GROWTH OF *COXIELLA BURNETII* IN MONOLAYER CULTURES OF CHICK EMBRYO ENTODERMAL CELLS

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Burnet (1938) and Cox and Bell (1939) cultivated *Coxiella burnetii* in tissue cultures of the Maitland type. Burnet reported satisfactory results with cultures of pooled chick embryo viscera, but not with skeletal or nervous tissues or single viscera. Cox and Bell noted that, of the chick embryo tissues, yolk sacs yielded best and most consistent growth. However, in both series of experiments, multiplication of the rickettsiae proceeded slowly and reached a peak during the second week after inoculation. Even under the best conditions, tissue cultures appeared to be less susceptible to infection than embryonated eggs.

This investigation was prompted by the belief that tissue cultures, although not the most satisfactory means of cultivating *C. burnetii*, would yield valuable information on the factors influencing its growth. Monolayer cultures of entodermal cells, derived from the avascular membranes of 4-day-old chick embryos, proved to be of value for the study of viruses of the psittacosis group (Weiss and Huang, 1954). The same type of cultures were used for the development of a quantitative method of study of *C. burnetii*. This paper describes the method and reports a few experiments which contributed to its formulation and improvement. It also illustrates possible applications.[3]

MATERIALS AND METHODS

The California strain of *C. burnetii* was used in these experiments. The strain was maintained in chick embryos and pools were prepared from infected yolk sacs. The chick embryo LD_{50} of the pools ranged from 10^{-5} to 10^{-6}. The chick embryo infectivity endpoints were not determined, but, on the basis of examination of smears of surviving embryos and of the work of Wagner and Gordon (1950), they were estimated to have been approximately 10^{-10}.

Fertile White Leghorn eggs and chicken serum and plasma were obtained from hens and roosters maintained on an antibiotic-free diet. Titrations of rickettsiae were carried out by yolk sac inoculation of 5-day-old embryos, which were then maintained and observed for a period of 14 days at 37 C. The procedures outlined by Scherer (1955) were followed for the preparation of balanced salt solution (BSS), chicken serum (CS), chicken plasma (CP), and embryo extract (EE). Medium Morgan 199 (199), horse serum (Ho), and HeLa cell nutrient (He), containing 25 per cent human serum, chick embryo constituents, BSS, and antibiotics, were obtained from Microbiological Associates, Inc. Sheep serum (Sh) was obtained locally.

The technique for the explantation of the avascular membranes of 4-day-old chick embryos was described by Weiss and Huang (1954). Unless otherwise indicated, the following procedure of inoculation and cultivation of the explants was used: The explants were divided in groups of 12, each group placed in a 125-ml Erlenmeyer flask and suspended in a mixture of 12 ml of nutrient medium (25 per cent chicken serum in balanced salt solution) and 3 ml of rickettsial pool appropriately diluted in BSS. The flasks were then shaken on a Brunswick mechanical shaker for 1 hr at a speed reduced to 90 oscillations per min. Each explant was then embedded on a 22 × 11 mm coverslip in an amount of plasma barely sufficient to insure adherence of the explant to the glass, placed in a Porter flask, 1.25 ml of the nutrient-inoculum mixture from the Erlenmeyer flask added, and incubated at 37 C. Single sheets of cells were usually formed by the third day; they expanded during the following 4 to 5 days to cover an area of approximately 1 cm^2. After variable periods of

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[3] Some of this material was presented previously (Weiss and Pietryk, 1954).

time, usually 7 days, the cultures were fixed, stained, and examined.

