**Georgia Journal of Science**

Volume 74 *No. 2 Scholarly Contributions from the Membership and Others*

Article 9

2016

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**Cover Page Footnote**
ACKNOWLEDGMENTS This work was supported in part by funds from the Valdosta State University (VSU) Department of Biology, as well as grants to J.T. from the VSU Faculty Research Seed Grant Program, the VSU Graduate Faculty Scholarship Fund, and the VSU Center for Applied Research. This research was part of a thesis submitted by K.H.V. in partial fulfillment of the requirements for the Master of Science degree in Biology. We are grateful to Jonathon Audia, Archna Bhasin, and James Nienow for helpful discussions.

# VIABILITY OF *Rickettsia prowazekii* AFTER RICKETTSIA-MEDIATED KILLING OF GAMMA INTERFERON-PRETREATED, MACROPHAGE-LIKE RAW264.7 CELLS

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## ABSTRACT

*Rickettsia prowazekii*, the bacterium that causes epidemic typhus, mainly grows within endothelial cells and can also infect macrophages. Previous studies showed that pretreatment of cultured, murine macrophage-like RAW264.7 cells with gamma interferon, followed by *R. prowazekii* infection, leads to the death of many of the macrophages within several hours after infection. The present study examined the fate of the rickettsiae after macrophage death. Rickettsiae released from gamma interferon-pretreated, infected macrophage cultures, in which 83 ± 4% (mean ± standard deviation) of the macrophages were trypan blue-positive (dead), remained viable, as judged by their ability to infect and grow in untreated Vero cells (originally established from the kidney of an African green monkey). The growth of these rickettsiae was comparable to the growth of rickettsiae released from untreated, infected macrophage cultures, in which 1 ± 1% of the macrophages were trypan blue-positive. These data raise the possibility that gamma interferon, which is known to be an anti-rickettsial host defense, may, in some instances, contribute to the spread of *R. prowazekii* infection within a host.

***Keywords:*** rickettsia, interferon, macrophage

## INTRODUCTION

*Rickettsia prowazekii*, an obligate intracellular bacterium and the cause of louse-borne epidemic typhus in humans, multiplies within the cytoplasm of its host cells. It grows mainly in the microvascular endothelial cells; however, it can also grow in macrophages. Features of epidemic typhus include a high fever, headache, swelling of and damage to the endothelial cells, vasculitis, perivascular lesions consisting of macrophages and lymphocytes, increased permeability of the endothelium, edema, decreased blood volume, hemorrhages of the small blood vessels, and a skin rash (Bechah et al. 2008a, 2008b; Dumler 2012).

After *R. prowazekii* initially enters a host cell through induced phagocytosis, the rickettsia lyses the phagosomal membrane and generally grows to large numbers in the cytoplasm before eventually killing the host cell and spreading to other cells (Bechah et al. 2008a; 2008b; Sahni and Rydkina 2009). In some circumstances, however, *R. prowazekii* may kill its host cells prematurely--before large numbers of rickettsiae accumulate. For example, in gamma interferon-pretreated, mouse macrophage-like RAW264.7 cell cultures, many of the macrophages die (become permeable to trypan blue dye) within several hours after *R. prowazekii* infection (Turco and Winkler 1997). Premature host cell death also occurs in cultures of cytokine-treated, *R. prowazekii*-

infected, mouse fibroblastic L929 cells; however, it occurs later and is less extensive (Turco and Winkler 1984, 1993). This early host cell death is interesting because gamma interferon (IFN-γ) protects animals against rickettsial infections (Walker and Ismail 2008; Sahni et al. 2013), and because cytokine treatment of cultured cells inhibits intracellular growth of and kills *R. prowazekii* (Turco and Winkler 1988, 1997).

