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Conconavalin A-Stimulated Bovine T-Cell Supernatants Inhibit Growth of *Cowdria ruminantium* in Bovine Endothelial Cells In Vitro

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Conconavalin A-stimulated bovine T-cell supernatants inhibited the growth of *Cowdria ruminantium* in bovine endothelial cells in vitro but did not affect their entry. This finding represents one mechanism by which T cells may control *C. ruminantium* multiplication and hence affect the severity of disease.

Cowdria ruminantium is the etiological agent of an economically important disease of livestock called heartwater or cowdriosis (22), which affects a large area south of the Sahara in Africa (22) and three islands in the Caribbean (22). *C. ruminantium* is an obligate intracellular rickettsia which is transmitted via *Amblyomma* ticks and in vivo has a predilection for vascular endothelial cells, macrophages, and neutrophils (10). Although the mortality rate due to heartwater is often high (22), animals that survive the infection are immune to further challenge (22). The mechanisms of protective immunity that are operational in recovered animals are unknown. Since *C. ruminantium* is essentially an intracellular organism, it is presumed that cell-mediated immunity will be essential in control and clearance of the organism and in eliciting protection. In other rickettsial infections, such as those caused by *Rickettsia typhi* and *Rickettsia prowazekii*, cytotoxic T-lymphocyte-mediated immune responses ($CD4^+$ and $CD8^+$) have been demonstrated in vitro (2, 3). In addition, several rickettsias induce the production of gamma interferon (IFN-γ) in vivo (7, 9, 12, 13), and growth of rickettsias in vitro can be inhibited by antigen- and mitogen-stimulated T-cell supernatants (11, 18, 24). Mitogen-stimulated mouse, bovine, and human T-cell supernatants are known to contain many cytokines, including IFN-γ and interleukin-2 (4, 14, 15, 23). The active rickettsial growth-inhibitory molecule in these supernatants has been shown to be immune interferon, or IFN-γ (8, 17, 19). The mechanism by which IFN-γ mediates its effects on growth inhibition of these rickettsias is not fully understood. However, IFN-γ can prevent entry of *R. prowazekii* into macrophage-like RAW264.7 cells (20, 21), cause lysis of infected cells (16, 19, 24), and prevent rickettsial replication and cause clearance of intracellular rickettsias (17–19, 24). In addition, IFN-γ caused clearance of intracellular *Rickettsia tsutsugamushi* and was toxic to infected cells (6). Since *C. ruminantium* is primarily an intracellular rickettsia and has host cell predilections which are similar to those of the rickettsias mentioned above, the effects of mitogen-stimulated bovine T-cell supernatants (cytokines) on its growth and entry into bovine endothelial cells were examined in vitro.

Crude bovine cytokines were prepared by conconavalin A (ConA) stimulation of T cells in peripheral blood leukocytes (PBL) obtained from an adult bovine which had no previous exposure to heartwater. PBL from heparinized bovine blood were separated on Ficoll-Paque gradients (Sigma) by a previously described method (5). PBL (2×10^6/ml) were cultured in Leibovitz's-15 (L-15) complete medium (L-15 [pH 6.5], 0.45% glucose, 10% tryptose phosphate broth, 292 μg of L-glutamine per ml, 100 IU of penicillin per ml, and 10% fetal calf serum) and ConA at 5 μg/ml for 72 h at 37°C in a 5% carbon dioxide-in-air atmosphere. The cell culture supernatants were harvested by centrifugation at 1,000 × *g* for 20 min to pellet the cells. One gram of α-methyl-mannoside was added to each 50 ml of the supernatant to inactivate any residual ConA. The culture supernatants containing cytokines were filtered through a 0.45-μm-pore-size filter, aliquoted, and stored at −40°C until use. When needed, various concentrations of the cytokines (20 to 1%) in L-15 medium were used in studying their effects on *C. ruminantium* growth.