Most of the cultures were fixed in absolute methanol and stained by the May-Gruenwald-Giemsa procedure as outlined by Scherer (1955). Macchiavello's staining technique was adapted to the staining of cultures as follows: The cultures were fixed in their Porter flasks in 10 per cent neutral formalin for 1 hr or longer. They were then washed in 3 changes of tap water and stained in Columbia staining jars with basic fuchsin, 0.25 per cent aqueous buffered to pH 7.4, for ½ to 1 hr at 37 C. Differentiation was accomplished by immersing the cultures in 2 changes of 0.5 per cent citric acid for 2 to 10 min and was stopped with phosphate buffer, pH 7.4. The cultures were then counterstained in 0.5 per cent aqueous buffered methylene blue, dehydrated and cleared in 2 changes each of acetone, acetone-xylene, and xylene, and mounted on slides with permount.

EXPERIMENTAL RESULTS

Morphological observations. C. burnetii appeared to grow exclusively in the cytoplasm of the entodermal cells. In a few cases they were detected as early as 3 days after inoculation, but usually they were not recognized until the fourth day, and did not appear to be numerous in the infected cells until the fifth day.

In most cases the individual rickettsiae remained fairly well separated (figure 1), but packets of organisms were not uncommonly seen. Infection appeared to start in a large vacuole of the host cell. In contrast to feline pneumonitis virus (Weiss and Huang, 1954), *C. burnetii* produced definite cytopathological changes throughout the course of infection. The many small vacuoles of the host cells coalesced to form few large ones and the foamy structure of the cell was gradually converted into one large vacuole loaded with rickettsiae. The nucleus was apparently compressed by the surrounding infected vacuoles, decreased in size, eventually became pyknotic and disappeared. A fully infected cell is shown in figure 1.

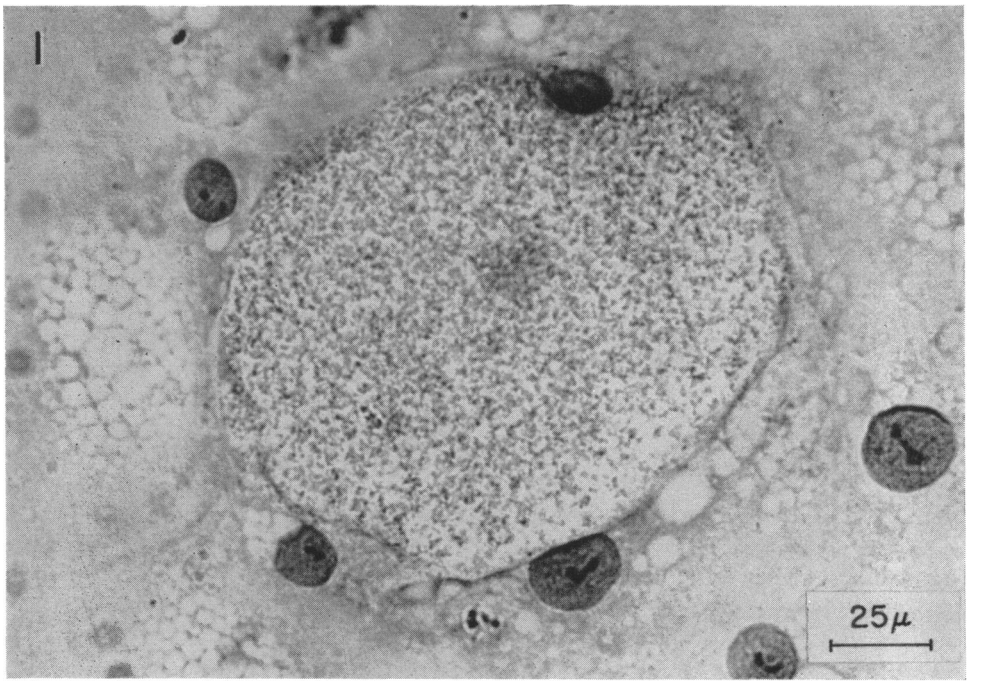
When dilute inocula were used, infected cells appeared to be well isolated during the first few days of infection. Subsequently, neighboring cells were also infected. Cell to cell infection apparently occurred at variable times during the course of intracellular multiplication and proceeded at a variable speed. However, infection usually remained confined to a few contiguous cells and discrete foci of infection were formed (figure 2). Cells appeared to provide an environment progressively less favorable for rickettsial growth and in undisturbed cultures the foci of infection did not generally enlarge after 10 days.

