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An attenuated *Ehrlichia ruminantium* (Welgevonden stock) vaccine protects small ruminants against virulent heartwater challenge

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Abstract

Heartwater is a tick-borne disease of ruminants caused by the intracellular rickettsia *Ehrlichia ruminantium*. The only commercially available immunization procedure involves infecting animals with cryopreserved sheep blood containing virulent *E. ruminantium* organisms, followed by treatment with tetracyclines when fever develops. The virulent Welgevonden stock of *E. ruminantium* was attenuated by continuous propagation of the organisms in a canine macrophage-monocyte cell line (DH82), followed by re-adaptation to grow in a bovine endothelial cell line (BA 886). The material used for the present experiments consisted of the attenuated stock between passages 43 and 64 after re-adaptation. When inoculated into sheep or goats the attenuated organisms did not produce disease, and the only symptom observed was a rise in body temperature in most, but not all, animals. All sheep injected with 2 ml of culture suspension were subsequently found to be fully protected against a lethal needle challenge with the virulent homologous stock or with one of four different heterologous stocks (Ball 3, Gardel, Mara 87/7, Blaauwkrans). Titrations of elementary body suspensions showed that 2 ml of a 1:10,000 dilution of culture suspension injected into sheep or goats was still sufficient to trigger an immune response which resisted a lethal needle challenge with the virulent Welgevonden stock. Adult *Amblyomma hebraeum* ticks, fed as nymphs on sheep immunized with DH82-derived organisms of passage 111, were able to transmit the attenuated stock to a naïve sheep, which was found to be protected against a subsequent lethal homologous needle challenge.

Keywords: *Ehrlichia ruminantium*; Heartwater; Attenuated vaccine

1. Introduction

Heartwater is an infectious, non-contagious, tick-borne disease of both wild and domestic ruminants caused by the rickettsia *Ehrlichia ruminantium*. The disease is prevalent in sub-Saharan Africa [1] and the Caribbean [2,3], from where it poses a threat to livestock in North and South America [4].

Field observations and experiments under laboratory conditions have shown that cattle, sheep and goats are capable of developing a protective immunity against heartwater after surviving a virulent infection [5]. In South Africa this led to the development of an "infection and treatment" type of immunization where animals are injected with fully virulent *E. ruminantium* organisms of the Ball 3 stock and are subsequently treated with tetracyclines to prevent serious disease [6]. Although this procedure is expensive and dangerous it has been the only commercially available "vaccine" for more than 50 years. Unfortunately, the spectrum of protection of the Ball 3 blood vaccine stock against other *E. ruminantium* stocks is limited. In contrast, the Welgevonden stock has been shown to stimulate protective immunity against several virulent South African stocks and, should therefore, be more suitable for immunization purposes than the Ball 3 stock [7].

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However, its high virulence has precluded it from being used for immunization [8]. Attempts to develop an attenuated vaccine were first reported using a Senegalese stock of *E. ruminantium*, which became attenuated after a limited number of passages in vitro [9]. This attenuated stock, while providing immunity to homologous challenge [9], did not provide efficient cross-protection against other virulent stocks [10]. Another stock, from Guadeloupe, has also been attenuated, although more than 200 passages were required [11], but efforts to attenuate other stocks have proved unsuccessful until recently. We successfully attenuated the virulent Welgevonden stock of *E. ruminantium* by continuous passage in a canine macrophage-monocyte cell line [12]. After more than 50 passages the cultured organisms did not produce disease when inoculated into mice or sheep, and the animals were immune to subsequent lethal homologous needle challenge [7]. Here we report the successful use of the attenuated Welgevonden stock to vaccinate sheep and goats under controlled experimental conditions.

