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TECHNICAL MANUSCRIPT 286

ASSAY OF COXIELLA BURNETII
BY ENUMERATION
OF IMMUNOFLUORESCENT INFECTED CELLS

Nicholas Hahon
Kenneth O. Cooke

MARCH 1966

UNITED STATES ARMY
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13. ABSTRACT (Continued)

The study of signal processing techniques for resolution improvement has continued and preliminary results of an azimuth compression technique are presented. Optical data processing techniques have been investigated for use in identifying obstacles.

Mathematical System Modeling for Computer Simulation

A digital simulation of an intermediate frequency amplifier has been accomplished which incorporates limiting and bandpass filtering. A simple horn antenna has been simulated which can be used as the building block for more complex aperture illumination functions.

Millimeter Radar Experiments

The statistical analysis of radar data obtained at Ballistics Research Laboratory, Aberdeen, Maryland, has been completed and the probability density functions are presented. The data has been correlated with the power spectral densities published in ECOM-01253-2.

The millimeter radar has been completed and is described in detail. Initial experiments have indicated that the system is very stable and can be used for reliable clutter and obstacle measurements. Data taken with the radar, at 70 GHz, is published and pertinent characteristics are discussed.

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ASSAY OF COXIELLA BURNETII BY ENUMERATION
OF IMMUNOFLUORESCENT INFECTED CELLS

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Project 1C622401A071

March 1966

ABSTRACT

A method for quantitative assay of C. burnetii was developed based on immunofluorescent staining of infected L cell monolayers and enumeration of cells containing fluorescent rickettsiae within 48 hours after inoculation. Adsorption of rickettsiae onto coverslip cell cultures was accelerated and highly efficient when augmented by centrifugal force. By this procedure, a proportionality was demonstrated between the number of infected cells and the volume of inoculum. The incubation period of 48 hours for inoculated cell cultures was established from sequential observations of the development of rickettsial infection of cells and from counts of infected cells. The relationship between rickettsial concentration and cell-infecting units of rickettsiae was linear; the distribution of infected cells was random. Compared with yolk sac inoculation of chick embryonated eggs, the infected cell-counting assay of C. burnetii was more rapid, precise, and sensitive.

I. INTRODUCTION

The development of quantitative and rapid procedures for the assay of viruses is one of many fundamental advances that have resulted from the use of cell cultures in virological studies. The usefulness of cell culture systems for the assay of rickettsiae, however, has been limited because of the slow growth of these infectious agents in cell monolayers and their apparent inability to produce readily discernible signs of infection, e.g., cytopathic effects or plaques. Recently, assays have been developed for a number of viruses¹⁻⁷ that are based on fluorescent antibody staining of infected cell monolayers and enumeration of cells containing fluorescent viral antigen. The ability to detect viral antigen in infected cells, usually within 24 hours, is an outstanding feature of the fluorescent cell-counting technique. In view of this and other advantages, an attempt was made to develop an assay of Coxiella burnetii, the rickettsial etiologic agent of Q fever, by employing this procedure.

The need for a rapid and quantitative assay of C. burnetii is evident from an examination of procedures presently employed for the purpose. Assays carried out in chick embryonated eggs, mice, or guinea pigs require prolonged intervals of incubation after inoculation, ranging from 14 to 40 days, before signs of infection are manifested and titration values may be determined.^{8,9} Weiss and Ritzky¹⁰ succeeded in developing a "focus count" method of titrating C. burnetii by inoculating chick embryo endodermal cell monolayers. Foci of infection were counted, however, from 5 to 10 days later. The feasibility of using an infected cell-counting assay of C. burnetii was supported by evidence that the rickettsiae may grow in a variety of cell cultures^{9,11-16} and that they are amenable to staining by fluorescent antibody.^{11,16,17}

This report describes the development and standardization of a quantitative assay of C. burnetii based on immunofluorescent staining of infected L cell monolayers and enumeration of cells containing fluorescent rickettsiae within 48 hours after inoculation.

