Evaluation of Primary Blood Monocyte and Bone Marrow Cell Culture for the Isolation of *Rickettsia rickettsii*

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Received for publication 13 August 1975

*Rickettsia rickettsii* was isolated from experimentally infected guinea pigs by culture of blood monocytes and bone marrow cells, and from experimentally infected rhesus monkeys by blood monocyte culture. Rickettsiae were identified in monocyte-macrophage monolayers stained by Giménez or fluorescent antibody techniques. A total of 78 culture attempts were made from 20 guinea pigs and 16 monkeys. The success of isolation of *R. rickettsii* in culture was positively correlated with the numbers of rickettsiae present in the blood and bone marrow. In cultures derived from infected guinea pigs, rickettsiae were usually observed after 5 to 7 days of culture, and in monkey monocyte cultures they were usually observed within 3 to 5 days. Positive cultures were derived from guinea pigs and monkeys as early as the first day of fever and 1 to 3 days before the appearance of other clinical signs. Monocyte cultures became negative with the resolution of rickettsemia and concomitantly with the appearance of serum antibody. Monocyte culture isolation of *R. rickettsii* may be as sensitive for the detection of rickettsiae in blood and marrow as the intraperitoneal inoculation of guinea pigs or the plaque assay technique. Because of the simplicity of the method and because rickettsiae were often identified within 3 to 5 days after initiation, the monocyte culture technique may be useful in the early diagnosis of human rickettsial disease.

Currently the laboratory diagnosis of rickettsial disease is based upon the demonstration of increases in serum antibodies or the isolation of the infecting agent by animal inoculation (6, 8). In the patient, seroconversion does not occur for 7 to 14 days after the onset of illness and is thus of little value in management during the early stages of the disease (4, 6). Identification of the etiology by animal inoculation is also a prolonged process. Because the initial signs and symptoms of rickettsial disease may not be sufficiently distinctive to permit the physician to make a clinical diagnosis (5, 6), the importance of an early laboratory diagnosis is apparent. Serious secondary sequelae and fatality in rickettsioses such as Rocky Mountain spotted fever usually occur when there has been a delay in diagnosis and initiation of specific antibiotic therapy.

Heretofore, tissue culture techniques generally have not been found useful for the primary isolation of rickettsiae, presumably because of the small numbers of organisms present in the blood and the relatively slow rate of multiplication in cell culture (2, 8). However, Wike and Burgdorfer (15) have reported application of the plaque assay technique to the primary isolation of the etiological agent of Rocky Mountain spotted fever, *Rickettsia rickettsii*, from the blood of infected guinea pigs. In a preliminary report (3) we described the isolation and cultivation of *R. rickettsii* in tissue cultures of bone marrow cells and blood monocytes derived from infected guinea pigs. We identified rickettsiae as early as 3 to 5 days after the cultures were initiated, prompting our interest in this technique as a possible method for the more rapid diagnosis of human rickettsial infections.

In the present report we extended our experience using the blood monocyte culture system for the isolation of *R. rickettsii* from experimentally infected guinea pigs and evaluated its usefulness for the diagnosis of induced disease in rhesus monkeys. We also studied primary bone marrow cell cultures from infected guinea pigs since in our previous investigations we found that rickettsiae appeared earlier in marrow than in blood cultures. Success of culture isolation was related to the appearance of clinical signs, level of rickettsemia, and...
appearance of serum antibodies. Sensitivity of the culture techniques was compared with primary isolation by guinea pig inoculation and plaque assay isolation.

**MATERIALS AND METHODS**

**Animals.** Male Hartley guinea pigs, 400 to 500 g, were used. Rectal temperature and clinical signs were recorded daily. Animals were fed commercial guinea pig feed. Rhesus monkeys (Macaca mulatta) of both sexes and approximately 1 to 3 years old were offered commercial monkey feed supplemented with fruit daily.

**Microorganism.** *R. rickettsii*, Sheila Smith strain, was cultivated in embryonating chicken eggs. Yolk sac material was harvested, homogenized with Snyder I diluent to make a 20% (wt/vol) suspension, and stored at −70°C for no more than 150 days. Test guinea pigs were infected with this preparation by the intraperitoneal inoculation of 10^5 to 50% guinea pig intraperitoneal infectious doses (GPIPID). Brain heart infusion broth at 4°C was used as the diluent in all guinea pig studies (16).