Macrophage-like RAW264.7 cells are useful models for studying *R. prowazekii*-macrophage interactions. Like human monocyte-derived macrophages (Gambrill and Wisseman 1973), untreated RAW264.7 cells support abundant growth of the virulent Breinl strain of *R. prowazekii*, but they markedly restrict the growth of the avirulent Madrid E strain (Turco and Winkler 1982).

After the discovery that IFN-γ-pretreated RAW264.7 cells are rapidly killed after infection with *R. prowazekii* (Turco and Winkler 1984), some additional studies focused on how this cell death might be occurring. Examination of the possible role of tumor necrosis factor alpha (TNF-α) revealed that both untreated and IFN-γ-pretreated RAW264.7 cells produce TNF-α during incubation with *R. prowazekii* bacteria (Turco and Winkler 1994). However, antibody-mediated neutralization of the TNF-α generated by IFN-γ-pretreated, infected RAW264.7 cells does not prevent macrophage death (Turco and Winkler 1994). The rapid killing of IFN-γ-pretreated, *R. prowazekii*-infected macrophages also does not depend on the macrophage respiratory burst (Turco et al. 1989), nor does it depend on nitric oxide synthase (Turco and Winkler 1994). In fact, pretreatment of macrophage-like RAW264.7 cells with both bacterial lipopolysaccharide and IFN-γ is associated with nitric oxide synthase-dependent inactivation of *R. prowazekii* and protection of the macrophages (Turco and Winkler 1994; Turco et al. 1998). Furthermore, exposure of rickettsiae to nitric oxide inactivates the rickettsiae and prevents them from infecting and killing IFN-γ-pretreated RAW264.7 cells (Turco et al. 1998). Thus, the mechanisms responsible for the rapid killing of IFN-γ-pretreated RAW264.7 cells after *R. prowazekii* infection are currently unknown.

Although the rapid killing of IFN-γ-pretreated RAW264.7 cells after infection requires viable rickettsiae (Turco and Winkler 1994), the fate of the rickettsiae themselves after the death of the macrophages is unknown. If the rickettsiae are killed along with their host cells, the premature macrophage death (if it occurs in vivo) would most likely benefit the host. On the other hand, the release of viable rickettsiae from dead macrophages could worsen the rickettsial infection. In the present study, we report that the rickettsiae remain viable after the death of the IFN-γ-pretreated, infected RAW264.7 cells.

## MATERIALS & METHODS

### Cultured Mammalian Cells and IFN-γ

The mouse fibroblastic L929 cell line and the mouse macrophage-like RAW264.7 cell line were provided by Jonathon Audia and Herbert Winkler (University of South Alabama). The Vero cell line, which was originally established from the kidney of an African green monkey, was provided by Dallas Ingram (Tifton Veterinary Diagnostic and Investigational Laboratory, University of Georgia). Recombinant murine IFN-γ derived from *Escherichia coli* ($4.7 \times 10^6$ units $mg^{-1}$) was a gift from Genentech Inc.

Cultured cells were grown at 35 °C in a humidified atmosphere of 5% carbon dioxide in air. Culture media were as follows: L929 cells, Eagle Minimal Essential Medium plus 10% newborn bovine serum (NBS) (Life Technologies Corporation); Vero cells, Eagle Minimal Essential Medium supplemented with nonessential amino acids (MEMNeaa) plus 5% fetal bovine serum (PAA Laboratories) and 5% NBS; RAW264.7 cells, high glucose Dulbecco Modified Eagle Medium (DUL) plus 10% NBS.