II. MATERIALS AND METHODS

A. RICKETTSIAL STRAIN

The AD (California bovine) strain of Coxiella burnetii was used in this study. To prepare a stock suspension of rickettsiae, 2×10^3 ELD₅₀ (egg LD₅₀) were inoculated into the yolk sacs of 7-day chick embryonated eggs. Yolk sacs were harvested aseptically from embryos dying on the

7th to 9th days, made into a 20% suspension with distilled water, and purified by dextran sulfate precipitation.¹⁸ The rickettsial suspension was stored in 1-ml amounts at -60 C and had a titer of $10^{5.9}$ ELD₅₀/ml.

B. CELL STRAIN

The L strain of mouse fibroblasts was employed in the assay of rickettsiae. Nutrient medium for the cells consisted of mixture 199 containing 0.5% lactalbumin hydrolyzate and 10% heat-inactivated calf serum. Cells were maintained in mixture 199 and 5% calf serum. For the assay, cells were cultivated on circular coverslips (15-mm diameter) inserted in flat-bottomed glass vials (18 x 100 mm). One ml of cell suspension, containing 1.5×10^5 cells, was introduced onto coverslips that were then incubated at 35 C for 24 hours or until a complete cell monolayer was formed.

C. ASSAY OF RICKETTSIAE

Assays were usually carried out in triplicate. Dilutions of rickettsial suspension were prepared in maintenance medium and introduced in 0.2-ml volumes directly into vials containing coverslip cell cultures. Routinely, rickettsial adsorption was carried out by centrifugation at 500 x g for 15 min, at 23 to 25 C. For this procedure, vials were placed in slotted cups containing tube adapters, sealed with a screw-dome cover, and mounted on a 4-place, pin-type head. Centrifugation was performed in an International centrifuge, size 2, model V. Coverslip cultures were rinsed twice with maintenance medium following adsorption of rickettsiae; 1 ml of the medium was added then to each vial. After incubation at 35 C for 48 hours, coverslips were rinsed twice with cold phosphate-buffered saline (PBS), pH 7.2, fixed with cold (-60 C) acetone, and either prepared immediately for immunofluorescent staining and cell-counting or stored at -60 C for subsequent examination. In infected cell cultures stored for as long as 4 weeks under these conditions, rickettsiae did not exhibit any appreciable loss of fluorescence on staining.

D. IMMUNOFLOUORESCENT TECHNIQUES

From guinea pigs that had survived infection by *C. burnetii*, antiserum was pooled and conjugated with fluorescein isothiocyanate.¹⁹ The complement-fixing (CF) titer of the antiserum was 1:32. To reduce autofluorescence, the conjugated antiserum was passed through a column of Sephadex G-25.²⁰ A double conjugate stain consisting of 0.1 ml rhodamine bovine albumin and 1 ml undiluted fluorescein isothiocyanate-conjugated antiserum, diluted 1:3 with PBS, was also effective for detecting infected cells.

The direct fluorescent antibody technique was employed to demonstrate immunofluorescence of rickettsiae in infected cells. Fixed cell cultures were washed three times with PBS and stained with conjugate for 30 min. The coverslip cell cultures were then rinsed in two changes of PBS to remove excess conjugate and mounted in 20% glycerol in PBS.

Coverslip cell cultures were examined with an American Optical microscope equipped with a Fluorolume illuminator, model 645, Corning 5850 and Schott BG-12 exciter filters, and a barrier filter (E.K. 15).

At 645 X magnification, 1280 microscopic fields were contained in the area of a 15-mm coverslip with the optical system employed. For each coverslip cell culture, 50 microscopic fields were examined for cells containing fluorescent rickettsiae. To calculate the number of cell-infecting units (CIU) of rickettsiae per ml, the average number of cells containing fluorescent rickettsiae per field was multiplied by the number of fields per coverslip, the reciprocal of the dilution of inoculum, and a volume factor (for conversion to ml).

