IN VITRO DEMONSTRATION OF THE HEMOLYTIC, CYTOTOXIC ACTIVITIES AND INDUCTION OF APOPTOSIS IN ORIENTIA TSUTSUGAMUSHI INFECTED L929 MOUSE FIBROBLAST CELLS

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Abstract. Using cultured mouse fibroblast L929 cells, this study demonstrated the hemolytic and cytotoxic activities and induction of apoptosis in cells infected with *Orientia tsutsugamushi*. Low levels of hemolytic activity were detected using heavily infected cells. No hemolysin or cytotoxin were detected in the infected culture fluid regardless of the pathogenicity of the *O. tsutsugamushi* strains in mice. Using propidium iodide uptake assay, acridine orange/ethidium bromide staining and terminal deoxynucleotide transferase-mediated dUTP-digoxigenin nick-end labeling assay, apoptosis was observed in L929 cells infected with Karp and Gilliam strains.

INTRODUCTION

Scrub typhus infection, which is caused by *Orientia tsutsugamushi*, is a major cause of febrile illness throughout the Asia-Pacific region (WHO, 1993). The basic pathology of scrub typhus is reported to be focal vasculitis and perivasculitis of the small blood vessels, involving a number of organs. Multiplication of the organisms in the endothelial cell lining of the small blood vessels causes endothelial proliferation and perivascular inflammatory cell infiltration, which results in hemorrhage, thrombosis and widespread vasculitis. The mechanisms causing infections and deaths of animals or humans are obscure.

One important virulence factor in some bacterial pathogens is the production of toxin and membranolytic substances such as hemolysins and cytotoxins. Hemolysins have often been implicated as virulence factors for a variety of human pathogens (Chippendale *et al,* 1994: Galan, 1996) including the typhus group Rickettsiae (Hackstadt, 1996). Organisms secreting low levels of cytotoxins may be more virulent once they are in an intracellular location. The detection and identification of such rickettsial components has not been demonstrated. Apoptosis has been shown to be an important mechanism of cell death in animal models of sepsis and endotoxemia (Wang *et al,* 1994; Rogers *et al,* 1996; Xu *et al,* 1997) and may contribute to multiple organ dysfunction in sepsis (Bone, 1996; Hotchkiss *et al,* 1997). In the scrub typhus research, prominent apoptotic changes have been demonstrated in lymphocytes in the regional lymph nodes and spleens of mice with rickettsial infections (Kasuya *et al,* 1996).

Mouse fibroblast L929 cells are the most frequently used for the growth and isolation of *Orientia tsutsugamushi*. In this study, heavily infected L929 cells and soluble fractions of *O. tsutsugamushi* cultures were tested for their cytotoxicity and hemolytic activity. This study also aimed to determine whether apoptosis occurs in these infected cells.

MATERIALS AND METHODS

O. tsutsugamushi strains

For cytotoxicity and hemolytic activity detection, Karp, Kato, Gilliam, and four local *O. tsutsugamushi* (OT) isolates, R1, R2 (both of

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which caused <50% deaths in infected mice), R6 and R8 (both of which caused >50% deaths in infected mice) were used. For the detection of apoptosis, three *O. tsutsugamushi* prototype strains: Karp, Kato and Gilliam, were used.

Growth of L929 cells

L929 (ATCC CCL-1) mouse fibroblast cells were maintained in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum (FBS) without antibiotics. Cells were grown routinely in 75 cm^2 flasks at 37ºC. Confluent stock cultures were trypsinized, counted and suspended in growth medium to a concentration of $3x10^5$ cells/ml.

Infection of L929 cells with *O. tsutsugamushi*

A 500 µl sample of a 20% (w/v) yolk sac homogenate heavily infected with *O. tsutsuga-mushi* strains was added to 10 ml of L929 cells held in a Corning tissue culture flask. After rickettsiae were allowed to adhere at room temperature for 3 hours with rocking, the infected L929 cells were grown in antibiotic-free RPMI 1640 tissue culture medium with 10% heat-inactivated FBS at 37ºC. The integrity of the cell layers was observed daily. The intensity of infection was examined in Giemsa-stained smears, and heavily infected cells were harvested by scraping the cell layer using a scraper. Rickettsiae were harvested by centrifugation at 6,000 rpm at 4ºC for 10 minutes. The pellet was resuspended in PBS, pH 7.2, to a concentration of $2x10^5$ cell/ml. Both supernatant and cell suspension were used immediately for the detection of cytotoxicity and hemolytic activity.