Rhesus monkeys also were infected with the Sheila Smith strain of *R. rickettsii*. The source of *R. rickettsii* was either duck embryo cell cultures (12) or yolk sac material. Seed stocks were stored as 50% suspensions in sucrose PG at −70°C. Monkeys were inoculated intravenously with rickettsiae diluted in sucrose PG and quantitated by titration in embryonating chicken eggs. Doses ranged from 10^3 to 10^7. 50% embryo lethal doses. Thirty-one of the sixteen monkeys received doses between 10^0 and 10^7.

**Cell cultures.** Blood monocyte cultures were prepared from heparinized blood (20 U/ml). A 20-ml portion was obtained by cardiac puncture of anesthetized guinea pigs or 10 ml was obtained from the femoral vein of monkeys. Blood was centrifuged in 10-ml glass buffy-coat tubes (Vir Tis, Arthur H. Thomas Co., Philadelphia, Pa.) or in 15-ml plastic round-bottomed tubes at 500 × g for 7 to 15 min at 4°C. Buffy-coat cells were aspirated and resuspended in autologous plasma (5 ml in guinea pigs and 3 to 4 ml in monkeys), and 1.0-ml amounts of the suspension were placed in Leighton-type culture tubes with cover slips (35 by 9 mm).

Bone marrow cell cultures were prepared by aseptically removing both femurs from euthanized guinea pigs and flushing the marrow cavities with 7 ml of medium consisting of Eagle minimum essential medium with Earle salts, 30% normal guinea pig serum, and 0.1 mmol of L-glutamine per ml. Marrow cells were suspended in the medium by vigorous pipetting, and the suspension was used to seed five to six Leighton tubes.

Cultures of blood monocytes and bone marrow cells were incubated at 35°C for 18 to 24 hr, washed twice with Hanks balanced salt solution, and fed with 1.0 ml of tissue culture medium consisting of Eagle minimum essential medium, 30% normal guinea pig serum or rhesus monkey serum (Grand Island Biologicals, Grand Island, N.Y.), and 0.1 mmol of L-glutamine per ml. Tissue culture medium was changed every 48 to 72 hr. Incubation was continued at 35°C.

**Examination of cultures for rickettsiae.** At 3, 5, 7, and occasionally 4 and 10 days of culture cover slips were removed, washed three times with sterile Hanks balanced salt solution, and rapidly air dried. Cover slips were either heat fixed for staining by the Giménez method (10) or acetone fixed for 10 min at 20°C for fluorescent antibody (FA) staining. Conjugates used for the FA test included a homologous fluorescein-conjugated anti-*R. rickettsii* rabbit anti-serum and, as controls, anti-*R. tsutsugamushi* anti-serum and conjugated normal rabbit serum. Conjugates were incubated on cover slips for 30 min at 37°C in a humidified chamber, washed twice for 5 min each in phosphate-buffered saline, air dried, and mounted with buffered glycerin (pH 8.0). FA-stained cover slips were examined by using standard methods for ultraviolet light microscopy.

Giménez-stained cover slips were examined for the presence of rickettsiae at a magnification of ×1,000 for a period of 30 min per cover slip. In most cases this involved observing 500 to 1,000 cells. All cultures were first examined by using the Giménez stain. If rickettsiae-like organisms were seen, the results were confirmed by the FA technique. FA staining was used as a primary stain only if Giménez staining did not reveal rickettsiae within 7 days of culture or if bacterial organisms not resembling rickettsiae were observed in cultures.

Occasionally the infectivity of a particular culture was determined by the intraperitoneal inoculation of a guinea pig with 1 to 2 ml of tissue culture medium. Only the fluid component of the culture was inoculated.

