Protective Immunity to Heartwater (Cowdria ruminantium) Infection Is Acquired after Vaccination with In Vitro-Attenuated Rickettsiae

FRANS JONGEJAN

Department of Tropical Veterinary Medicine and Protozoology, Institute of Infectious Diseases and Immunology, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands

Received 4 June 1990/Accepted 8 November 1990

A Senegalese (S) stock of Cowdria ruminantium was passaged on bovine umbilical endothelial cells with an average interval of 13.9 days (range, 8 to 34 days) between passages. The virulence of infected bovine umbilical endothelial cultures was tested in susceptible goats and sheep by intravenous inoculation of culture supernatant from passages 2 (51 days in vitro), 3 (69 days), 11 (229 days), 14 (264 days), and 16 (291 days). Both animals inoculated with passages 2 and 3 died of heartwater. However, clinical reactions were completely absent in goats and sheep that were inoculated with C. ruminantium from passages 11, 14, and 16. High antibody titers were detected, with immunofluorescence in all vaccinated animals, and a strong signal was found against a 32-kDa Cowdria protein in Western blots (immunoblots). Moreover, the vaccinated animals proved solidly immune when challenged with virulent Cowdria sp.-infected blood stablitate (S strain), whereas all control goats died. No attenuation of a second Cowdria stock (W) was achieved after 226 days in culture, at which time passage 17 was tested in a recipient goat which died of typical heartwater. This is the first report of vaccination with live attenuated C. ruminantium. These attenuated organisms may replace vaccination with virulent blood currently in use in areas where heartwater is endemic.

The tick-borne rickettsia Cowdria ruminantium is the causative agent of heartwater, a serious infectious disease of ruminants (16). The disease is endemic throughout sub-Saharan Africa (14) and since 1980 has also been known to occur in the Caribbean region (13). Protective immunity against heartwater can be induced in domesticated ruminants by infection with virulent blood and subsequent treatment of the reaction with antibiotics to prevent a more serious course of the disease (17). This type of immune prophylaxis is practiced in South Africa by using virulent sheep blood vaccine or Amblyomma hebraeum nympha suspensions infected with Ball 3 stock, followed by treatment with tetracyclines (12). Although such live vaccination material has been useful to control the disease, the vaccine is far from ideal, as its application is cumbersome and risky (12). In addition, the existence of distinct antigenic differences between Cowdria isolates (5, 8, 11) may explain the occurrence of clinical heartwater in animals that were vaccinated with this vaccine on the basis of one stock (7).

Recently, the in vitro cultivation of C. ruminantium in an irradiated endothelial cell line (E5) has been reported (1, 3). I have found that cultures of nonirradiated endothelial cells established from bovine umbilical cord arteries provide a more convenient, still reproducible in vitro system for the propagation of Cowdria isolates (9). In this study, the sequential passage of C. ruminantium in bovine umbilical endothelial (BUE) cell cultures and the resulting attenuation observed in one Cowdria stock are reported.

Two C. ruminantium stocks were used: a Senegalese isolate, designated S (11), and the Welgevonden stock, designated W (6). Both stocks were stored as infected blood stablitate in liquid nitrogen (17). The infectivity of the isolates was tested in susceptible goats (Saanen breed) by intravenous inoculation of 2-ml aliquots of thawed blood stablitate Cr111 (S stock) or stablitate Cr123 (W stock). Brain squash smears were prepared from any animal which had died to confirm death due to heartwater. Two goats (no. 8740 and 8759) were infected with stablitate Cr111 and subsequently treated with terramycin to obtain positive control sera.

Saanen goats and Tesselara sheep were used to test the virulence of cultivated rickettsiae. The animals were monitored by daily temperature records, clinical inspections, and collection of blood samples for serology and Western blotting (immunoblotting) analysis. Sera were obtained from vaccinated and control animals at approximately weekly intervals from day 6 until day 43 postinfection (p.i.). All vaccinated animals were challenged on day 30 postinoculum with virulent blood stablitate Cr111, which caused fatal heartwater in all nonvaccinated control animals (Table 1).