Four bovine endothelial cell lines which supported *C. ruminantium* growth were used in this study: three bovine pulmonary artery cell lines (BPA 191, BPA 291, and BPA 593/1) and one bovine aorta cell line (BA 987). These cell lines were isolated from bovine blood vessels (1) obtained from different animals from a local abbatoir. The cells were routinely grown in 25-cm^2 flasks in Glasgow's minimum essential medium with supplements as described earlier (1). Before inoculation with *C. ruminantium*, the Glasgow's minimum essential medium was changed to L-15 maintenance medium with supplements and 5% fetal calf serum (1). Inoculation of these cell lines with *C. ruminantium* (Crystal Springs, Plumtree, and Mbizi strains from Zimbabwe) was conducted as follows: the medium was poured off, and the infective material was added (usually 1 ml of supernatant from ongoing infected cultures which were at a stage when the endothelial cells were lysed by *C. ruminantium* infection) and incubated at 37°C for 2 h on a rocking platform. The inoculum was poured off, and 4 ml of fresh L-15 maintenance medium with or without the cytokines was added, after which the flasks were returned to the rocking platform in the incubator. The cultures were routinely monitored for development of cytopathic effects and sampled for quantitation of the percentage of infected cells on Giemsa-stained smears. Attempts to quantitate the number of rickettsias per cell were unsuccessful, as *Cowdria* organisms grow in very tightly packed colonies. The onset of cytopathic effects usually followed the microscopic detection of infected cells on smears. Each experimental group contained a set of five 25-cm^2 endothelial cell flasks, and an average of five readings and the standard deviation were calculated for plotting the respective graphs.

Prior to studying the effects of T-cell cytokines on *C. ruminantium* infection in vitro, uninfected bovine endothelial

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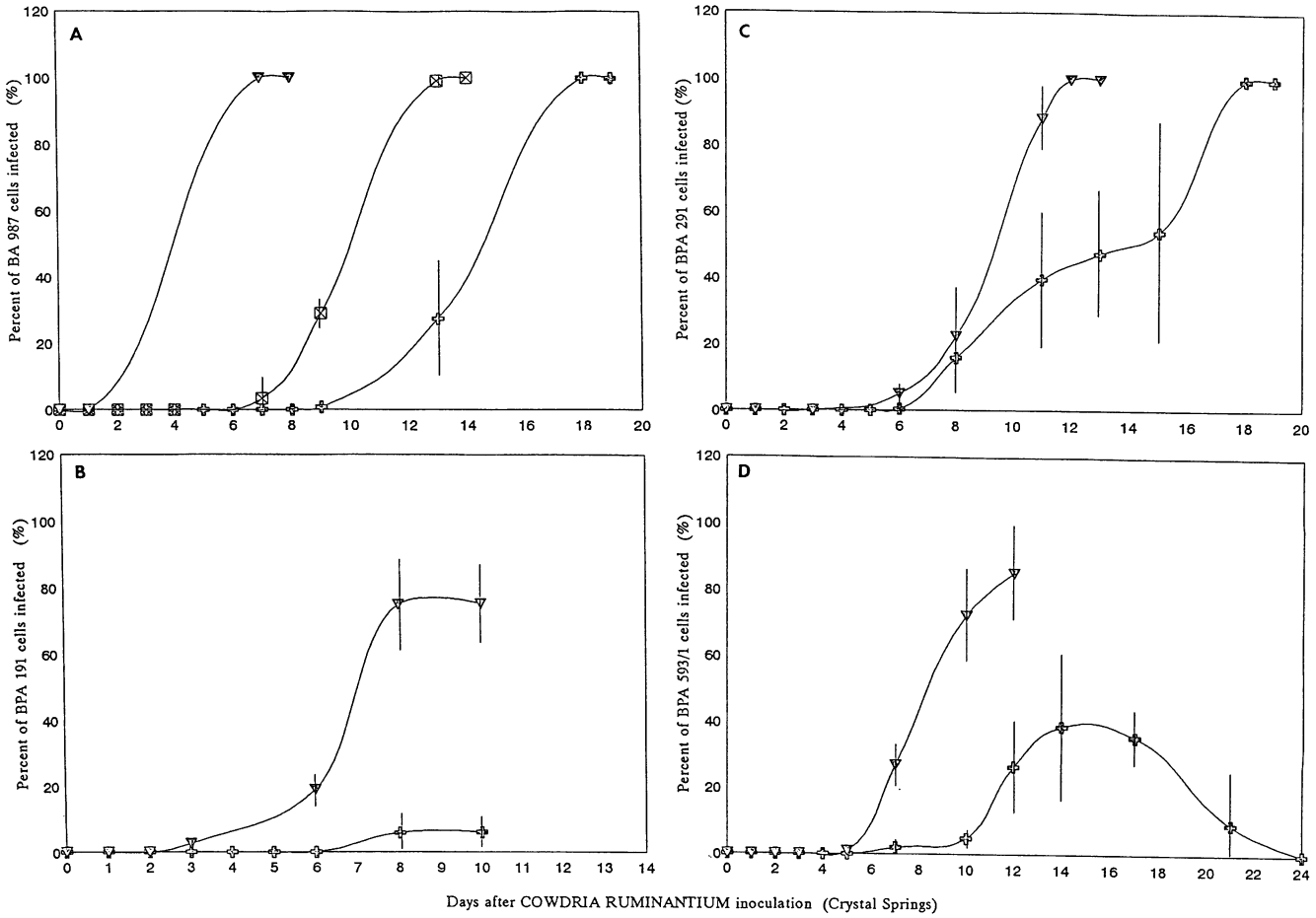