III. RESULTS

A. STANDARDIZATION OF ASSAY

1. Adsorption of Rickettsiae

Centrifugation of rickettsial inoculum onto coverslip cell cultures was investigated because previous studies with viruses indicated that the procedure promoted adsorption of inoculum.^{4,6,7,21} An experiment was performed to determine the rate of rickettsial adsorption onto cell monolayers during centrifugation (500 x g, 24 C) and stationary incubation (35 C). Two groups of vials containing coverslip cell cultures were inoculated with 0.2 ml of a 1:50 dilution of the stock rickettsial suspension; each group was subjected to a different condition of adsorption. At designated intervals, vials were removed and residual inoculum from each vial was introduced onto additional cell monolayers to measure unadsorbed rickettsiae. Residual inoculum was adsorbed onto cell monolayers by centrifugation at 500 x g for 30 min. Cell cultures exposed to initial or residual inocula were rinsed twice with maintenance medium and incubated with fresh medium at 35 C for 48 hours.

Results in Table 1 attest to the efficiency and rapidity of centrifugation for the adsorption of C. burnetii onto cell monolayers. Approximately 97% of the rickettsial inoculum was adsorbed within 15 min, although only 63% was adsorbed during 120 min of stationary incubation. Extension of the centrifugation period to 30 min, increasing the centrifugal force to 1000 x g, or centrifuging inoculum at 500 x g for 15 min followed by stationary incubation at 35 C for 2 hours did not appreciably enhance the quantity of rickettsiae adsorbed.

Further evidence of the efficiency of centrifugation for adsorption of rickettsial inoculum is shown in Table 2. The results reveal a proportionality between the number of cells containing fluorescent rickettsiae and volume of inoculum. In a similar experiment no proportionality was demonstrable between the number of infected cells and volume of inoculum when rickettsial inoculum was maintained at stationary incubation (35 C, 2 hr).

TABLE 1. CENTRIFUGATION VS. STATIONARY INCUBATION FOR ADSORPTION OF C. BURNETII ONTO L CELL MONOLAYERS

Minutes	Per Cent Inoculum Adsorbed	
	Centrifugation (500 x g)	Stationary (35 C)
5	78	30
10	93	ND ^a
15	97	39
30	98	41
60	ND	52
90	ND	60
120	ND	63

a. Not determined.

TABLE 2. PROPORTIONALITY BETWEEN VOLUME OF INOCULUM
AND CELL-INFECTING UNITS OF C. BURNETII^a

Volume, ml	Infected Cells/ 50 Fields	CIU, $\frac{b}{10^6/ml}$
0.1	201	2.5
0.2	414	2.5
0.4	800	2.5
0.5	1031	2.6
1.0	1970	2.4

- a. Rickettsiae adsorbed onto L cell coverslip cultures by centrifugation at 500 x g for 15 minutes, 24 C.
b. Cell-infecting units of rickettsiae.

2. Incubation Period

The incubation period is the interval between adsorption of rickettsiae and the appearance in primary infected cells of quantities of rickettsiae sufficient to be readily discerned by fluorescent antibody staining. It was determined from sequential observations of rickettsial growth and infected cell counts. To insure that only primary infected cells were counted, anti-Q immune serum was employed to prevent secondary infections through cell-to-cell spread of extracellular rickettsiae. A group of coverslip cell cultures were inoculated in the prescribed manner with a 10^{-2} dilution of rickettsial suspension and incubated at 35 C. Four hours later, the maintenance medium in one-half of the cell cultures was replaced with 1 ml of 1:10 dilution of anti-Q monkey immune serum. At 16, 21, 24, 48, and 72 hours of incubation, both cell cultures treated with maintenance medium or immune serum were fixed, stained with fluorescent antibody, and examined for rickettsiae or signs of intracellular infection.

The appearance of rickettsiae in cells was equivocal at 16 hours of incubation. At 21 and 24 hours, infected cells could be recognized by the presence of small, clear, well-defined cytoplasmic vacuoles containing fluorescent rickettsiae. Occasionally, fluorescent rickettsiae were noted adjacent to the cell nucleus or in small clusters in the cytoplasm. Vacuolated cells were more numerous and the vacuoles were larger and contained numerous rickettsiae at 48 hours (Figures 1, 2). Sometimes a cell contained more than one cytoplasmic vacuole. At 72 hours, the nucleus in some infected cells was distorted by an enlarged cytoplasmic vacuole that crowded it against the cell membrane. Fluorescent rickettsiae in vacuoles varied from a few to uncountable numbers. There



Figure 1. Typical Appearance of L Cell Infected with C. burnetii 48 Hours after Inoculation. Note cytoplasmic vacuole containing clump of fluorescent rickettsiae. Stained with fluorescein isothiocyanate-labeled anti-Q guinea pig serum. X 253.