BUE cells were isolated from umbilical cord arteries (18), as described previously (9). Briefly, the cells were grown until confluency in RPMI 1640 medium supplemented with antibiotics, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (20 mM, pH 7.0 to 7.3), L-glutamine (2 mM), and 10% newborn calf serum, and they were subcultured with trypsin-EDTA. Confluent BUE cell monolayers were inoculated with blood infected with C. ruminantium (S or W stock), diluted with two parts of sucrose-phosphate-glutamate buffer (4), and incubated overnight on a slowly rocking platform. Thereafter, the cultures were rinsed with Hanks balanced salt solution, and Cowdria growth medium (Glasgow minimal essential medium supplemented with penicillin [100 IU/ml], streptomycin [100 µg/ml], amphotericin B [1.25 µg/ml], HEPES buffer [20 mM; pH 7.0 to 7.2], L-glutamine [2 mM], and 10% newborn calf serum) was added and incubated further at 37°C on the rocking platform. Samples of BUE cells were scraped from the bottom of the culture flask, smeared onto a glass slide, and examined for Cowdria inclusions after staining with Diff-Quik (Merck & Dada AG, Düdingen, Switzerland).

The growth cycle of C. ruminantium consisted of reticulate bodies within BUE cells, resulting in elementary bodies (EB) which were released into the culture medium. The infection of BUE cell cultures was scored as follows for
TABLE 1. Typical reactions of C. ruminantium (S and W stocks) in untreated Saanen goatsa

<table>
<thead>
<tr>
<th>Stock</th>
<th>Mean incubation period ±SD (days)</th>
<th>Mean maximum temperature ±SD (°C)</th>
<th>Mean febrile period ±SD (days)</th>
<th>Mean days to death ±SD</th>
<th>Mortality rateb</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>11.6 ± 1.7</td>
<td>41.2 ± 0.6</td>
<td>4.4 ± 1.2</td>
<td>16.2 ± 2.8</td>
<td>5/5</td>
</tr>
<tr>
<td>W</td>
<td>10.0 ± 1.2</td>
<td>41.3 ± 0.6</td>
<td>3.2 ± 0.6</td>
<td>13.2 ± 1.1</td>
<td>5/5</td>
</tr>
</tbody>
</table>

a S and W stocks were stored as infected blood stabilites Crlll (S) and Crll25 (W). Five goats were infected in each case.

b Death resulting from heartwater was confirmed by brain smears.

reticulate bodies: 1+, scanty intracellular colonies, with less than 1% of BUE cells infected; 2+, approximately 10% of cells infected; and 3+, virtually all cells infected. Scoring for EB was as follows: 1+, scanty extracellular particles; 2+, present in large numbers coinciding with moderate cytopathic effect; and 3+, heavily infected culture supernatant coinciding with destruction of most BUE cells.

Cultures containing extracellular EB (score of 3+) were used to passage C. ruminantium onto other BUE cell cultures, with an average interval of 13.9 days (range, 8 to 34 days) between passages. After 10 months of continuous cultivation, passage level 20 was reached. BUE culture supernatant (20 ml) heavily infected with EB (score of 3+) of the S stock from passages 2 (51 days in vitro), 3 (69 days), 11 (229 days), 14 (264 days), and 16 (291 days) was inoculated intravenously into sheep or goats. In addition, culture supernatant (20 ml) infected with the W stock was also tested for infectivity in goats at passage levels 4, 9, and 17.

Sera were monitored by Western blotting analysis as follows: sonicates of endothelial cell culture supernatants infected with S isolates from passages 14 to 16 were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis on 7.5 to 20% polyacrylamide gradient gels, followed by Western blotting analysis (10). In addition, serological responses were monitored by indirect immunofluorescence with infected BUE cultures as the antigen. Cultures containing large numbers of extracellular EB (score of 3+) were collected, washed twice with phosphate-buffered saline (PBS), spotted onto microscopic slides, and then fixed in acetone. The slides were incubated with serial twofold dilutions of antisera from goats immunized against C. ruminantium. Fluorescein isothiocyanate-labeled rabbit anti-goat immunoglobulins or fluorescein isothiocyanate-labeled rabbit anti-sheep immunoglobulins (Nordic Diagnostic Laboratory, Tilburg, The Netherlands) were used as second antibodies.

Four animals (two goats and two sheep) injected with S isolates from passages 11, 14, and 16 did not show any clinical signs and were fully protected against homologous challenge with virulent blood stabilites (Table 2), shown to be lethal for all five nonvaccinated control goats (Table 1). However, S infection with passages 2 and 3 caused typical heartwater in one goat (no. 8911; Table 2) and one sheep (no. 48; Table 2). High antibody titers were detected with the immunofluorescent-antibody test in sera from the four vaccinated animals (Table 2) before the animals were challenged with virulent rickettsiae on day 30 p.i. Furthermore, a large number of antigens were recognized in Western blots with sera obtained before challenge from both vaccinated goats (8907 and 8910), with a strong signal directed against a previously reported immunodominant 32-kDa Cowdria protein (10). Moreover, the strong overall signal shown in Fig.

1, lane 9, may be due to the fact that this serum was collected after challenge.