**Quantitation of rickettsiae.** The numbers of infectious rickettsiae (GPIPID) in guinea pig blood or bone marrow suspensions used to initiate cultures were approximated by the intraperitoneal inoculation of guinea pigs with aliquots of the heparinized whole blood or bone marrow cell suspensions diluted in brain heart infusion broth. Dilutions of blood and bone marrow suspensions were the 10^-1 and 10^-3 dilutions in one guinea pig experiment and the 10^-1 and 10^-2 dilutions in the other two guinea pig experiments. Three guinea pigs were inoculated per dilution. Rectal temperature and clinical signs of guinea pigs inoculated at the 10^-1 dilution level were recorded daily for 10 days after inoculation. At 28 days after inoculation, surviving animals were sacrificed, and the serum was tested at a dilution of 1:40 for antibodies to *R. rickettsii*. All guinea pigs that died after inoculation had unequivocal gross signs of Rocky Mountain spotted fever infections.

Numbers of infectious rickettsiae in monkey blood were determined by the plaque assay technique described by Wike and Burgdorfer (15). Heparinized blood, either undiluted or diluted 10^-1 in cold brain heart infusion broth, was inoculated in 0.05-ml volumes onto chicken embryo cell cultures prepared as described by Wike et al. (17). Plaques appeared by day 5. A final agar overlay containing 0.1% neutral red was applied on day 6, and plaques were counted the following day.

**Serology.** Serum from guinea pigs was tested at a
dilution of 1:40 for antibodies to *R. rickettsii* by the indirect fluorescent antibody technique (7). Plasma from infected monkeys was tested at serial twofold dilutions by rickettsial microagglutination in microtiter plates as described by Fiset et al. (9). Rickettsiae for this test were grown in duck embryo cells in roller bottle cultures as described by Kenyon et al. (12). Rickettsiae were purified from cellular debris by differential centrifugation and two ether extractions. Rickettsial suspensions for antigen were standardized to 1 mg/ml. After incubation overnight at room temperature, a drop of acridine orange (1:5,000) was added to each well in the plate to facilitate reading.

**Experimental design.** In each of three separate experiments, eight to ten guinea pigs were inoculated with rickettsiae and one animal was sacrificed for culture of blood and marrow on days 3, 4, 5, 6, 7, 10, and 11 or 12 post-inoculation (p.i.). Cultures were prepared from at least one uninfected (control) guinea pig in each experiment. Serum was collected at the time of sacrifice and stored at −20 °C for indirect fluorescent antibody testing.

Monkeys infected with *R. rickettsii* were under study for a variety of reasons besides evaluation of monocyte cultures. Monkeys were infected in groups of four per experiment. Rectal temperature, clinical signs, and rickettsemia were determined daily. Monocyte cultures were prepared 3, 5, 7, and occasionally 10 days p.i. Serological determinations were performed at predetermined intervals after inoculation.

**RESULTS**

Mononuclear cells isolated from the buffy coat of guinea pigs and monkeys and from the bone marrow of guinea pigs had the morphological characteristics of cultured monocytes described by others (1, 13). Isolated monocytes and polymorphonuclear leukocytes readily attached to culture vessel surfaces. By 3 days of cultivation, the polymorphonuclear cells disappeared from the culture, leaving a population of monocytes that were avidly phagocytic and that underwent maturation to typical macrophages in vitro (Fig. 1A and B). The number of adherent cells appeared to be higher in cultures taken from animals with rickettsial infections than in control cultures.

A summary of the monocyte culture attempts is shown (Table 1). A total of 78 separate isolation attempts was made. Satisfactory cultures were defined as those without gross bacterial contamination, properly stained with Giménez and/or FA techniques, and with sufficient cells to enable counting of at least 250 macrophages. Unsatisfactory cultures could not be evaluated for a number of reasons, but the most common cause was an insufficient number of cells in the culture. Overall, four out of five cultures prepared were satisfactory. All results presented were obtained with cultures that were considered satisfactory.

Rickettsiae were readily visible in both Giménez- and FA-stained preparations; the latter is illustrated in Fig. 1C. The numbers of cells infected with rickettsiae and the number of rickettsiae per cell increased with time of culture. When rickettsiae were first observed, especially on days 3 and 4 of culture, careful searching was necessary to locate rickettsiae since often only one or two organisms were present in an infected cell. At this time, an estimated 0.5 to 2.0% of the cells was infected in positive cultures, whereas 5 to 50% was infected at 7 days of culture. Cells containing rickettsiae tended to be clustered as though there were spread from foci of infection in the monolayer. In several cases, monolayers 7 to 10 days old were destroyed completely by rickettsial infection. In a few cases, when Giménez-stained cultures could not be evaluated because of bacterial contamination, FA staining served as a satisfactory substitute for the primary observation as well as confirmation of *R. rickettsii* in cultures. However, because of the presence of nonspecific fluorescent material that could only be distinguished from *R. rickettsii* at higher powers of magnification (×400), we found that FA staining was no better for the primary observation of rickettsiae than was staining with Giménez.

