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Injury restricted to cells infected with spotted fever group rickettsiae in parabiotic chambers

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Summary

One chamber of paired parabiotic chambers separated by 0.2 μm pore-sized membrane filters which prevented passage of rickettsiae were infected with either *Rickettsia rickettsii* or *R. conorii*. Infected VERO cell monolayers underwent necrosis. Uninfected monolayers in adjoining chambers which shared the same extracellular milieu including soluble rickettsial products did not undergo necrosis.

Key words: *Rickettsia rickettsii; Rickettsia conorii;* rickettsial pathogenesis; parabiotic chamber.

Introduction

The hypothesis that cell and tissue injury are mediated by a rickettsial toxin has been suggested (Moe et al., 1976; Murray, 1980) although an exotoxin has never been demonstrated and rickettsial lipopolysaccharides do not have potent toxic activity (Schramek et al., 1977). Much of the confusion concerning rickettsial pathogenesis is the result of the name given to the phenomenon of the lethal effect of large doses of viable rickettsiae when inoculated intravenously into mice (Bell and Pickens, 1953; Gildenmeister and Haagen, 1940). Traditionally, this rickettsial laboratory assay has been termed the "mouse toxin phenomenon" although it cannot be produced by rickettsiae that are metabolically inactive or dead (Bovarnick and Allen, 1954 and 1957), and this toxicity has never been produced by a purified component of rickettsiae.

Recent investigations by Winkler and coworkers and in our laboratory have suggested that a rickettsial enzyme may mediate rickettsial injury to host

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cell membranes. Phospholipase activity appears to play an important role in *Rickettsia prowazekii*-induced hemolysis (Winkler and Miller, 1980), in the immediate cytotoxicity of a large inoculum of *R. prowazekii* on cell monolayers (Winkler and Miller, 1982), and in plaque formation by *R. rickettsii* (Walker et al., 1983). Phospholipase activity is a plausible hypothesis for explanation of the mouse toxicity phenomenon. In this study parabiotic chambers were employed to determine whether any soluble rickettsial product would injure uninfected cells sharing the same culture medium with cells infected and killed by *R. rickettsii.*

Materials and Methods

Twenty pairs of sterile parabiotic chambers (Bellco Glass, Vineland, NJ) were separated by 25 mm diameter cellulose triacetate membrane filters (Gelman Sciences, Ann Arbor, MI) with 0.2 μm pore size sealed between the chambers with silicone stopcock grease. Coverslips measuring 10.5×35 mm were placed in each chamber and were seeded with 5×10^5 VERO cells (CDC Tissue Culture Unit, Atlanta, GA). After incubation at 37° C in minimum essential medium with 5% heat-inactivated fetal calf serum and 10% tryptose phosphate broth for 24–48 h, monolayers were confluent. The medium was removed, and 11–40 plaque-forming units of *R. rickettsii* (Sheila Smith strain) were inoculated into one chamber of each of 13 pairs of parabiotic chambers. After 30–45 min for adsorption of inoculum, 10 ml of the same medium was added. Five pairs of chambers were not inoculated with rickettsiae. Coverslips from adjoining inoculated and uninoculated chambers were examined for evidence of cell death as determined by trypan blue staining (Garvey et al., 1977) on days 3, 4, 5, 6 and 7 postinoculation and for presence and distribution of *R. rickettsii* by direct immunofluorescence (Walker and Cain, 1980) on days 5, 6, 7 and 9 postinoculation. Uninoculated pairs of chambers were examined as controls on day 7 after inoculation. For a positive toxin control, one chamber of each of two pairs was inoculated with a fresh clinical isolate of *Pseudomonas aeruginosa* with examination of chambers on day 3 and on day 5 by trypan blue staining. In a subsequent experiment, 36–360 plaque forming units of *R. conorii* (strain 7) were inoculated in a similar manner into one of the matched pairs of parabiotic chambers containing coverslips with monolayers of VERO cells. The coverslips were examined on days 5 and 6 by phase contrast microscopy after trypan blue staining and then after acetone fixation by direct immunofluorescence for rickettsiae.