2. Materials and methods

2.1. Stocks of E. ruminantium

The Welgevonden stock was isolated from a male *Amblyomma hebraeum* tick, which was collected on the farm Welgevonden in the Northern Transvaal, South Africa [13]. Another four stocks of *E. ruminantium* were used for cross protection experiments in sheep. Three of these stocks were from South Africa: the Ball 3 stock [14], the Mara 87/7 stock [8] and the Blaauwkrans stock isolated from an eland near Port Elizabeth in 1996. In addition we used the Gardel stock, isolated in Guadeloupe [15].

2.2. Propagation of E. ruminantium

The medium used for all cultures, infected and uninfected, consisted of Dulbecco's modified Eagle's medium nutrient mixture Ham F-12 (DME/F-12) (Sigma, St. Louis, MO, USA; catalogue no. D 0547) containing 15 mM HEPES and 1.2 g l^{-1} sodium bicarbonate. It was further supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Propagation of the Welgevonden stock in DH82 cells, which were derived from a dog suffering from malignant histiocytosis [16], has been described in more detail elsewhere [12]. Briefly, infected DH82 cell cultures were harvested by resuspending the cells into the medium, which contained 1 μg ml^{-1} cycloheximide (Sigma). The cell cultures were subcultured at a ratio of 1:2 into new culture flasks containing uninfected DH82 cells. After 24 h all of the medium was discarded and replaced with 5 ml of fresh medium containing 1 μg ml^{-1} cycloheximide. The cultures were incubated at 37 °C.

2.3. Re-infection of BA 886 cells by DH82-derived Welgevonden elementary bodies

The attenuated Welgevonden stock was propagated in DH82 cells through 61 passages and infected cell cultures were harvested by resuspending the cells into the medium. The cell suspension was centrifuged (800 × *g*; 10 min; room temperature) and 2.5 ml of supernatant containing elementary bodies was distributed into a culture flask containing bovine endothelial cells (BA 886) [17] and 2.5 ml of fresh medium. Newly infected BA 886 cultures were harvested for subcultivation by scraping off the cell monolayer into the medium. Cells were dispersed by pipetting the suspension up and down and the suspension was then transferred at various ratios (between 1:2 and 1:5) on to new endothelial cell monolayers. After 24 h all of the medium was discarded and replaced with 5 ml of fresh medium. Subcultures were performed every 3 days. The culture medium used contained 0.25 μg ml^{-1} cycloheximide.

2.4. Experimental animals

We used a total of 63 six to eight month old Merino sheep and 25 Boer goats. The animals were purchased from the farm where they were born and raised, in Warden in the Free State (South Africa), a heartwater- and *Amblyomma*-free area [18]. DNA samples obtained from the blood of all animals used in these experiments were initially examined by PCR [19] and probed with the *E. ruminantium*-specific pCS20 probe [20], which confirmed that they were all negative for *E. ruminantium*. This test is the method of choice for the detection of *E. ruminantium* infection [21]. All animals were inspected daily for clinical symptoms of heartwater, and the rectal temperature was recorded daily. Rectal temperatures of 40 °C in goats and 40.5 °C in sheep were considered to constitute fever. Control animals were included in each experiment, and in compliance with the institute's animal ethics regulations, oxytetracycline was administered to any animal showing severe clinical symptoms, such as depression, laboured respiration, anorexia, recumbency or nervous signs (in-coordination).

2.5. Antibody analysis by an indirect fluorescent antibody test (IFAT)

Serum samples of all animals were subjected to a slightly modified IFAT which, as described elsewhere [22] detects anti-*Ehrlichia* antibodies. Serum was collected on day 0, before immunization, and on the respective day before challenge (Tables 1–3). Two-fold dilutions of the test sera were applied to the wells of antigen slides and were incubated at 37 °C for 30 min. The second antibody was rabbit anti sheep IgG or anti goat IgG, labelled with fluorescein isothiocyanate (Sigma), and diluted 1:80 in 0.1% Evans blue solution. Slides were mounted in 50% glycerol in phosphate buffered saline and the fluorescence was evaluated under a