## *R. prowazekii* Suspensions

An initial sample of the Breinl strain of *R. prowazekii* was kindly provided by Jonathon Audia and Herbert Winkler (University of South Alabama). Rickettsial suspensions for use in experiments were prepared from heavily infected L929 cells. After the infected cells [four plates (150 mm diameter) or flasks (150 $cm^2$ area)] were washed once with sucrose-phosphate-glutamate solution (SPG) (0.218 M sucrose, 3.76 mM $KH_2PO_4$, 7.1 mM $K_2HPO_4$, 4.9 mM potassium glutamate, pH 7.0), they were scraped into SPG and centrifuged at 9,000 × *g* for 10 min at 5 °C. The resulting pellet was suspended in SPG and an equal volume of sterile glass beads (1 mm diameter) was added. The beads and cells were mixed vigorously on a vortex mixer for a total of 4 min (1 min vortexing, 1 min on ice, repeated three times). After the beads had settled, the cell lysate was removed to a separate tube and centrifuged at 300 × *g* for 5 min at 5 °C. After subsequent centrifugation of the supernatant fluid at 9,000 × *g* for 10 min at 5 °C, the pellet was resuspended in SPG (~7 ml), dispensed into vials, and frozen in liquid nitrogen.

Samples of two different rickettsial preparations were used in these experiments. Each preparation was tested prior to conducting experiments to determine the dilution most suitable for infecting the RAW264.7 cells. The highest dilution that killed approximately 80% of the IFN-γ-pretreated RAW264.7 cells was used in experiments. The dilution used was 1/500 for one of the rickettsial preparations and 1/1,000 for the second preparation.

## Preparation of Cell Cultures in 24-Well Plates and Treatment with IFN-γ

Sterile, round glass cover slips were placed in some of the wells for evaluation of the rickettsial infection. Duplicate wells were used for each treatment in a given experiment. RAW264.7 cells (suspended in DUL plus 5% NBS at a concentration of 5.5 × $10^5$ cells $ml^{-1}$) and Vero cells (suspended in MEMNeaa + 5% fetal bovine serum at a concentration of 3.2 × $10^5$ cells $ml^{-1}$) were dispensed at 0.4 ml per well and incubated overnight at 35 °C. Then 0.1 ml of IFN-γ diluted in the appropriate medium plus serum (AMS) was added per well of RAW264.7 cells as required; the final IFN-γ concentration was 25 units $ml^{-1}$. Wells of Vero cells and untreated control wells of RAW264.7 cells were given 0.1 ml of the AMS. After incubation for 22 to 25 more hours, the cultures were washed once with the AMS before being infected.

## Infection of Cultured Cells and Evaluation of Rickettsial Infection and Growth

After the cells were washed, 0.5 ml of rickettsiae diluted in the AMS was added per well. After centrifugation at 500 × *g* for 15 min at room temperature, the plates were incubated at 35 °C for 45 min. To check the initial infection, cover slips were removed from some wells and air dried immediately (at 1 hour). To assess rickettsial

growth, cover slips were removed from other wells after incubation of the cells at 35 °C for 48 additional hours. Cultured cells on cover slips were fixed and stained for rickettsiae by a modification of the Giménez method, as previously described (Wisseman et al. 1974; Turco and Winkler 1983).

Stained cells were examined with the oil immersion objective of a light microscope, and the number of rickettsiae within each of 100 cells was determined for each cover slip. The percentage of cells infected and the number of rickettsiae per infected cell were calculated. At the 49-hour time point, it was sometimes necessary to estimate the rickettsial numbers in cells that were heavily infected. If a cell contained more than 100 rickettsiae, a value of 100 was used in the calculations.

**Host Cell Viability Assessment**

Viability of the RAW264.7 cells in some wells was checked at 4 hours after the addition of the rickettsiae. To stain the cells, 125 µl of 0.4% trypan blue was added to the existing medium in each well, and a pipet was used for gentle mixing. After incubation at 35 °C for 8-10 min, the mixture was removed, and a small amount of phosphate-buffered saline was added. An inverted light microscope was used to examine at least 100 cells per well, and the percent trypan blue-positive (dead) cells was calculated.

**Determination of Rickettsial Viability after Death of the IFN-γ-Pretreated RAW264.7 Cells**

Viability of the rickettsiae harvested from untreated and IFN-γ-pretreated RAW264.7 cultures was evaluated by determining their ability to grow in untreated Vero cells. These African green monkey cells were used because they support the growth of *R. prowazekii* and were not expected to be sensitive to any residual mouse interferons that might have been present in the media and lysates from the infected RAW264.7 cultures (Samuel and Farris 1977; Schroder et al. 2004).