Results

By day 3 after inoculation, foci of trypan blue-stained necrotic cells were present in the parabiotic chamber inoculated with *R. rickettsii.* Over the succeeding days, the trypan blue-stained foci appeared to enlarge progressively. By day 5 these foci consisted of 25–50 necrotic cells admixed with an equivalent quantity of viable cells. By day 7 the infection had become confluent and contained a majority of necrotic cells. The uninoculated monolayers both in chambers adjoining infected cytopathic cells and in control chambers exhibited a similar appearance with only a few ($<1\%$), single, randomly distributed trypan blue-stained cells. The presence and distribution of *R. rickettsii* as determined by immunofluorescence correlated with the distribution of cell necrosis. On days 5 and 7, monolayers were examined by trypan blue staining and subse-

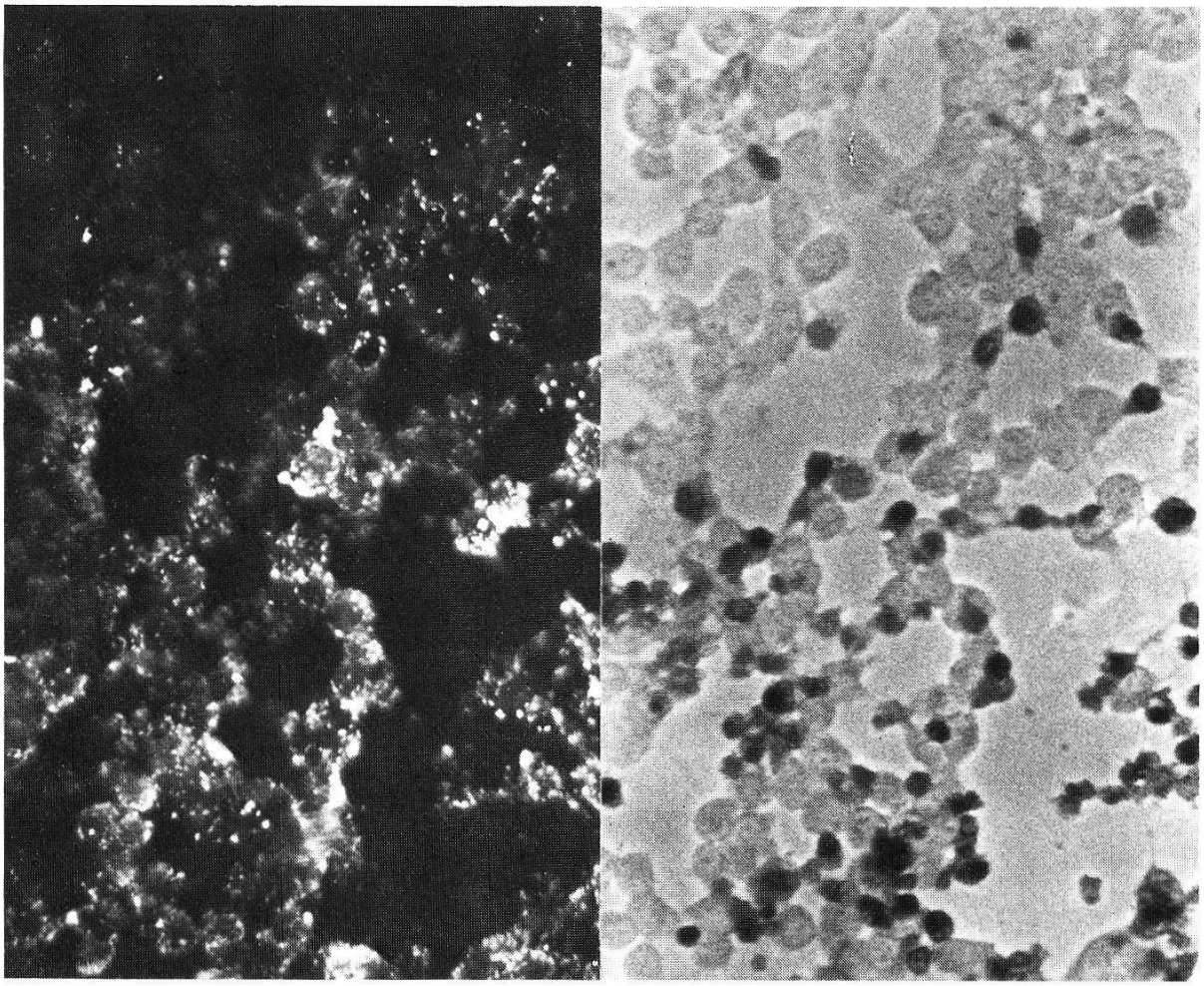

Fig. 1. Photomicrographs of the identical microscopic field of a monolayer in a parabiotic chamber 5 days after inoculation with *Rickettsia rickettsii*. Immunofluorescent demonstration of intense *R. rickettsii* infection in the lower portion of the field (left). Trypan blue-stained necrotic cells are numerous in the same area (right). FITC-labelled anti-*R. rickettsii* rabbit globulin (left) and phase contrast (right). ×300.