FIG. 1. Demonstration of inhibition of *C. ruminantium* growth in endothelial cells by T-cell cytokines. Effects of 1% (⊠) and 2% (✣) cytokine-supplemented media on *C. ruminantium* (Crystal Springs) growth in BA 987 (A), BPA 191 (B), BPA 291 (C), and BPA 593/1 (D) endothelial cells. The panels show a slower progression of *C. ruminantium* infection in the cytokine-supplemented cultures compared with that in normal-medium control cultures (▽). Standard deviations are shown by vertical bars.

cells were maintained in their presence to determine their toxicity for these cells. These experiments showed that the T-cell cytokines adversely affected the endothelial cell cultures at final concentrations greater than 2%. Hence, the effect of cytokines on *C. ruminantium* growth was examined by using media supplemented with 1 and 2% cytokines. The 1 and 2% cytokine-supplemented media added to BA 987 cultures immediately after *C. ruminantium* inoculation inhibited growth of *C. ruminantium* (Fig. 1A). Compared with that in the infected control cultures containing normal L-15 medium, complete lysis (when cells reached a 100% infection rate) of 1 and 2% cytokine-supplemented cultures was delayed by 5 and 11 days, respectively.

The reproducible inhibitory effect of 2% cytokine-supplemented medium on growth of *C. ruminantium* Crystal Springs, Plumtree, and Mbizi in other endothelial cell lines (BPA 191, BPA 291, and BPA 593/1) is shown in Fig. 1B to D and 2. A more complete suppression of *Cowdria* growth could be achieved if the 2% T-cell cytokine-supplemented medium was replenished repeatedly, i.e., every 3 to 4 days after inoculation (data not shown).

T-cell cytokines added to cultures that had been infected for 3 days (when no infection or cytopathic effect was detected) and 7 days (when the percentage of infected cells was 20 to 40%) did not appear to inhibit *Cowdria* growth, and neither did their replenishment on day 10 (data not shown).

The effect of the T-cell cytokines on entry of *C. ruminantium* into endothelial cells was examined in two cell lines, BPA 191 and BPA 593/1. These cells were maintained for 24 h in cytokine-supplemented medium prior to inoculation with *C. ruminantium*. The pretreatment appeared to have no inhibitory effect on entry of the Crystal Springs strain in BPA 191 cells (Fig. 3) or the Plumtree strain of *C. ruminantium* in BPA 593/1 endothelial cells (data not shown), as the infections in these cultures progressed at the same rate as in the control cultures. The greatest inhibition occurred in pretreated cultures which received cytokine-supplemented medium following *Cowdria* inoculation.