Detection of cytotoxic activity

The L929 cell line was grown in RPMI 1640 tissue culture medium, supplemented with 10% FBS without antibiotics. Confluent cultures were trypsinized, counted and suspended in growth medium to a final concentration of $2x10^5$ cells/ml. A volume of 0.1 ml of the cell suspension was pipetted into flat-bottomed, 96-well tissue culture plates (Costar, USA). Monolayers were established by incubation at 37ºC for 18-20 hours. The growth medium was then gently removed from each well, and 50 µl of maintenance medium was carefully layered over the monolayer

in each well. This was followed by 50 µl of the cell pellet or supernatant. The plates were incubated at 37ºC for 24-48 hours. Plates were examined microscopically with an inverted microscope at magnifications of 200x and 400x. Cells were inspected for changes in morphology such as swelling, granularity, rounding or floating for 48-96 hours.

Detection of hemolytic activity

Sheep blood was collected in Alsever's solution and centrifuged at 2,000 rpm for 10 minutes. The packed cells were washed twice with PBS, pH 7.2 and then resuspended in 99 ml of PBS. The hemolytic activity of *O. tsutsugamushi* strains was assayed by a modification of the microplate assay of Dominguez-Rodriguez *et al* (1986). Briefly, two-fold dilutions of infected cell suspension/supernatant were made in a microtiter plate with U-bottomed wells (Linbro, USA), in which 50 µl PBS had been placed. To each dilution, 100 µl of 1% washed erythrocytes was added. The plates were incubated at 37ºC for at least 48 hours. The hemolytic activity titer was expressed as minimal hemolytic units, which was the highest dilution at which hemolysis was detected. Uninfected L929 cells were used as the negative control for the assay.

Detection of apoptosis in *O. tsutsugamushi*-infected L929 cells

For apoptosis detection, eight-day-old infected cells were harvested from the tissue culture flask. The intensity of infection was examined in Giemsa-stained smears and heavily infected cells were harvested by spinning at 2,000 rpm at 4ºC for 5 minutes. The supernatant was discarded and the cells were resuspended in maintenance medium to a concentration of $2x10^5$ cell/ml. The cell suspension was then fixed on glass slides for propidium-iodide (PI) uptake, acridine orange/ethidium bromide (AO/EB) staining and terminal deoxynucleotide transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay.

Propidium iodide (PI) uptake assay. This was performed as described by Spector *et al* (1998). Briefly, a thin smear of infected L929 cells (approximately 10 ml of the cell suspension) was made on a pre-cleaned glass slide. The smear

was air-dried and fixed in acetone at 4ºC for 10 minutes. Cells were stained with 100 μg/ml of PI in PBS, pH 7.4 for 5 minutes. The slide was then washed 3 times, for 5 minutes each time, in PBS and air dried before mounting with 90% (v/v) glycerol. The cells were observed immediately under a fluorescent microscope. Apoptotic cells appear as red fluorescent cells with reduced cell size, necrotic cells as red fluorescent cells with size similar to viable cells.

Acridine orange/ethidium bromide (AO/EB) staining. This was performed as described by Spector *et al* (1998). Briefly, a 25 μl cell suspension (0.5x10⁶-2.0x10⁶ cell/ml) was mixed gently with 1 μl of 100 μg/ml of AO/EB solution. A volume of 10 μl of the cell suspension was then placed onto a microscopic slide, covered with a glass coverslip, and examined for apoptotic cells under a fluorescent microscope. AO is a vital dye and will stain both live and dead cells. EB will stain only cells that have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will incorporate EB and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin. This method gives information on early, versus late, apoptosis and is somewhat subjective but definitive.