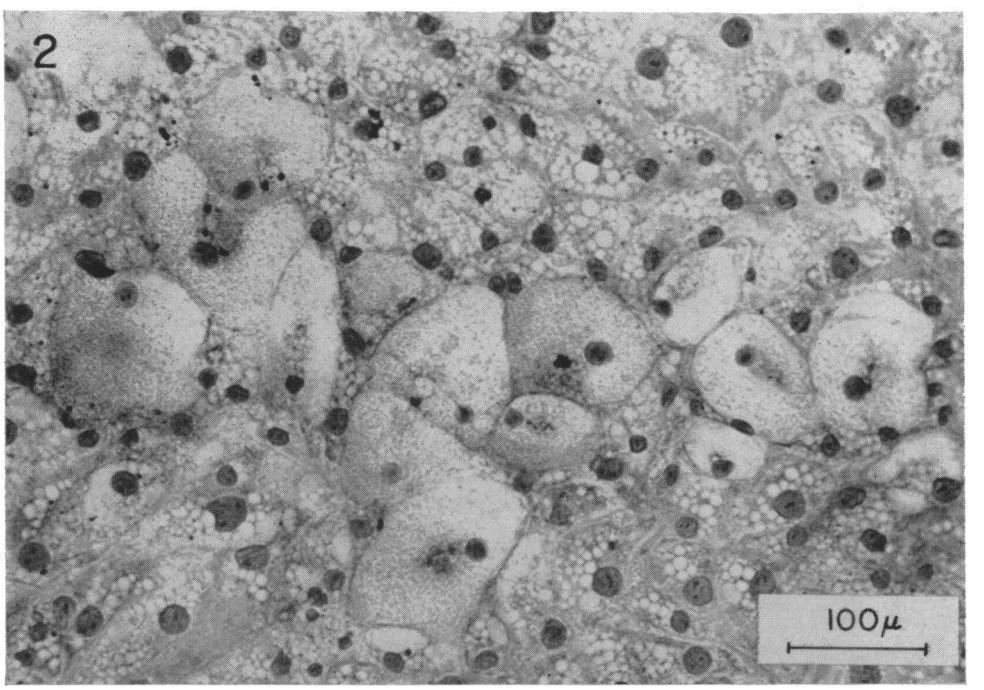
Entodermal cells obviously varied in their susceptibility to infection. Possibly, cells with large vacuoles and cells adhering to the original explant were more frequently infected than others, but a consistent association between morphological cell type and rickettsial growth could not be established.

When stained by the May-Gruenwald-Giemsa method, the rickettsiae retained some of the eosin as well as azure II and assumed various shades of purple. In many cases excellent contrast between the rickettsiae and tissue elements was obtained by the Macchiavello stain, but occasionally the rickettsiae failed to retain the basic fuchsin.

The focus count method of titration (FC). The above described observations prompted the development of a tissue culture method of quantitation of *C. burnetii*. The method was based on the assumption that a focus of infection generally represented a clone and, therefore, the number of foci of infection was proportional to the concentration of the rickettsiae in the inoculum. Since the time varied at which cell to cell infection began, foci of infection, rather than infected cells were counted. Foci of infection included sometimes 1, often 2 to 10, occasionally more than 10 contiguous infected cells. Since foci were not always clearly separated from each other in heavily infected cultures, infected cells in immediate proximity to each other were arbitrarily considered as part of one focus, while infected cells separated by at least one uninfected cell were counted as two foci. The seventh day after inoculation was selected as the time of examination of the cultures, although, because of the slow progress of the infection, other time intervals could have been equally satisfactory. In some cases, cultures were examined at 5 or 10 days.