The data presented here indicate that crude preparations of T-cell cytokines derived following ConA stimulation of bovine PBL inhibit the progression of *C. ruminantium* infections in bovine endothelial cells in vitro. This inhibition was greatest when the cytokines were added immediately after inoculation of cultures with *C. ruminantium*. Such cytokines, if induced during heartwater infections in ruminants, would be expected to have an antagonistic effect on *C. ruminantium* multiplication and hence affect the severity of the disease. The inhibitory effects of the T-cell cytokines were observed regardless of the

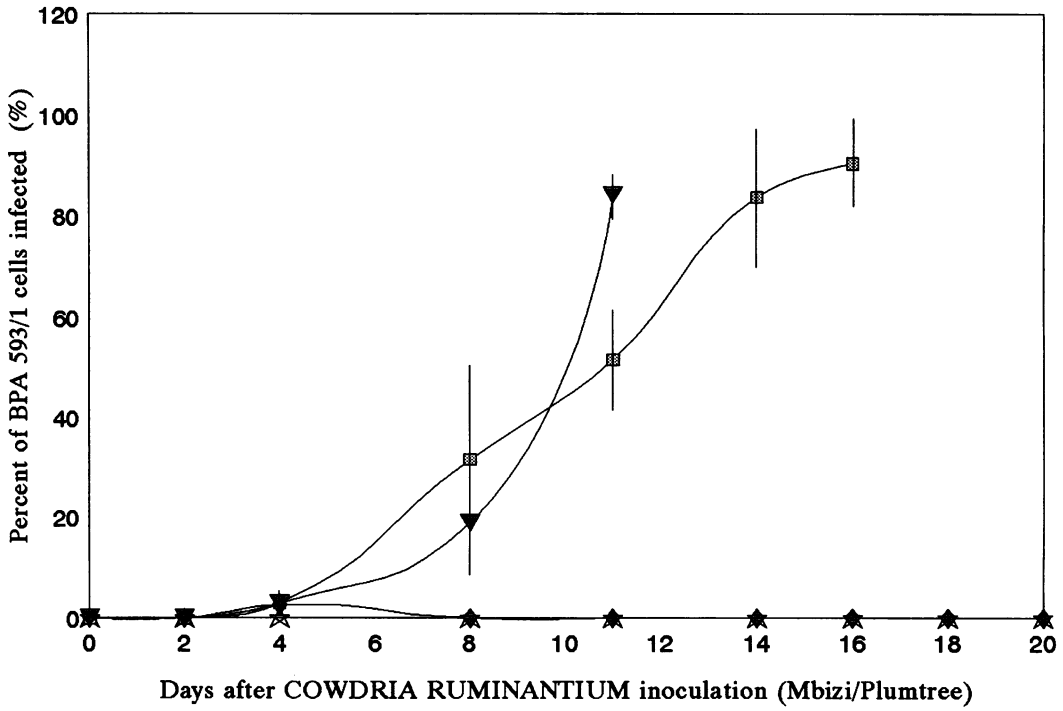

FIG. 2. Inhibition of *C. ruminantium* Plumtree and Mbizi by 2% T-cell cytokine-supplemented medium. Control Plumtree (▨) and Mbizi (▼) cultures received normal medium after inoculation with the two strains of *C. ruminantium*. Other Mbizi (◆)- and Plumtree (☆)-infected cultures were maintained in medium supplemented with 2% cytokine after inoculation. Standard deviations are indicated by the vertical bars.