Figure 2. L Cell Monolayer with Several Infected Cells Containing Cytoplasmic Vacuoles 48 Hours after Inoculation with C. burnetii. Stained with fluorescein isothiocyanate-labeled anti-Q guinea pig serum. X 129.

were signs that some vacuoles had ruptured to release rickettsiae into the extracellular surroundings. There was no evidence of intranuclear infection. The development of rickettsial infection in L cells was comparable to that described by Roberts and Downs.¹¹ In uninoculated cell cultures, an occasional cell possessed a cytoplasmic vacuole, but it was clear and contained no fluorescent particles. The most reliable criterion for distinguishing and enumerating infected cells was the presence of cytoplasmic vacuoles containing fluorescent rickettsiae. Counts of infected cells between cell cultures incubated with and without immune serum for 24 and 48 hours were equivalent. However, the number of infected cells at 48 hours was approximately twice the number at 24 hours. Infected cell counts from cell cultures incubated for 48 hours in the absence of immune serum were comparable to those of immune serum-treated cultures incubated for 72 hours. In 72-hour cell cultures that had not received immune serum, the number of infected cells was almost twice that obtained at 48 hours. The results indicate that 48 hours is an optimal incubation period for inoculated cell cultures to insure growth and accumulation of rickettsiae in primary infected cells.

That the reactions observed in cell monolayers were a consequence of infection with C. burnetii was confirmed further by serum-neutralization tests. Equal quantities of 1:50 dilution of rickettsial suspension and 1:10 dilution of anti-Q guinea pig or monkey immune serum were incubated at 35 C for 2 hours and then inoculated in 0.2-ml volume onto coverslip cell cultures in the prescribed manner. The number of vacuolated cells containing rickettsiae was reduced approximately 90% compared with cell cultures that received inoculum consisting of mixtures of rickettsiae and normal serum.

B. QUANTITATIVE EVALUATION OF ASSAY

1. Linearity with Dilution

Figure 3 demonstrates a linear relationship between twofold dilutions of rickettsial suspension and the number of cell-infecting units. These data suggest that cell infection was initiated by a single rickettsia and that only infected cells were enumerated.

2. Precision

In a single experiment, ten determinations were performed to estimate the precision of the assay for C. burnetii. Coverslip cell cultures were infected by a standard quantity of rickettsial inoculum and treated in the prescribed manner. The number of cell-infecting units of rickettsiae per ml of inoculum ranged from 1.0×10^6 to 1.3×10^6 with a mean of 1.1×10^6 and standard deviation of 0.11.