Table 1 gives two examples of titrations by the focus count (FC) compared to tissue culture infectivity and chick embryo lethality. The groups of cultures receiving a moderately high concentration of the inoculum, 10^{-5} and 10^{-6},





Figures 1 and 2. Photomicrographs from a 10-day-old culture infected with *Coxiella burnetii* and stained with May-Gruenwald-Giemsa, kindly taken by Dr. R. B. Williams, Pathology Division of the Naval Medical Research Institute. *Figure 1.* Infected cell. Magnification 540×. *Figure 2.* Focus of infection involving 15 cells. The infected cells are readily recognized by their granular appearance. Magnification 180×.

TABLE 1

The focus count (FC) compared to other methods of titration of Coxiella burnetii

Experiment No.	Inverse Log. Dilution	Focus Counts	No. Infected Cultures/Total No. Cultures	Per cent Infection Expected From FC	Inverse Log. Titer		
					Tissue Culture		Chick Embryo
					FC	ID_{50}	LD_{50}
I (Pool 1)	5	16, 20, 22, 22, 23, 24, 29, 43, 47, 47, 63 (Mean = 32*)	11/11	100	6.7	6.9	5.4
	6	(Numbers too small to	9/9	96			
	7	be significant)	4/11	28			
	8		1/11	3			
	9	0	0/11	<1			
II (Pool 2)	6	16, 24, 25, 28, 38, 49 (Mean = 30†)	6/6	100	7.7	7.7	6.0
	7	(Numbers too small to	8/8	95			
	8	be significant)	2/8	26			
	9	0	0/8	3			

* Chi square = 20.77. Probability of random distribution = 3 per cent.

† Chi square = 22.9. Probability of random distribution < 1 per cent.

pools 1 and 2, respectively, were used for the determination of the FC in each case. The other groups were used for the calculation of the tissue culture ID_{50} by the Reed and Muench method (1938). For purposes of comparison, the probability of infection in each group and the tissue culture ID_{50} were calculated from the FC averages, on the assumption of a Poisson distribution of the rickettsiae among the cultures of the various groups.

As shown in table 1, a close agreement was obtained between FC and infectivity titration in tissue cultures. The results support the hypothesis that each focus represented a clone of rickettsiae. With either pool the rickettsial titers obtained by tissue cultures were about 30 times higher than chick embryo LD_{50}, but considerably lower than the chick embryo ID_{50} (Wagner and Gordon, 1950). The variations (expressed by the chi square) among individual FC of the same group were relatively high and indicated that factors other than the normal distribution influenced the FC. Although this is not surprising, because of the nature of the experiments, a state of aggregation of the particles was possibly an important source of variation because crude yolk sac suspensions which had undergone only one light centrifugation were used. In spite of the above described shortcoming, it appeared that the FC was a far more sensitive and accurate method of titration of *C. burnetii* than the determination of the chick embryo LD_{50}.

Factors influencing the FC. The experiments reported below were carried out in the course of development of the FC method, but did not clarify the conditions favoring the growth of *C. burnetii*. Understanding these conditions was complicated by the fact that they did not necessarily coincide with those favoring the host cells. For example, in any one group of identically treated cultures the FC varied independently of the size of the colony and the physiological conditions of the cells, in contrast to the behavior of feline pneumonitis virus in the same type of host cell (Weiss and Huang, 1954).

As shown in table 2, it appeared that CS_{25} (chicken serum, 1 part; Hanks' balanced salt solution, 3 parts) was the most satisfactory and simplest of the media studied for both the host cells and rickettsiae. Growth was influenced, to some extent, by the concentration of chicken serum, 25 being somewhat more favorable than 5 per cent (experiment III, groups 1 and 2). However, serum was not essential, because in an experiment not shown in table 2, growth was obtained in 199 without serum. Host cells and rickettsiae were not influenced in the same fashion by the nature of the media. For example, CS_5 diluted in 199 instead of BSS, although producing a lower FC, supported colony development