For each experiment, a vial of *R. prowazekii* Breinl was thawed and a portion was diluted in DUL plus 5% NBS and used to infect the RAW264.7 cells. The remainder was held on ice for later use as a control in the Vero cell infection. Some of the RAW264.7 cells were stained with trypan blue to assess RAW264.7 cell death at 4 hours after addition of the rickettsiae. In most experiments, the infected RAW264.7 cells were mechanically disrupted to release the rickettsiae. In these cases, the infected RAW264.7 cells were scraped from the bottoms of the wells into the existing media, and the contents of two duplicate wells were added to a 1-ml vial half-filled with 2.5 mm glass beads. After disruption of the cells in a Mini BeadBeater (BioSpec Products, Inc.) at 4,800 rpm for 90 s (30 s beating, 30 s on ice; repeated two times), each lysate was added to two wells of washed Vero cells. In a few experiments, the scraped cells and media from IFN-γ-pretreated RAW264.7 cell cultures were vortexed (30 s vortexing, 30 s on ice; repeated two times) rather than being disrupted by bead-beating before addition to washed Vero cell cultures. Vortexing was used to break apart the debris from the dead macrophages without lysing any remaining viable macrophages. Media removed from infected RAW264.7 cell cultures were also tested for viable rickettsiae. This was done by incubating washed Vero cells with culture media collected from infected RAW264.7 cells at 1 hour or at 4 hours after addition of the rickettsiae. (Medium collected from one well of infected RAW264.7 cells was added to one well of

washed Vero cells.) In all experiments, the control rickettsial sample that had been held on ice (since initiation of the macrophage infection) was also diluted and added to other washed Vero cells. After centrifugation of the Vero cells for 15 min and incubation for 45 additional minutes as described earlier, cover slips were removed to check the initial (1 hour) rickettsial infection. After incubation of the remaining Vero cell cultures for 48 additional hours, cover slips were removed to assess rickettsial growth.

### Statistical Analyses

Means and standard deviations are from at least two independent experiments with duplicate determinations per experiment. For statistical analysis, a one-way analysis of variance (for a particular parameter by treatments) and multiple comparisons of the treatments were performed using the Assistant in Minitab 16 Statitstical Software (Minitab Inc).

## RESULTS

The method for assessing rickettsial viability in the infected macrophage cultures was designed to maximize rickettsial recovery in order to facilitate comparison with the control rickettsial samples that were held on ice. At 4 hours after addition of the rickettsiae to the RAW264.7 cell cultures, the macrophages were scraped from the bottoms of their wells into the existing media, and the mixtures were subjected to bead-beating or vortexing before being added to Vero cell cultures. At this 4 hour time point, the percentages of trypan blue-positive cells in the untreated, infected macrophage cultures and in the IFN-γ-pretreated, infected macrophage cultures were 1 ± 1 and 83 ± 4 (means ± SD), respectively. Bead-beating was used to physically lyse any remaining, viable macrophages so that the growth of rickettsiae from untreated and IFN-γ-pretreated macrophages could be compared in Vero cells. Vortexing was used to break apart the debris from the dead, IFN-γ-pretreated macrophages without lysing any remaining viable macrophages. Comparison of the data for the vortexed and bead-beaten, IFN-γ-pretreated macrophage cultures was used to assess what portion of the rickettsiae that infected and grew within the Vero cells had come from the IFN-γ-pretreated macrophages that had died.