The success of isolation of rickettsiae and the day of first observation of rickettsiae in culture were compared with the level of rickettsemia in guinea pigs and monkeys (Table 2). In all cases the percentage of cultures infected with rickettsiae increased proportionally to the level of rickettsemia. Rickettsiae were observed in all monocyte cultures prepared when the level of rickettsemia was greater than 10^2 GPIPIDo or plaque-forming units (PFU)/ml. In cultures derived from infected guinea pigs, there appeared to be no definite correlation between level of rickettsemia and the day of first observation of organisms in culture, but in monkey monocyte cultures such a correlation was apparent (Table 2).

The relationship of monocyte culture results to clinical signs, level of rickettsemia, and appearance of serum antibody in infected guinea pigs was evaluated. Fever occurred on the second or third day p.i. Rickettsiae could be isolated from blood and bone marrow both by inoculation of guinea pigs and by monocyte culture on the first or second day of fever, which was 2 to 3 days before the onset of other clinical signs of disease. Mean levels of rickettsiae in blood and marrow were relatively constant.
FIG. 1. (A) Blood monocyte culture 3 days after isolation from a guinea pig infected with *R. rickettsii*
Most of the cells have the morphology of mature macrophages. Monocytes isolated from the blood of rhesus monkeys had similar morphology. May-Grunwald Giemsa stain; original magnification, ×195. (B) Guineapig bone marrow cells after 7 days in culture. May-Grunwald Giemsa stain; original magnification, ×78 (C) *R. rickettsii* stained by specific fluorescent antibody in 7-day guinea pig bone marrow cell culture. Original magnification, ×780.
VOL. 12,

pigs on day 7 p.i., and at period (mean, \(10^{1.9}\) GPI/PID in/ml). During the 3- to 7-day p.i. period, 22 out of 24 cultures (92%) were positive. Seroconversion occurred in one of three guinea pigs on day 7 p.i., and at 10 and 11 days all animals that were examined were seropositive by indirect fluorescent antibody testing. Rickettsiae could not be recovered by inoculation after day 10 p.i., and only four of nine cultures (44%) prepared after day 7 were positive. The decrease in numbers of positive blood and marrow cultures correlated with defervescence of fever and progression of clinical signs to the late sequelae of \(R.\) rickettsiae infection. However, of eight cultures prepared from guinea pigs with indirect fluorescent antibody titers greater than 1:40, five were positive.

Out of a total of 33 cultures obtained from guinea pigs, three bone marrow cultures were negative for rickettsiae when the aliquots simultaneously inoculated into other guinea pigs revealed rickettsiae, and two cultures (one blood and one bone marrow) contained organisms when animal inoculations were negative. In the remaining 28 cultures, both cell cultures and animal inoculations were positive in 23 instances, and both were negative in five instances. Close observation of guinea pigs inoculated with blood or marrow revealed that animals that became ill developed fever by 4 to 5 days p.i. and developed typical clinical signs by 6 to 8 days p.i. In all of seven such attempts, inoculation of tissue culture medium from cultures in which rickettsiae were observed visually resulted in typical \(R.\) rickettsiae infection in guinea pigs.