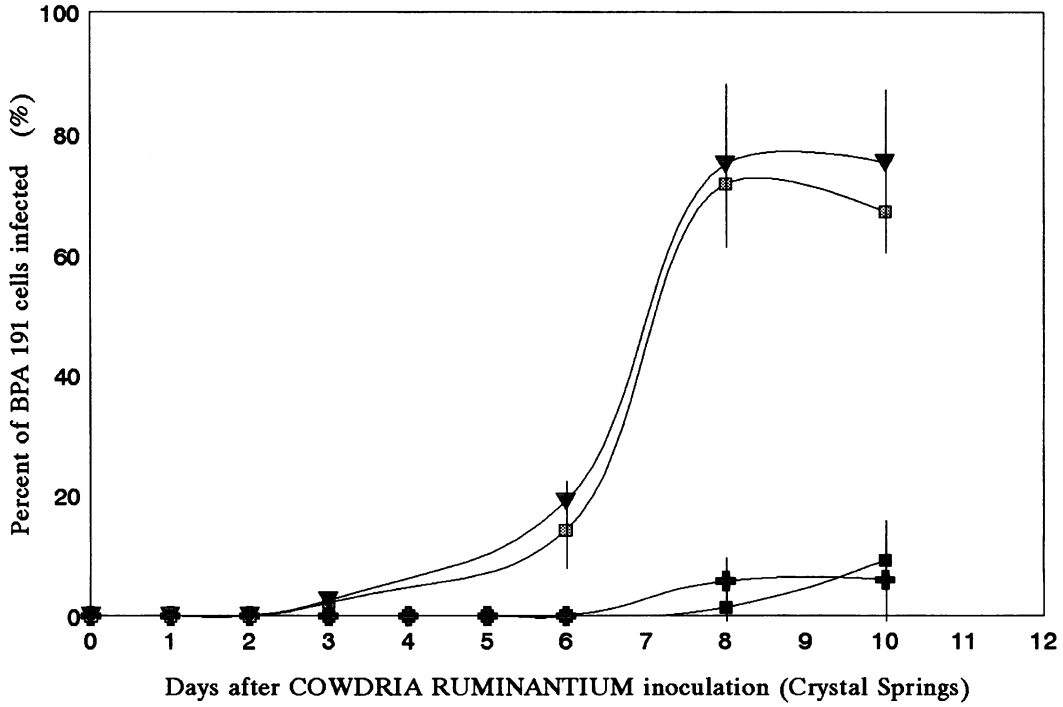

FIG. 3. Effect of 2% cytokine-supplemented media an entry of *C. ruminantium* into cells. The BPA 191 endothelial cells were pretreated for 24 h with 2% cytokine-supplemented medium and then maintained with either normal (▨) or cytokine-supplemented (■) medium following inoculation with the Crystal Springs strain of *C. ruminantium*. Pretreatment and then normal medium did not affect entry of *Cowdria* organisms into the endothelial cells. Pretreatment and then cytokine-supplemented medium gave the most inhibitory effect on *Cowdria* growth. ▼, control cultures maintained with normal medium; ✚, 2% cytokine-supplemented medium. Standard deviations are indicated by the vertical bars.

endothelial cell lines and *C. ruminantium* strains used in the study, although the degree of inhibition varied depending upon the cell line or the *C. ruminantium* strains that were being used. This may have been due to a direct or indirect effect (via the host cells) of the cytokines on *C. ruminantium.*

The probable mechanisms by which these cytokines caused inhibition of *C. ruminantium* growth are (i) inhibition of early multiplication of the organisms and (ii) lysis of intracellular *Cowdria* organisms or specific lysis of infected cells. It was not possible to differentiate between these two effects because *C. ruminantium* grows in very tightly packed colonies, making its quantitation on a per-cell basis impossible. The cytokines did not inhibit the entry of *C. ruminantium* into endothelial cells, nor did they have an effect once the infection had been allowed to establish to a certain stage (i.e., 3 or 7 days after inoculation).

By extrapolation from similar studies of other rickettsias, the most likely cytokine to be involved in this inhibition is IFN-γ (6, 17–21, 24). It is a priority to identify the active inhibitory molecule(s) within the crude T-cell cytokine preparation. Bovine IFN-γ and anti-IFN-γ antibodies could be tested to define their role in inhibition of these in vitro *C. ruminantium* infections. The roles of other cytokines, such as IFN-α/β and tumor necrosis factor, could also be analyzed. In addition, correlations should be made between the development of protective immunity in ruminant hosts and production of T-cell cytokines antagonistic to *C. ruminantium*. These questions will be the focus of our future studies of immune mechanisms against *C. ruminantium* infections.

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