Terminal deoxynucleotide transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay. This was performed using a commercial kit (DeadEnd Colorimetric Apoptosis Detection System, Promega, USA). The TUNEL assay is based on the *in situ* labeling of DNA fragmentation sites in nuclei of intact fixed cells. A thin smear of infected L929 cells was made on a pre-cleaned glass slide, air dried and fixed in 10% buffered formalin in a Coplin jar at room temperature for 25 minutes. This was followed by washing twice in fresh PBS for 5 minutes. The cells were then per-

meabilized by immersing the slide in 0.2% Triton X-100 solution in PBS for 5 minutes. The slide was rinsed twice in fresh PBS for 5 minutes. Excess liquid was removed from the slide and the cells were covered with 100 μl of equilibration buffer for 5-10 minutes at room temperature. A positive control was included, by treating a sample with 100 μl of DNAse I buffer containing 1 unit/ml of DNAse I to cause DNA fragmentation. The equilibrated areas were then blotted with tissue paper before adding 100 μl of TdT reaction mix (containing biotinylated nucleotide mix and TdT enzyme). The cells were covered with a plastic coverslip to ensure even distribution of the reagent and incubated at 37ºC for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur. The reaction was terminated by immersing the slide in 2x SSC in a coplin jar for 15 minutes. The slide was then washed in fresh PBS three times to remove unincorporated biotinylated nucleotides and endogenous peroxidase blocked by immersing the slide in 0.3% hydrogen peroxide at room temperature for 5 minutes. The slide was then washed thrice in fresh PBS. A volume of 100 μl of streptavidin HRP solution, diluted 1:500 in PBS, was then added and incubated at room temperature for 30 minutes. The slide was washed in fresh PBS three times and stained with 3′3 diaminobenzidine tetrahydrochloride solution. The slide was then rinsed several times in deionized water before counter-staining with 1.0% methyl green, mounted in Permount mounting medium and read under a light microscope. Using this procedure, apoptotic nuclei are stained dark brown and normal cells stain bluish-green.

RESULTS

O. tsutsugamushi-infected cells began to dislodge from the tissue culture flask on day 4 onwards and the cell monolayer was disintegrated by day 8. Control cells, on the other hand, appeared as a uniform adherent monolayer. However, no cytopathic effect was noted with the supernatant of the infected cells. The cells remained intact after 96 hours of incubation. Also, very low levels of hemolytic acti-
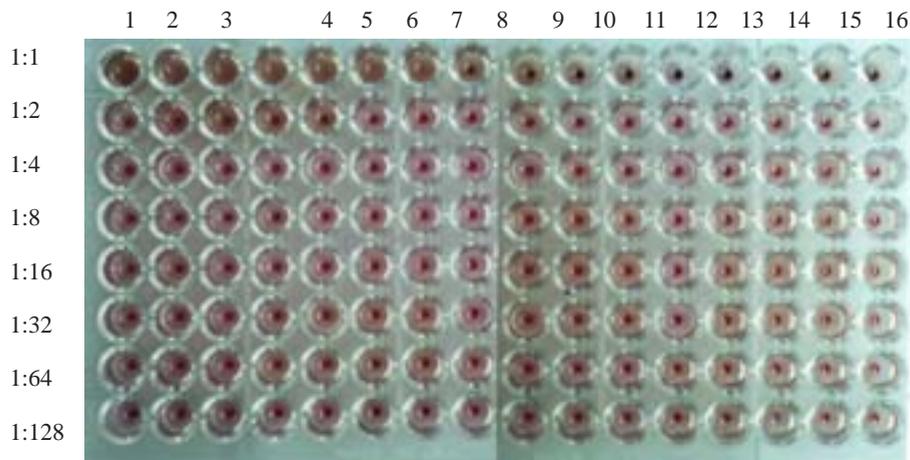
Fig 1–Detection of hemolytic activity of *O. tsutsugamushi* strains using sheep erythrocytes in a microplate assay. Lanes 1-7: cell pellet from *O. tsutsugamushi*-infected L929 cells diluted serially from 1: 1 to 1:128. Lane 8: cell pellet from uninfected L929 cells. Lanes 9-15: culture fluid from *O. tsutsugamushi*-infected L929 cells diluted serially from 1: 1 to 1:128. Lane 16: culture fluid from uninfected L929 cells.
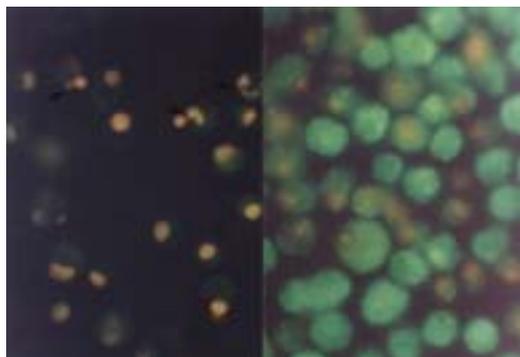