Of the 16 monkeys from which monocyte cultures were prepared, we simultaneously determined rickettsetemia by plaque assay in ten monkeys and performed serial determination of antibody titers in five of these ten. Results of monocyte culture isolation of \(R.\) rickettsiae from infected rhesus monkeys have been partially summarized (Tables 1 and 2). Monkeys had a more acute disease after inoculation of \(R.\) rickettsiae than did guinea pigs. The clinical, laboratory, and histopathological results of the experimental infection of these rhesus monkeys with \(R.\) rickettsiae will be reported separately. Generally, animals became febrile 1 to 3 days p.i. and clinical signs, including depression, anorexia, dehydration, and hyperemia of mucous membranes, were observed by 2 to 4 days p.i. Monkeys were often rickettsemic 1 or 2 days p.i. before the first monocyte cultures were prepared on day 3. Monocyte cultures initiated 3

### Table 1. Summary of blood monocyte and bone marrow cell attempts

<table>
<thead>
<tr>
<th>Culture source</th>
<th>Total no. of culture attempts</th>
<th>Culture results</th>
<th>Satisfactory (no.)</th>
<th>Positive (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig blood (20)</td>
<td>20</td>
<td>12 (60)</td>
<td>11 (55)</td>
<td></td>
</tr>
<tr>
<td>Guinea pig bone marrow (20)</td>
<td>20</td>
<td>20 (100)</td>
<td>14 (70)</td>
<td></td>
</tr>
<tr>
<td>Rhesus monkey blood (16)</td>
<td>38</td>
<td>30 (78)</td>
<td>20 (67)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate number of animals.

* Numbers in parentheses indicate percentage.

* Satisfactory cultures were often obtained from animals without detectable rickettsetemia, as determined by methods other than monocyte culture.

### Table 2. Success of isolation of \(R.\) rickettsiae in monocyte and bone marrow cell cultures related to level of rickettsetemia in blood or marrow

<table>
<thead>
<tr>
<th>Culture source</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; GPI/PID in/ml or PFU/ml</th>
<th>No. of positive cultures/no. of satisfactory cultures</th>
<th>Day of culture rickettsetemia was first observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig blood</td>
<td>&lt;1.0</td>
<td>2/3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.0-2.0</td>
<td>3/4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt;2.0</td>
<td>6/6</td>
<td>2</td>
</tr>
<tr>
<td>Guinea pig bone marrow</td>
<td>&lt;1.0</td>
<td>1/5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.0-2.0</td>
<td>9/11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt;2.0</td>
<td>4/4</td>
<td>3</td>
</tr>
<tr>
<td>Monkey blood</td>
<td>&lt;1.0</td>
<td>4/10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.0-2.0</td>
<td>5/8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt;2.0</td>
<td>6/6</td>
<td>3</td>
</tr>
</tbody>
</table>

* Level of rickettsetemia in blood or marrow expressed as log<sub>10</sub> GPI/PID in/ml (guinea pigs) or log<sub>10</sub> PFU/ml (monkeys).

* Number of cultures.

* NE, Not examined on this day.
and 5 days p.i. were positive in 11 of 14 and 7 of 9 cultures initiated at those times, respectively. In only two of five attempts were cultures positive 7 days p.i. A comparison of the success of monocyte culture isolation to plaque assay isolation revealed that three monocyte culture attempts failed to detect rickettsiae when plaque assay was positive, and in four cases monocyte cultures revealed rickettsemia when plaque assay was negative (<10⁶ PFU/ml). The relationship between the appearance of serum antibodies to *R. rickettsii* and the success of monocyte culture isolation from infected monkeys is shown (Table 3). As serum antibody titers increased to significant levels (>1:4), we were unsuccessful in isolating rickettsiae in monocyte cultures, even though rickettsemia persisted in one of five monkeys. Monocyte cultures from infected rhesus monkeys usually had more cells per cover slip than did blood monocyte cultures from infected guinea pigs, and positive monkey cultures were recognized earlier after cultures were initiated (Table 2).

**DISCUSSION**

In a preliminary investigation (3), we demonstrated the isolation of *R. rickettsii* in blood and bone marrow monocyte cultures from infected guinea pigs and observed that rickettsiae were identified earlier in marrow cultures. This difference, which was probably due to experimental variation, was not observed in the present study. Bone marrow cultures were, however, more often satisfactory than were peripheral monocyte cultures. Most cultures that could not be evaluated had insufficient numbers of cells, which is a problem that we have encountered in our laboratory during attempts to cultivate blood monocytes from a variety of animal species. These failures may be related to the need for a minimum number of glass-adherent cells in the population used to seed the culture. The use of special gradients, such as Ficoll-hypaque, for the separation of blood mononuclear cells might help to overcome this problem, but the delay inherent in the technique as well as the procedures involved may reduce the chances of recovering viable rickettsiae. Since all marrow cultures could be evaluated, the bone marrow may be a better source of adherent cells for the primary cultivation of monocytes and rickettsial isolation. Bacterial contamination was a problem only in the preparation of guinea pig blood monocyte cultures, when bacteria may have become incorporated in the sample during cardiac puncture.