quent immunofluorescence on the same monolayer utilizing a microscope designed for both phase contrast and fluorescent microscopy. The areas of intense rickettsial infection and areas of trypan blue staining coincided (Fig. 1). No rickettsiae were detected in adjoining chambers that were separated by the 0.2 μm filter. The monolayers infected with *R. conorii* showed severe cytopathic effect but with less necrosis than *R. rickettsii* infected monolayers although nearly all of the cells were infected. In contrast, the adjacent uninoculated monolayers appeared without cytopathic effect. Validation of the parabiotic chamber toxin model was provided by demonstration of progressive destruction of the monolayers in the chamber infected with *P. aeruginosa* and in the uninfected chamber when examined on days 3 and 5.

Discussion

In this model of severe cell injury and cell death caused by spotted fever group rickettsiae, uninfected cells of the same type as those injured and killed by rickettsiae were exposed to the same extracellular milieu including concentration of soluble rickettsial products which would have passed freely along with other macromolecules through the 0.2 μm pore filter. None of these exposed yet uninfected cells exhibited any more cellular necrosis than control monolayers in which both parabiotic chambers were not infected with rickettsiae. These data argue strongly against the existence of an important rickettsial exotoxin or soluble enzyme analogous to the phospholipase of *Clostridium perfringens* in the pathogenesis of cell injury by *R. rickettsii.*

These data are compatible with the proposed phospholipase-associated penetration mechanism of cell injury by rickettsiae. *R. prowazekii* requires attachment to erythrocytes for accomplishing rickettsial hemolysis (Ramm and Winkler, 1973 and 1976; Winkler, 1977). *R. rickettsii* appears to require attachment to cells by the cholesterol-containing receptor in the plaque model to exert cell injury (Walker et al., 1983). Thus, prevention of passage of spotted fever group rickettsiae from one chamber to the adjoining chamber by the 0.2 μm pore-size filter would limit the phospholipase-associated penetration mechanism to the infected chamber. The more extensive frank necrosis with *R. rickettsii* infection than with *R. conorii* correlates with the greater incidence of complications and mortality in Rocky Mountain spotted fever than in boutonneuse fever.

This in vitro experiment does not exclude the possibility of a role for endotoxin in Rocky Mountain spotted fever although the data for a lipopolysaccharide with in vivo endotoxin activity for rickettsiae are weak. Classical endotoxin pathogenic mechanisms involve in vivo host-mediated mechanisms dependent on polymorphonuclear leukocytes and coagulation; these host-mediated pathogenic elements were not tested in this parabiotic chamber model.

This parabiotic chamber model was chosen over exposure of uninfected monolayers to filtered supernatant of rickettsia-infected cells. Since the parabiotic system compares the effect of the products of rickettsial infection over the same time course and concentrations as of the monolayer with rickettsial cytopathic effect, its negative results may be interpreted as valid. In contrast, acute exposure of monolayer to filtered supernatant would not reflect such a dynamic interaction.

The formation of enlarging foci of infection and necrosis occurs in this model with fluid medium that allows release of rickettsiae from infected cells into the medium and spread to infect randomly any cell of the monolayer. The contiguity of most infected and injured cells suggests that cell-to-cell spread of rickettsiae may be important in the pathogenesis of spotted fever group rickett-

sioses. This contiguous distribution of rickettsia-infected and injured cells is analogous to our observations of rickettsial distribution in human Rocky Mountain spotted fever (Adams and Walker, 1981; Walker et al., 1978) and is compatible with the proposed mechanism of injury by the phospholipase-associated penetration mechanism. Further experiments should be designed to explore this and other direct rickettsial mechanisms of cytotoxicity other than soluble exotoxins and enzymes.

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