The rickettsiae from both untreated and IFN-γ-pretreated macrophages infected and grew well within the untreated Vero cells, as did the rickettsiae in the control samples that were held on ice from the time of the macrophage infection (Figure 1). In all cases, the average numbers of rickettsiae per infected Vero cell at 49 hours (which ranged from 50.7 to 61.0) were significantly greater than the corresponding averages at 1 hour (which ranged from 9.3 to 10.3) ($p < 0.01$). In addition, at a given time point, there were no significant differences between treatments for rickettsiae per infected cell. The average percentages of Vero cells infected with rickettsiae in these experiments ranged from 70.0 to 82.6. Rickettsiae harvested from bead-beaten macrophage cultures (whether untreated or IFN-γ-pretreated) were similar to the rickettsiae in the control samples in their ability to infect and grow in Vero cell cultures. Thus, recovery of rickettsiae from the bead-beaten macrophage cultures was very good. The similarities between the data for Vero cells incubated with rickettsiae from vortexed, IFN-γ-pretreated RAW264.7 cell cultures and the corresponding data for Vero cells incubated with rickettsiae from bead-beaten, IFN-γ-pretreated RAW264.7 cells indicated that most

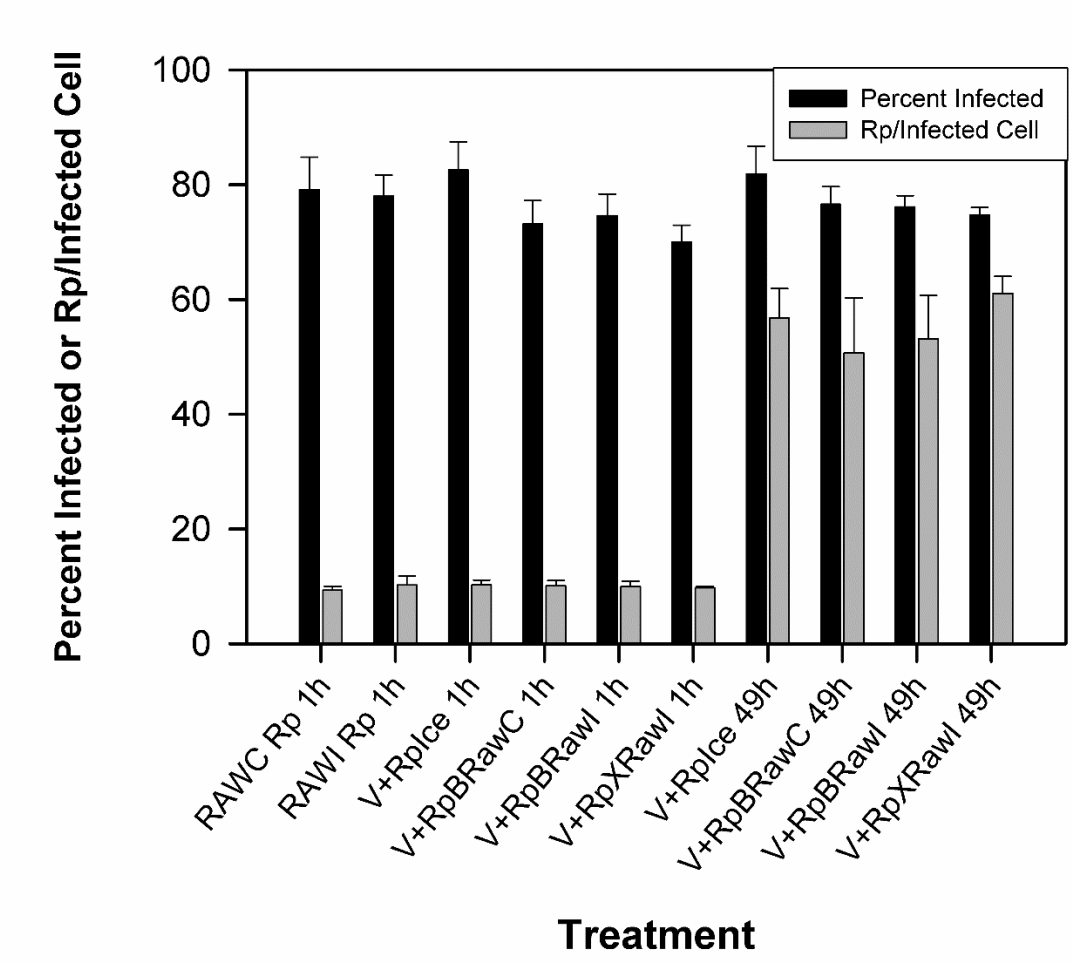


***Figure 1.*** Viability of *R. prowazekii* Breinl harvested from untreated and IFN-γ-pretreated RAW264.7 cell cultures, as determined by their subsequent growth in untreated Vero cells. To allow comparison, the first two pairs of bars show the untreated and IFN-γ-pretreated RAW264.7 cells. Rp, *R. prowazekii*; V, Vero cells; RAW, RAW264.7 cells; C, untreated control; I, IFN-γ; RpIce, Rp that was held on ice before dilution and addition to Vero cells; RpBRaw, Rp released from bead-beaten RAW cells; RpXRaw, Rp released from vortexed RAW cells; 1h, 1 hour after additions to Vero cells (or RAW cells); 49h, 49 hours after additions to Vero cells. Each value represents the mean ± standard deviation of four independent experiments with duplicate determinations, except for V+RpIce (three experiments with duplicate determinations) and V+RpXRawI (two experiments with duplicate determinations).

of the rickettsiae that infected and grew within the Vero cells must have come from the IFN-γ-pretreated RAW264.7 cells that had died. The statistical analysis revealed small, but statistically-significant, difference in percentage infected at the 1-hour time point between the Vero cell cultures incubated with the control rickettsial samples (82.6 ± 4.9), and the Vero cell cultures incubated with rickettsiae obtained from vortexed, IFN-γ-pretreated RAW264.7 cell cultures (70.0 ± 2.9) ($p < 0.01$). However, no significant differences in percentage infected were observed at a given time point for