TABLE 2

Effect of nutrient on focus count

Experiment No.	Group	No. Cultures	Medium	Focus Count: Mean ± standard error	Significant Differences (with Group 1)
III	1	8	$CS_{25}BSS$	80 ± 9.3	
	2	9	CS_5199	46 ± 7.8	*
	3	8	$CS_{10}199$	58 ± 15.6	
	4	9	$CS_{25}199$	72 ± 21.3	
IV	1	9	$CS_{25}BSS$	33 ± 5.0	
	2	10	$Ho_{25}BSS$	45 ± 8.8	
	3	10	$He_{25}BSS$	10 ± 3.1	*
	4	10	$Sh_{25}BSS$	12 ± 3.7	*

as well as CS_{25} diluted in BSS. Conversely, host cells bathed in $CS_{25}199$ (group 4) were more intensely stained and produced larger colonies than those receiving $CS_{25}BSS$ (group 1), but the FC were approximately the same. Experiment IV has another example of the same phenomenon. Entodermal cells were far more stable and developed into considerably larger colonies in homologous than in heterologous serum. However, the FC of group 2 nourished by horse serum was at least as high as that of the group receiving chicken serum. Human and sheep sera, however, were less favorable for the rickettsiae. The human serum medium contained 50 units each of penicillin and streptomycin, which might have influenced growth.

TABLE 3

Effect of tissue-nutrient ratio on focus count (Experiment V)

Group	No. Cultures	No. Explants per Culture	Focus Count: Per explant: Mean ± standard error	Per explant: Probability (with group 1)	Per culture (calculated): Mean ± standard error	Per culture (calculated): Probability (with group 1)
1	7	1	41±5.6		41±5.6	
2	7	2	28±4.5	0.10	56±9.0	0.20
3	6	3	23.5±3.3	0.02	70.5±9.9	0.03

Entodermal cells upon cultivation *in vitro* apparently lost some of the factors which supported absorption and growth of rickettsiae. In four preliminary experiments, the cultures were grown for 4 to 6 days prior to inoculation with the rickettsiae. The FC or ID_{50} were the same or somewhat lower than the chick embryo LD_{50}, while in all the experiments in which the explants were inoculated prior to embedding, the FC or ID_{50} were uniformly higher. Infection spread more rapidly among the densely packed cells near the original explant than at the periphery of the colony.

The influence of yolk sac constituents on the growth of the microorganisms is also illustrated in tables 3 and 4 (experiments V and VI). In experiment V the tissue-nutrient ratio was varied while the inoculum was maintained constant. Cultures in group 1 contained one explant as in previous experiments, those of the other two groups 2 and 3 explants, respectively, embedded one on the coverslip and the others directly on the Porter flasks. Only the colonies on the coverslips were examined, but since the explants were infected before embedding and all developed to approximately the same extent, it was assumed that the FC of the colonies grown on the Porter flasks were comparable to those embedded on the coverslips. As shown in table 3 the differences in the three groups were not very great, but it appeared that the same inoculum distributed

TABLE 4

Effect of yolk sac constituents on growth of entodermal cells and focus count (Experiment VI)

Group	Procedure of Inoculation	No. Cultures	Area of Cultures (mm^2): Mean ± standard error	Area of Cultures (mm^2): Probability (with group 1)	Focus Count: Mean ± standard error	Focus Count: Probability (with group 1)
1	Explants shaken in complete medium plus inoculum (1.25 ml/explant)	7	50 ± 8.1		42 ± 8.6	
2	Explants shaken in inoculum only (0.25 ml/explant)	9	65 ± 9.6	0.28	54 ± 4.8	0.25
3	Explants shaken in inoculum previously used for group 2	10	90 ± 8.6	<0.01	76 ± 7.6	<0.01

among a larger number of explants yielded a smaller FC per explant, but a higher total FC per culture. The results therefore indicated that infection was favored by a high tissue-nutrient ratio.