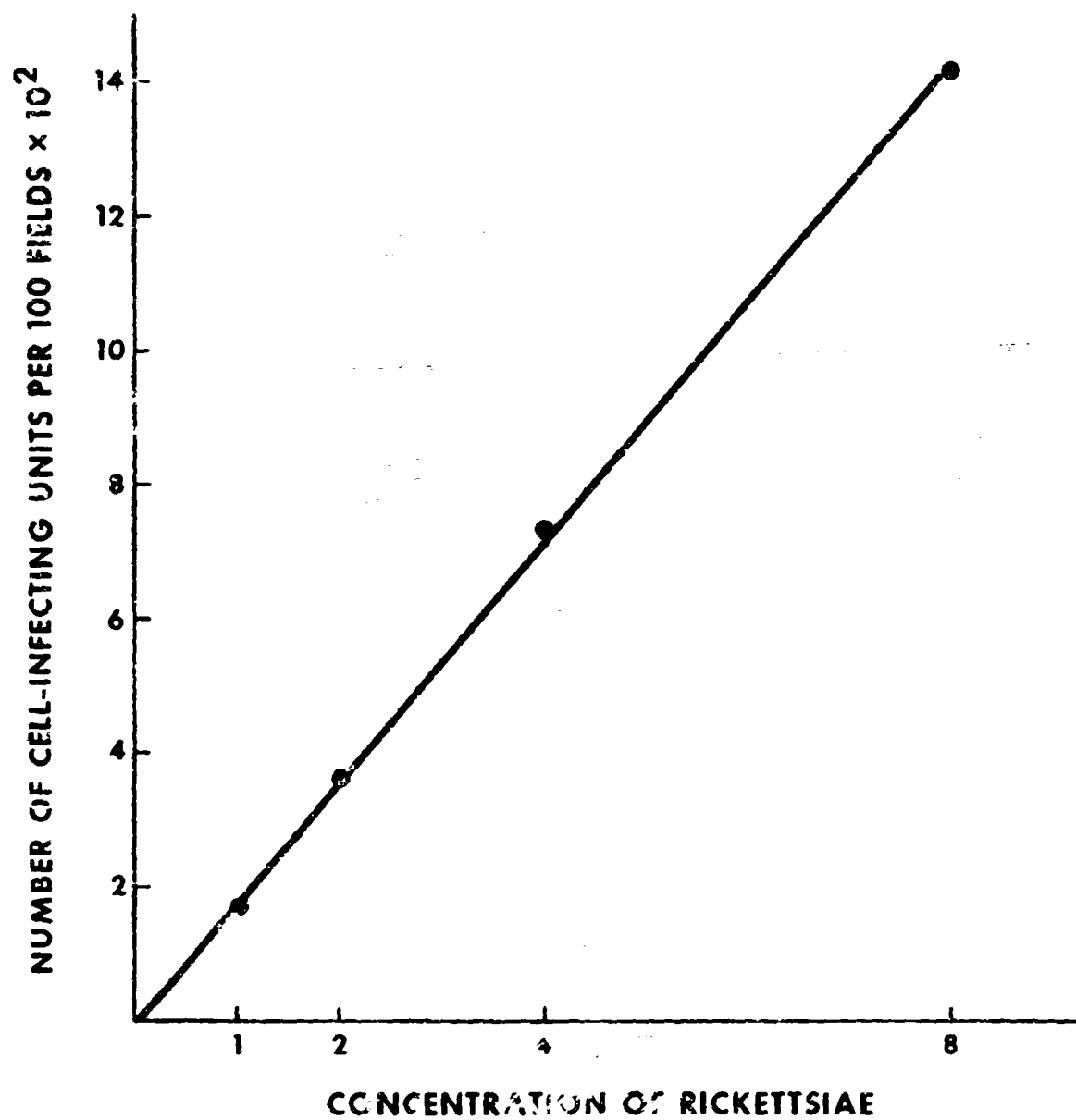


Figure 3. Linear function between the Number of Cell-Infecting Units and Concentration of *C. burnetii*.

3. Distribution of Infected Cells

The mode of distribution of cells containing fluorescent rickettsiae on a coverslip cell monolayer was determined by examining 100 random microscopic fields. The frequencies of fields containing infected cells correspond closely to the theoretical frequencies (Figure 4). The X^2 test of goodness of fit of the observed data to the theoretical Poisson distribution showed no significant difference (Probability slightly greater than 0.70 at d.f. = 6). Cells containing fluorescent rickettsiae were randomly distributed in infected cell monolayers.

4. Sensitivity

The sensitivity of the infected cell-counting procedure was compared with that of yolk-sac inoculation of chick embryonated eggs. The results in Table 3 indicate that the assay based on immunofluorescent staining and enumeration of infected cells exhibited less variability and gave a slightly higher mean titration value than the method of yolk-sac inoculation. The rapidity of the former procedure (48 hours) over that of the latter (14 days) was a singular advantage.