Vero cell cultures infected with control rickettsial samples or with rickettsiae from bead-beaten macrophages, whether untreated or IFN-γ-pretreated. Taken together, the results indicate that the rickettsiae remained viable after the death of their IFN-γ-pretreated host macrophages.

Centrifugation was used in this study to increase the efficiency of infection of the cultured cells by the rickettsiae. However, to be certain that the viable rickettsiae detected in the macrophage cultures (Figure 1) did not represent rickettsiae that had remained in the culture media rather than having become associated with the RAW264.7 cells, additional experiments were performed in which media removed from infected RAW264.7 cultures were added to Vero cells to check for the presence of viable rickettsiae. These media were collected from the untreated and IFN-γ-pretreated RAW264.7 cells at 1 hour and 4 hours after addition of *R. prowazekii*. For the macrophage culture media collected at the 1-hour time point, none of the Vero cell cultures had detectable infected cells (Figure 2). For the media collected from the untreated and IFN-γ-pretreated RAW264.7 cell cultures at the 4-hour time point, only one of four Vero cell cultures had detectable infected cells; values for percent infected Vero cells (means ± SD) were 3.3 ± 6.5 and 7.3 ± 14.5 (respectively) at 1 hour; and 1.5 ± 3.0 and 2.3 ± 4.5 (respectively) at 49 hours. There was little evidence of rickettsial growth in the one of four Vero cell cultures in which rickettsiae were detected; values for rickettsiae per infected Vero cell were 6.2 and 9.8 (respectively) at 1 hour and only 14.0 and 15.9 (respectively) at 49 hours. The values for percent infected were dramatically and significantly lower than the corresponding percentages observed in Vero cell cultures incubated with the control rickettsiae that had been held on ice (86.0 ± 2.0 at 1 hour and 84.3 ± 6.1 at 49 hours) ($p < 0.01$). In addition, as expected, the control rickettsiae that had been held on ice grew well in the Vero cells; the rickettsiae per infected cell at 49 hours (53.6 ± 5.5) was significantly greater than the corresponding value at 1 hour (10.8 ± 0.5) ($p < 0.01$). Overall, these data indicate that most of the rickettsiae were associated with the untreated and IFN-γ-pretreated RAW264.7 cells at 1 hour and 4 hours after addition of the rickettsiae. Therefore, the viable rickettsiae that were detected in the bead-beaten and vortexed macrophage cultures (Figure 1) represented rickettsiae that had become associated with the RAW264.7 cells during the first hour (centrifugation for 15 min plus incubation for 45 min), and that remained macrophage-associated during the next 3 hours, rather than rickettsiae that had remained in the culture media.