Fig 2–Acridine orange/ethidium bromide staining of Karp-infected L929 cells. Nuclei of cells infected by Karp strains in late apoptosis were condensed and fragmented (arrows) and stained bright yellow-orange by ethidium bromide (left). Uninfected cells were stained green by acridine; the scattered yellow granules visible in the cytoplasm were aggregated RNA granules (right) (Magnification, x400).
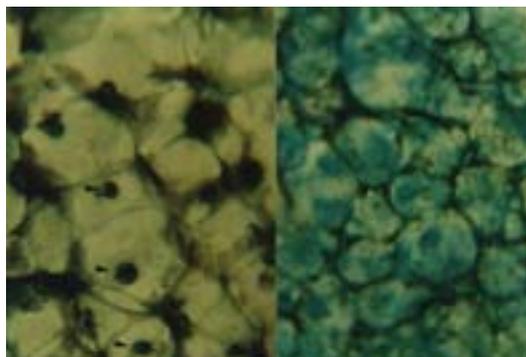

Fig 3–TUNEL assay of Gilliam-infected L929 cells. Apoptotic nuclei were stained dark brown (arrows) in Gilliam-infected L929 cells (left) but not in Kato-infected cells (right) (Magnification, x1000).

vity of *O. tsutsugamushi* were demonstrated by high concentrations of infected L929 cells, but no hemolytic activity was detected in the culture fluid (Fig 1).

The nuclear morphology of *O. tsutsugamushi*-infected cells on day 6, assessed by PI staining, showed a decrease in the size of the nucleus of some Karp- and Gilliam-infected cells compared to the control cells. Infected L929 cells demonstrated vacuolations in the perinuclear regions of L929 cells, while control cells had a well-defined, almost spherical, smooth plasma mem-

brane. At the later stage (8 days) of *O. tsutsuga-mushi* infection, the host cells were round and degradation of the nucleus in most of the infected cells was observed. Examination of AO/EB staining showed that the nuclei of most of the Karp- and Gilliam infected cells were stained bright yellow-orange by ethidium bromide (Fig 2), whereas normal cells were stained green. The Karp- and Gilliam-infected cells demonstrated condensed and fragmented nuclei. In the TUNEL assay, a large number of Karp- and Gilliam-infected cells were found to take up the label, with the apoptotic nuclei stained dark-brown (Fig 3), as compared to the uninfected (not demonstrated) and Kato-infected cells.

DISCUSSION

Because of experimental limitations imposed by the obligate intracellular nature of rickettsiae, little is known about specific virulence determinants utilized by this and other *Rickettsia* spp. All the eight *O. tsutsugamushi* strains used in this study caused cytopathic effects in L929 cells. However, a toxic effect was not demonstrated in any of the culture fluid of the infected L929 cells in this study. Low hemolytic activity was detected using approximately 10^4-10^5 rickettsiae-infected cells, but not the culture fluid of the infected cells. This suggests that the hemolytic and cytotoxic activities of *O. tsutsugamushi* are most likely cell-bound. There was no difference in the level of hemolytic and cytotoxic activities demonstrated by the *O. tsutsugamushi* strains regardless of the pathogenicity of the *O. tsutsugamushi* strains in mice (Fig 1).

Induction of apoptosis was observed in the Karp- and Gilliam-infected L929 using the PI uptake assay, AO/EB staining and TUNEL assay. Distinct morphological changes were observed in the nuclei of Karp- and Gilliam-infected cells. Future work will involve the identification of individual apoptosis-inducing *O. tsutsugamushi* components and the mechanism by which they induce apoptosis in L929 cells. This may add further knowledge of the pathogenicity of *O. tsutsugamushi*.

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