IV. DISCUSSION

The feasibility of employing an infected cell-counting procedure for the rapid assay of C. burnetii was established by the studies described here. The technique is based on immunofluorescent staining of infected L cell monolayers and enumeration of cells containing fluorescent rickettsiae. The adsorption of C. burnetii onto coverslip cell monolayers was accelerated and more efficient in comparison with stationary incubation. By the former procedure, 97% of the rickettsial inoculum was adsorbed within 15 min; by the latter, 63% was adsorbed within 2 hours. In the shortest contact time employed by Roberts and Downs¹¹ for the attachment and invasion of L cells by C. burnetii, they found that rickettsiae may enter L cells within 30 min. With the application of centrifugal force, a contact time of 5 min was sufficient to initiate cellular infection by a majority of C. burnetii in the inoculum (Table 1). Studies with another rickettsia, R. tzutsugamushi,¹² indicated that rickettsial penetration into mammalian cells was linear for the first 15 min and that invasion continued into cells that were already infected when lengthy periods of adsorption were employed. By the use of centrifugal force, this latter event is precluded because the time required to establish contact between rickettsiae and cells is markedly diminished and the resultant adsorption

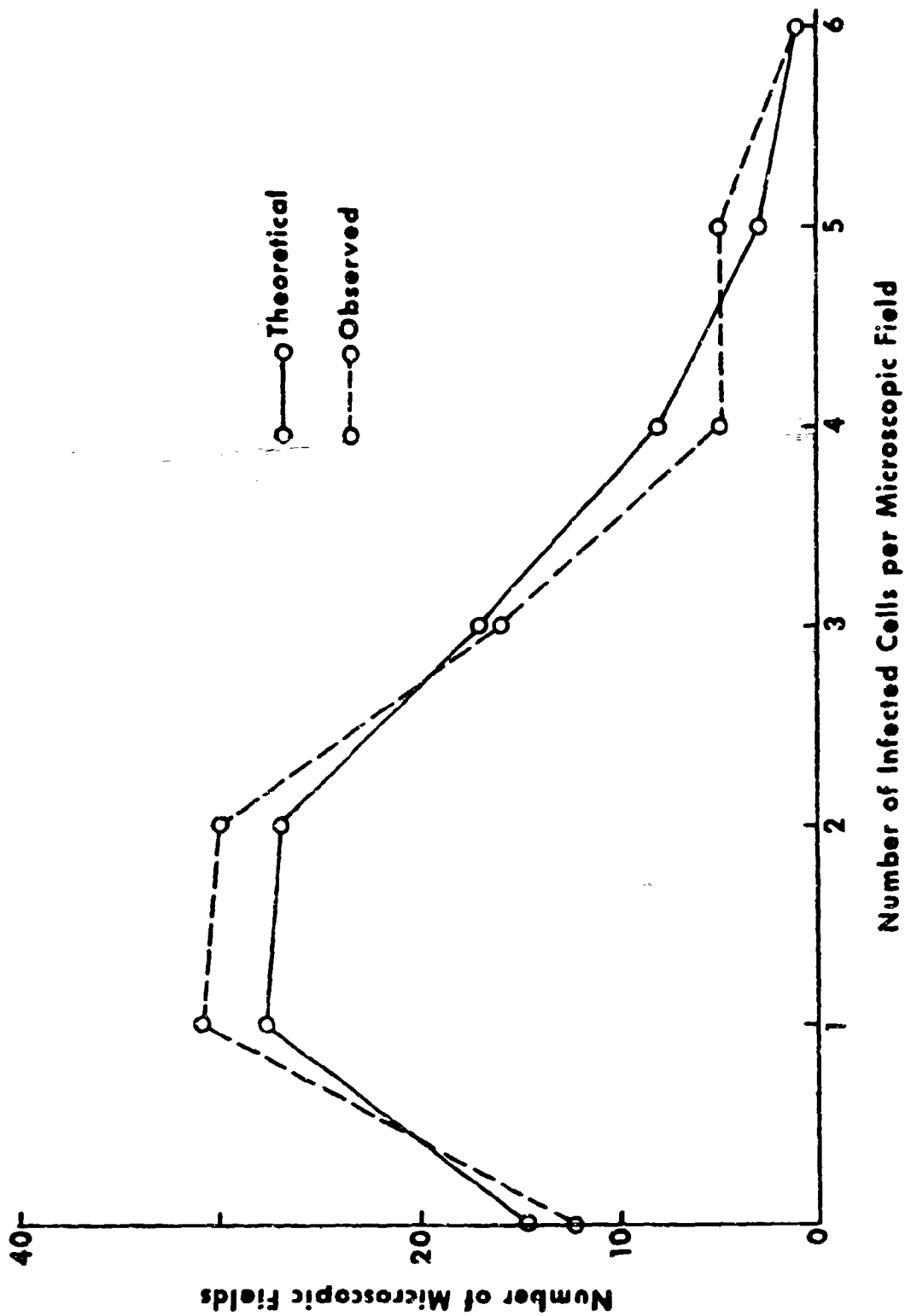


Figure 4. Frequency Distribution of L Cells Infected with G. burnetii.

