| Parturition   | 10 | 10 | 10 | 10 |
| Postpartum   | 160 | 80 | 80 | 160 |

Blood collected at birth before nursing. Blood collected 5 days after parturition.

Data are expressed as reciprocal of plaque reduction-neutralization (PRNT80 antibody titer). Lamb No identifies ewe and letter designates her respective lamb or lambs.
tible viremia at birth or thereafter, and virus was not detected from cotyledons from ewe 3. The virus was not isolated from tissues of the other abortuses, except from cotyledons from ewe 3. The virus was isolated from all ewes.

Clinical hematologic and serum enzyme analyses—On PID 1, group-A ewes had WBC counts of 3 to $4.5 \times 10^3$ cells/dl. Values returned to within base-line values (7 to $9 \times 10^3$ cells/dl) by PID 4, except for cwe 11 that had a WBC count that decreased to $1.4 \times 10^3$ cells/dl on PID 2 and gradually returned to baseline by PID 10. Absolute lymphopenia coincided with leukopenia. Mean lymphocyte counts decreased from $5.5 \times 10^3$ cells/dl at the time of virus inoculation to $1.1 \times 10^3$ cells/dl at PID 2. Other hematologic values remained within base-line limits. Ewe 11, which aborted on PID 5, had increased AST, γ-glutamyl transferase, and LD values on PID 2 through 10 (Fig 4). Except for a transient increase in serum AST values in ewe 3 and serum creatine kinase values in ewe 8, serum enzymes of ewes 3 and 8 that aborted later did not deviate markedly from their baselines. The increased LD values of ewe 11 were the result of an increased LD-1 fraction. In contrast, after MV P12 virus inoculation, hematologic and serum enzyme values for all group-B ewes remained within base-line limits.

Virus isolation—The RVFV was isolated from all ewes. At least 1 isolate from each ewe and abortus was identified as RVFV by RVFV fluorescent-antibody staining. Viremia was not detected beyond PID 4. Serum and plasma virus titers were usually equal; however, on several occasions, low viremia titers (<2.5 log$_{10}$ PFU/ml) were detected in the plasma fraction of EDTA-treated whole blood, but could not be recovered from serum collected at the same blood collection. Virus also was isolated from cellular blood fractions, but only when RVFV was detected in serum. Virus was not recovered from oral or rectal swab samples collected through PID 10 or from tissues collected from the ewes at necropsy.

High virus titers were present in organs and whole blood collected from the fetus aborted by ewe 11 (Table 4). Virus was not isolated from tissues of the other abortuses, except from cotyledons from ewe 3. The ELISA seemed to have greater sensitivity in detecting RVFV antigen in 10% tissue homogenates than did DFA staining of sectioned tissues.

Assays of identical tissues from lambs born to the group-B ewes revealed no evidence of viral antigen by DFA or ELISA. As described, lambs from group-B ewes had no detectable viremia at birth or thereafter, and virus was not isolated from their tissues at necropsy.

Of 100 mosquitoes that fed on the MV P1-inoculated ewes, 19 became infected, whereas none of 32 mosquitoes

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**Table 3—Comparison of neutralizing antibody titers in serum and CSF collected at necropsy**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Group-A ewes (MV P1)</th>
<th>Group-B ewes (MV P12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>3 8 11</td>
<td>4 9 10</td>
</tr>
<tr>
<td>CSF</td>
<td>80 20</td>
<td>20 80</td>
</tr>
<tr>
<td>Serum/CSF ratio</td>
<td>64 64 32</td>
<td>256 64</td>
</tr>
</tbody>
</table>

Data are expressed as reciprocal of plaque-reduction neutralization 100% antibody titer.
feeding on the MV P12-inoculated ewes became infected. The group of mosquitoes, all of which were infected, which fed on ewe 11 at 48 hours and the group of 16 mosquitoes, only one of which was infected, which fed on ewe 8 at 72 hours transmitted virus to hamsters. Viral titers of infected mosquitoes were 10^4 to 10^5 (mean = 10^{1.2}) PFU/mosquito. There was significant correlation (P < 0.01; r = 0.8, Spearman's rank correlation coefficient) between mosquito infection and sheep viremia. For example, at 48 hours' inoculation, none of 27 mosquitoes that fed on ewe 8 (1.6 log_{10} PFU/ml of serum), 9 of 12 that fed on ewe 3 (4.0 log_{10} PFU/ml of serum), and all 6 that fed on ewe 11 (5.6 log_{10} PFU/ml of serum) became infected.

Postchallenge exposure response—At PID 63, group-B ewes were inoculated sc with 1 x 10^6 PFU of ZH-501 strain RVFV and were monitored for 10 consecutive days. Clinical illness, pyrexia, or viremia was not detected in these sheep. Serum neutralizing antibody titers to RVFV in all ewes increased from 1:80 to 1:160 to 1:5,120 by postchallenge exposure day 10, when ewes were euthanized. Because of logistic constraints, nonvaccinated control sheep were not included in this phase of the study.

Discussion

Immunization of susceptible hosts to RVFV serves a 2-fold purpose by limiting the devastating impact of RVF on domestic livestock production and preventing the infection of additional vectors, thus reducing the risk of human and animal infection. Conventional killed and modified-live virus vaccines are effective in providing a degree of immunity, but each has practical or theoretic disadvantages. Chemical mutagen-induced attenuation of the virulence properties of a virus may provide a novel alternative in vaccine development. However, the probability of reversion to virulence must be determined.