## DISCUSSION

In the Vero cell cultures infected with rickettsiae in this study, there were significant and marked increases (approximately 5.5-fold) in the average numbers of rickettsiae per infected cell during the 2-day incubation period (Figure 1). The average percentages of Vero cells infected ranged from 70.0 to 82.6 for this 2-day incubation period; thus, no dramatic differences were observed for this parameter. The data collected are therefore consistent with the idea that the viability of the rickettsiae released from the IFN-γ-pretreated RAW264.7 cells is comparable to the viability of the control rickettsiae as well as the viability of the rickettsiae released from the untreated RAW264.7 cells.

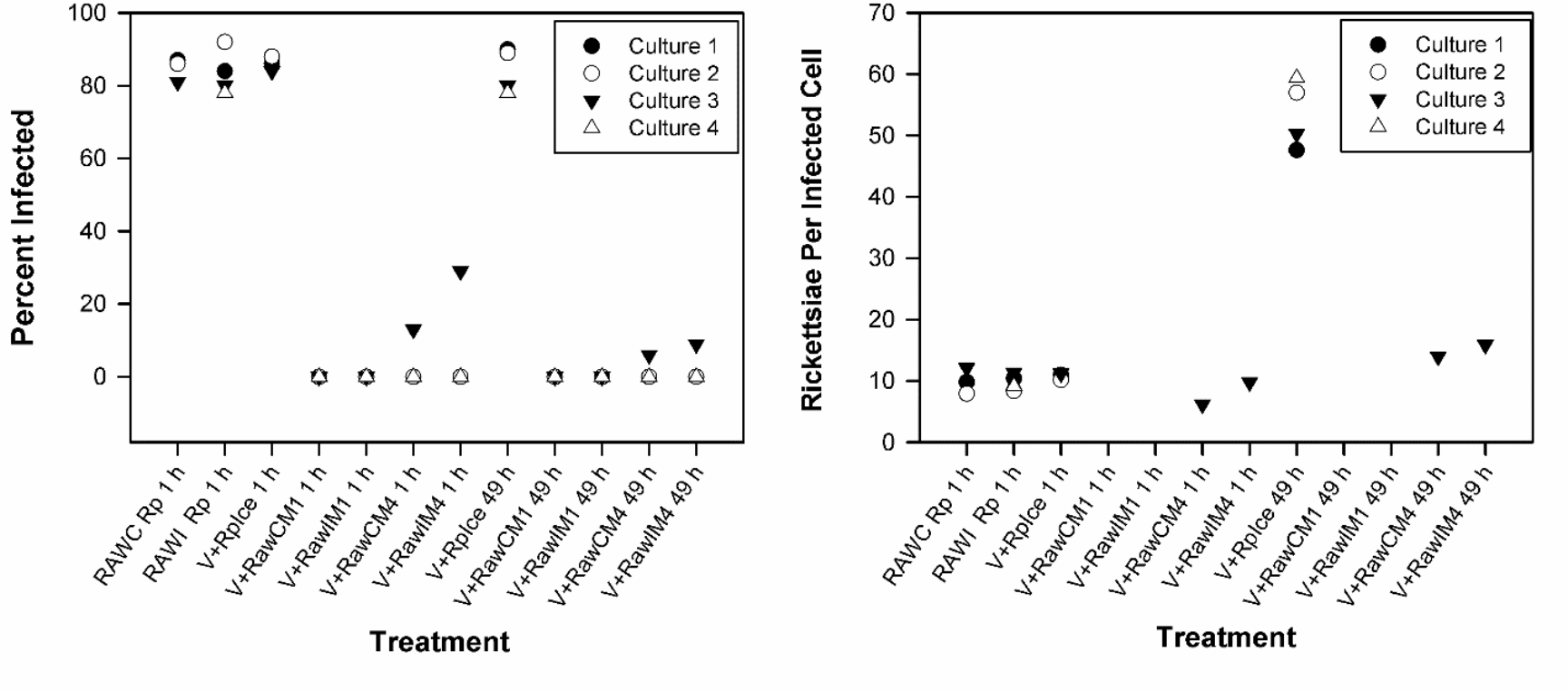


***Figure 2.*** Rickettsial infection in Vero cell cultures incubated with culture media collected from *R. prowazekii*-infected RAW264.7 cell cultures. To facilitate comparison, the first two columns in each chart show the untreated and IFN-γ-pretreated RAW264.7 cells; the third column shows the Vero cells incubated with the rickettsiae that were held on ice. Rp, *R. prowazekii*; V, Vero cells; RAW, RAW264.7 cells; C, untreated control; I, IFN-γ; RpIce, Rp that was held on ice before dilution and addition to Vero cells; RawM1 and RawM4, media removed from infected RAW cells 1 hour or 4 hours after initial addition of Rp; 1 h, 1 hour after additions to Vero cells (or RAW cells); 49 h, 49 h after additions to Vero cells.

It is important, however, to note some limitations of the methods used in this study. For example, rickettsial growth rates were not determined in the infected Vero cell cultures from the data collected because the Vero cells were dividing as ricketssial growth was occurring, and the numbers of Vero cells were not determined at the end of the incubation period. Other limitations include the following factors: (i) it was sometimes necessary to estimate the numbers of rickettsiae in heavily infected Vero cells; and (ii) a Vero cell with more