of rickettsiae is highly efficient. The possibility that synchronous infection of cells may be achieved by this procedure assumes obvious importance, particularly in kinetic studies that attempt to define the early stages of rickettsial infection and growth. The finding that the relative efficiency of rickettsial adsorption as a function of the volume of inoculum when augmented by centrifugation is relevant also to the detection of rickettsiae from dilute suspensions and contributes to the sensitivity of the assay.

TABLE 3. COMPARISON OF TWO PROCEDURES
FOR THE ASSAY OF C. BURNETII

Assay Procedure	Mean $10^5/\text{ml}$	SD	SE Mean
Infected cell-counting	13.0 ^a /	± 0.27	± 0.20
Yolk sac inoc. chick embryos	8.7 ^b /	± 1.26	± 0.43

- a. Cell-infecting units of rickettsiae based on 7 titrations, determined at 48 hours.
- b. LD₅₀ units of rickettsiae based on seven titrations. Six-day embryonated eggs inoculated with 0.2 ml inoculum and incubated at 35 C for 14 days. LD₅₀ calculated by Reed and Muench formula.

In agreement with previous observations,¹¹ rickettsial infection of L cells was detected within 21 hours after inoculation. It is significant that the rickettsial inoculum used in this study, derived directly from infected eggs, elicited in an equivalent time period a specific cellular response comparable to that of rickettsial inoculum that had undergone 11 transfers in L cells.¹¹ The incubation period, the interval between inoculation of cell monolayers, and the appearance of readily recognizable signs of rickettsial infection of cells, was established as 48 hours. Sufficient numbers of rickettsiae accumulated in cytoplasmic vacuoles of inoculated L cell cultures at this time so that they could be easily distinguished by immunofluorescent staining. This was compatible with observations reported on the growth of C. burnetii in different cell lines.^{13,14} In addition to sequential observations of rickettsial manifestations of cellular infection, counts of infected cells helped to define the optimal period of incubation. Experimental use of anti-Q immune serum in inoculated cell cultures, to limit the spread of extracellular rickettsiae to other cells, insured

that only primary infected cells were counted. At 48 hours, infected cell counts, in the presence or absence of immune serum, were comparable with each other and with counts from immune serum-treated cell cultures incubated for 72 hours. In cultures that had not received immune serum and were incubated for 72 hours, infected cell counts were much higher, indicating that a secondary cycle of infection had occurred at least 24 hours earlier. It may be safely assumed that only primary infected cells were counted at 48 hours even though rickettsiae may have been released from infected cells shortly before this time. The few hours time that elapsed following the liberation of rickettsiae was too short to permit the development of visual signs of secondary cellular infection. The successful neutralization of extracellular rickettsiae in culture fluids and of rickettsial infectivity in vitro by immune serum, resulting in a significant reduction of infected cells in the latter instance, augurs the practicability of developing a rapid fluorescent cell-counting neutralization test to measure Q fever serum-neutralizing antibodies.

Paralleling the findings of fluorescent cell-counting techniques for viruses,¹⁻⁷ a linear relationship was demonstrated between the concentration of C. burnetii and the number of infected cells; the distribution of infected cells in cell monolayers followed the Poisson law. The evidence suggests that infection of individual cells was initiated by single rickettsia or aggregates not divisible by dilution. In precision, the infected cell-counting assay of C. burnetii was comparable to similar techniques employed with viral agents.^{4,6,7} On the basis of sensitivity and rapidity, the infected cell-counting procedure was superior to the method of yolk-sac inoculation of chick embryonated eggs. The assay technique described in this report may prove useful in experimental studies that attempt to elucidate quantitatively the complex mechanisms involved in rickettsial infection, growth, and nutrition at the cellular level.

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