The ovine fetus is highly susceptible to RVF infection, with abortion rates in infected pregnant ewes approaching 100%. Consequently, inoculation of RVF-susceptible pregnant ewes is a stringent test of the degree of attenuation of RVFV vaccine candidates. Seemingly, the serially mutagenized ZH-548 strain RVFV used in the present study had greatly reduced pathogenicity in 2 pregnant ewes, was nonabortogenic, and protected those ewes against subsequent challenge exposure with virulent ZH-501 strain RVFV. Because sheep that develop serum-neutralizing antibody titers of ≥ 20 after vaccination with an inactivated RVFV vaccine may be resistant to challenge exposure with virulent RVFV, the lack of control ewes was not considered critical to this phase of the study.

Clinical features of RVFV infection in the ewes infected with the parent RVFV ZH-548 were characterized by early lymphopenia and viremia coincident with the febrile period, the appearance of neutralizing antibody in conjunction with termination of viremia, and abortions. During the brief febrile period, ewes seemed depressed but were not anorectic and had hyperemia of mucous membranes. The apparent increased susceptibility of ewe 11 to parent virus infection, evidenced by a higher and more prolonged viremia, more pronounced lymphopenia, abnormal serum chemical values, and an earlier abortion than that in the 2 other ewes in that group may be similar to differences seen in inbred rat strains in which resistance seems to be determined by a single Mendelian dominant gene. The RVFV was recovered from all tissues examined from the earlier abortus, with high viral titers detected in whole blood, placental cotyledons, and CNS tissue. Advanced autolysis probably accounted for the inability to recover virus or detect antigen by DFA in tissues from the late abortions, but did not prevent antigen detection by ELISA. Further studies are required to standardize and evaluate this rapid assay system under field situations in which RVFV infection is suspected, but tissues are so badly deteriorated that virulent virus can no longer be isolated. Results of DFA staining were similar to results of virus isolation.

The detection of CSF neutralizing antibody in all group-A ewes and in 2 group-B ewes may represent antibody of CNS origin in response to virus replication in the brain, in vivo leakage of serum antibody because of virus-related vasculitis, or perhaps serum contamination at the time of collection, although each CSF specimen was collected with no evidence of gross blood contamination. Evidence of encephalitis or retinal complications was not noticed by clinical examination during life or postmortem histologic examination.

On 5 occasions, virus was isolated from plasma and not serum from both groups of ewes when virus titers were low. If this strain of RVFV was predominantly plasma associated, higher virus titers would be expected in plasma than in serum, but this was not the case. With the exception of those times when virus was isolated from plasma alone, the serum and plasma virus titers were equivalent.

The parent RVFV was capable of inducing viremia titers high enough to infect mosquitoes, and some of these mosquitoes did transmit virus to susceptible hamsters. These data also may indicate that viremia induced by the mutagenized virus does not reach titers high enough to infect C. pipiens. Generally, by gestation day 100, the fetal lamb's immune system will generate an antibody response to sev-

<table>
<thead>
<tr>
<th>Tissue</th>
<th>VT*</th>
<th>DFA†</th>
<th>ELISA</th>
<th>VT*</th>
<th>DFA†</th>
<th>ELISA</th>
<th>VT*</th>
<th>DFA†</th>
<th>ELISA</th>
<th>VT*</th>
<th>DFA†</th>
<th>ELISA</th>
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<tbody>
<tr>
<td>Liver</td>
<td>&lt; 1.4</td>
<td>+</td>
<td>Pos</td>
<td>&lt; 1.4</td>
<td>+</td>
<td>Pos</td>
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<td>+</td>
<td>Pos</td>
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<tr>
<td>Cerebrum</td>
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<td>+</td>
<td>Pos</td>
<td>&lt; 1.4</td>
<td>+</td>
<td>Pos</td>
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<td>Pos</td>
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<td>Whole blood</td>
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<td>7.1</td>
<td>+</td>
<td>Pos</td>
<td></td>
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</table>

* Data are expressed as log_{10} PFU/g of tissue. † Tissues were macerated and difficult to interpret.

TABLE 4—Virus detection by isolation (VT), direct fluorescent antibody (DFA) staining, and ELISA in tissue homogenates and aborted fetuses.

eral mammalian viruses if infected in utero. Group-B ewes were inoculated with mutagenized virus approximately on gestation day 90 to 110, yet all 4 lambs were seronegative for RVFV at birth, indicating that there was no in utero virus contact. By the 5th day of life, the lambs had appreciable serum antibody values, presumably a result of passive transfer, because their dams' colostral antibody titers were ≥ 1:320, and all lambs nursed within 3 hours after parturition.

Although numbers in each group were small, the uniformity of results in each group indicates that this mutagenized strain of RVFV may offer a means of effectively immunizing pregnant ewes to RVFV without abortogenic effects. In utero protection by this vaccine to RVF infection remains to be evaluated. Although minimal antibody titers (≥ 1:20) may be sufficient to protect the ewe, but not the fetus, the higher neutralizing-antibody titers (≥ 1:80) induced by MV P12 may be adequate to afford full protection to both.

References