Levels of rickettsiae as assayed by guinea pig inoculation were roughly comparable in guinea pig blood and bone marrow cell suspension. However, guinea pig marrow suspensions were prepared by flushing two femoral marrow cavities with 7 ml of tissue culture medium. Assuming a marrow volume of 0.7 ml for both femurs of a 450-g guinea pig (11,18), this represents a 1:10 dilution and suggests that bone marrow may contain approximately 10-fold the number of viable rickettsiae found in peripheral blood. On several occasions we attempted, without success, to observe rickettsiae in Giménez-stained direct smears of guinea pig marrow suspensions. The possible advantage of examining bone marrow aspirates for the etiological agent in suspected rickettsial infection deserves consideration. Such examinations should include culture of marrow monocytes.

In both infected guinea pigs and monkeys, rickettsiae were isolated by monocyte culture, guinea pig inoculation, or plaque assay from specimens obtained on the first or second day of fever and 1 to 3 days before clinical signs became apparent. Also of diagnostic importance is the time necessary to confirm the presence of rickettsiae after inoculation of the specimen. Guinea pigs did not develop clinical signs presumptive for rickettsial infection until 6 to 8 days p.i. and, in those guinea pigs examined, serum antibodies were often not apparent until day 10 p.i. In our experience and as reported by Wike et al. (17), plaques caused by *R. rickettsii* can be observed with the plaque assay technique after 5 to 6 days of culture. Presumably, careful microscopic examination of cells removed from plaques at this time would reveal rickettsial organisms that could be definitively identified by FA staining. In more than 50% of all positive monocyte cultures prepared by us, rickettsiae were identified by day 5 of culture. Of monocyte cultures derived from infected

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**Table 3. Relationship between seroconversion and success of monocyte culture isolation of R. rickettsii in five monkeys studied serologically**

<table>
<thead>
<tr>
<th>Day p.i.</th>
<th>No. of monkeys serologically positive</th>
<th>No. of positive cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0/3</td>
<td>2/3</td>
</tr>
<tr>
<td>5</td>
<td>2/4</td>
<td>3/4</td>
</tr>
<tr>
<td>7</td>
<td>4/4</td>
<td>0/2</td>
</tr>
<tr>
<td>8–11</td>
<td>5/5</td>
<td>0/2</td>
</tr>
</tbody>
</table>

*Denominator signifies the number of animals having antibody determination and monocyte cultures on the day indicated. Serology was performed by microagglutination.

*Denominator signifies the number of satisfactory culture attempts made on the day indicated.
monkeys, more than 75% were recognized as being positive within 3 to 5 days. Further, it appears that the monocyte culture technique may be as sensitive for the detection of rickettsia as are guinea pig inoculation and the plaque assay. However, the dilutions used by us for animal inoculation and plaque assay allowed recovery of rickettsiae only at levels equal to or greater than $10^{0.5}$ GPPID$_{50}$/ml and $10^{1}$ PFU/ml, respectively.

The technique of culturing monocytes for the isolation of rickettsiae might be of greater potential diagnostic value if methods are found to reduce the time necessary to identify organisms in cultured cells. Use of bone marrow as the source of cells may provide a means whereby the species of rickettsia can be identified within 1 to 2 days after sampling of the patient. However, in its present form the technique may provide an inexpensive and simple method of laboratory diagnosis and should obviate the need for maintenance of animals or special cell cultures for rickettsial isolation. The limited availability of commercially prepared diagnostic reagents (14) makes the development and further study of alternative diagnostic methods, such as monocyte cultivation, of utmost importance.

ACKNOWLEDGMENTS

We wish to thank Leonard Beller, Thomas Johnson, and Alan Kleiman for excellent technical assistance, and E. H. Stephensen for help in preparation of the manuscript.

LITERATURE CITED