than 100 rickettsiae was assigned a value of 100 for the calculations. Vero cells with 100 or more rickettsiae per cell were observed only at the 49 hour time point, and less than 0.2% of the cells had 100 or more rickettsiae at that time. Therefore, it was determined that this issue would have had a very minor impact (if any) on the data collected in this study.

Despite the limitations discussed, the results of this study indicate that the macrophage-associated *R. prowazekii* organisms remain viable after macrophage death has occurred in cultures of IFN-γ-pretreated, infected RAW264.7 cells. This finding is highly interesting since IFN-γ activates macrophages, which ideally function to destroy invading pathogens (Schroder et al. 2004). Thus, premature death of macrophages (if it occurs in vivo), could contribute to rickettsial pathogenesis by aiding rickettsial survival and possibly rickettsial dissemination. However, released, viable rickettsiae could be harmed by other host defenses in vivo. For example, anti-rickettsial antibodies could facilitate the eventual destruction of the rickettsiae by phagocytic cells (Beaman and Wisseman 1976; Turco and Winkler 1982; Keysary et al. 1989). Experiments are currently underway to reveal the mechanisms responsible for the premature destruction of the IFN-γ-pretreated macrophages by *R. prowazekii* and to determine if endothelial cells (the major cell type infected in vivo) are similarly affected.

## ACKNOWLEDGMENTS

This work was supported in part by funds from the Valdosta State University (VSU) Department of Biology, as well as grants to J.T. from the VSU Faculty Research Seed Grant Program, the VSU Graduate Faculty Scholarship Fund, and the VSU Center for Applied Research. This research was part of a thesis submitted by K.H.V. in partial fulfillment of the requirements for the Master of Science degree in biology. We are grateful to Jonathon Audia, Archna Bhasin, and James Nienow for helpful discussions.

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