In goats 8740 and 8759, which had been immunized against C. ruminantium S by infection and treatment with blood stabilites, antibody titers reached 5,210 on day 36 and 10,240 on day 49, respectively. Moreover, sera from goat 8740 recognized the same pattern of antigen bands in Western blots as sera from the vaccinated goats, but in the vaccinated goats the antibodies appeared somewhat earlier (Fig. 1).

A second strain of Cowdria (W) passed on BUE cell

FIG. 1. Western blot analysis with sera from goats vaccinated with in vitro-attenuated C. ruminantium S stock. Lane 1, Positive antisera collected on day 49 p.i. from goat 8759 immunized with virulent infected blood; lane 2, noninfected control serum from goat 8650; lanes 3 to 6, sera from goat 8740 immunized with virulent infected blood collected on days 8, 15, 20, and 36 p.i., respectively; lanes 7 to 9, sera from goat 8907 vaccinated with S stock (Table 2) collected on days 22, 29, and 43 p.i., respectively; lanes 10 to 13, sera from goat 8910 vaccinated with S stock collected on days 6, 13, 21, and 27 p.i., respectively. Molecular mass markers are indicated on the left. Both vaccinated goats were challenged with virulent rickettsiae from stabilites Crlll on day 30 p.i.

TABLE 2. Vaccination of sheep and goats with in vitro-grown C. ruminantium (S stock)

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Passage</th>
<th>Days in culture</th>
<th>Result</th>
<th>Challenge</th>
<th>Outcome</th>
<th>IFA titerti ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8911</td>
<td>2</td>
<td>51</td>
<td>Heartwaterb</td>
<td>NDc</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>8907</td>
<td>14</td>
<td>264</td>
<td>No reaction</td>
<td>Crllll1</td>
<td>Immune</td>
<td>5,210</td>
</tr>
<tr>
<td>8910</td>
<td>16</td>
<td>291</td>
<td>No reaction</td>
<td>Crllll1</td>
<td>Immune</td>
<td>10,420</td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>3</td>
<td>69</td>
<td>Heartwaterb</td>
<td>NDc</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>229</td>
<td>No reaction</td>
<td>Crllll1</td>
<td>Immune</td>
<td>2,560</td>
</tr>
<tr>
<td>50</td>
<td>16</td>
<td>291</td>
<td>No reaction</td>
<td>Crllll1</td>
<td>Immune</td>
<td>5,210</td>
</tr>
</tbody>
</table>

a IFA (immunofluorescent-antibody) titers were determined 2 to 4 days prior to challenge with virulent rickettsiae.

b Confirmed by demonstration of rickettsial colonies in brain smears.

c ND, Not done.
cultures caused typical heartwater at passages 4 (67 days in culture), 9 (141 days), and 17 (226 days) (Table 3).

Nevertheless, protection of sheep and goats against lethal heartwater challenge proved possible by vaccination with in vitro-attenuated rickettsiae by using the S strain. This provides a basis for the further development of a cell culture vaccine against heartwater. The apparent loss of virulence was demonstrated by the complete absence of clinical reactions after vaccination. The fact that the rickettsiae retained their immunogenicity was clearly shown by the 100% protection acquired by sheep and goats against challenge with virulent rickettsiae. Attenuation in vitro of rickettsiae is not as common as that of viral pathogens. However, there are a few examples. The virulent Madrid strain of *Rickettsia prowazekii* (the causal agent of human louse-borne epidemic typhus) loses its virulence during egg passage and can be subsequently used as a live attenuated vaccine (E strain) (19). Vaccination of cattle by using live attenuated strains of another rickettsia, *Anaplasma marginale*, has also been practiced on a significant scale (15).

The attenuation of stock S was unexpected on the basis of previous results with W stock passaged on irradiated cultures of an endothelial cell line (ES) by Bezuidenhout (2). This stock remained virulent for at least 97 days in culture, and I showed that it remained virulent up to passage 17 (226 days). Although these initial results are encouraging, several questions have to be answered before the potential of attenuated *Cowdria* strains for vaccination can be fully assessed. (i) It remains to be shown whether the animals become rickettsemic after vaccination, which is relevant with respect to the spread of rickettsiae through *Amblyomma* ticks feeding on them. (ii) If so, it is important to know whether avirulent rickettsiae remain so after passage through ticks. (iii) It remains to be shown whether the loss of virulence in culture is stable. (iv) Titration to determine the number of infectious units in an inoculum has to be developed. (v) Finally, more data on cross-protection against other stocks under laboratory and field conditions have to be collected, since cross-immunity is often incomplete (11).

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### REFERENCES