In experiment VI (table 4) the method of inoculation was varied and that, in turn, affected the concentration of host cell constituents in the cultures. The explants of group 1 were shaken in inoculum added to the nutrient (10 explants in 12.5 ml of fluid) as in other experiments. The explants of group 2 were shaken in the same concentration of inoculum without nutrient (10 explants in 2.5 ml of fluid). After 1 hr the explants were embedded and given fresh nutrient. The explants of group 3 were shaken in the inoculum already used for group 2 and treated in identical fashion. The cultures of group 3 developed to a considerably larger size than those of group 1. A possible explanation for this difference was the contact of the explants of group 3 with nutritional constituents released by the explants of group 2 in the inoculum. The FC of groups 1 and 2 were approximately the same and indicated that adsorption and growth were not materially affected by the above described differences in procedure. The FC of group 3, however, was significantly higher than that of group 1. This was surprising because the inoculum used for that group had already lost infectious particles to group 2. The results suggested that (a) not all and possibly only a fraction of the potentially infective particles actually infected cells under the conditions of most of these experiments; (b) infection, as well as cell growth, was enhanced by the constituents released by the explants of group 2.

One attempt (experiment VII) was made to study the effect of temperature on adsorption. Six groups of explants were prepared and shaken frequently by hand for 1 hr at 5, 22, 30, and 37 C and one shaken mechanically at 22 C. Differences in FC did not appear to be significant with a possible exception of a lower FC obtained in the group shaken at 5 C.

Infectivity of cultures. The tissue culture method used in this investigation was not well adapted to the production of large numbers of rickettsiae. However, an effort was made to determine whether growth of rickettsiae in this type of culture was in any way comparable to that occurring in the chick embryos (experiments VIII and IX).

In experiment VIII, 18 cultures were prepared by the routine method, but each given 2.5×10^2 chick embryo LD_{50} or an inoculum 100 times greater than that used in FC experiments. Seven days after preparation of the cultures, 6 were fixed, stained, and found to contain, as expected, foci too numerous to be counted. The other 12 cultures were pooled, homogenized in their nutrient fluids, and titrated in chick embryos. The equivalent of each culture was found to contain 1.5×10^4 chick embryo LD_{50}, or an amount 60 times greater than that inoculated. This value is possibly not a complete measure of the rate of rickettsial growth, because Experiment VI suggested that under the conditions of this experiment many rickettsiae remained in the supernatant and presumably did not multiply.

In experiment IX the procedure was changed to permit a larger amount of rickettsial growth. The experiment consisted of only one Erlenmeyer flask with 10 explants and 12.5 ml of the usual nutrient-inoculum mixture containing a total of 2.5×10^3 chick embryo LD_{50}. The explants were shaken in the flask as in previous experiments, but the flask was incubated at 37 C without any further manipulations. On the fourth day, 7.5 ml were withdrawn and at 3- to 4-day intervals thereafter, 5 ml of fluid were withdrawn and replaced by 5 ml of fresh nutrient. On the eighth and fifteenth days 10 additional explants were added. Most of the explants, except for those added on the fifteenth day, adhered to the flask and formed colonies comparable to those obtained in Porter flasks, lasted approximately 15 days, and then disintegrated. All fluids withdrawn from the cultures were immediately titrated in chick embryos. On the twenty-fifth day the experiment was terminated and the explants emulsified in their own nutrients and titrated.

The rickettsial titer of the culture increased slowly, but consistently. The amount recovered on the twenty-fifth day was 3×10^6 chick embryo LD_{50}, which compared favorably with the optimal titers obtained in eggs. If losses of rickettsiae during nutrient replacement procedures are taken into account, it can be estimated that a 10^5-fold increment in the numbers of infective rickettsiae had taken place in 25 days, a rate of increment considerably lower than that occurring in eggs. The low tissue content of the culture, especially during its first eight days, was possibly a factor contributing to the low rate of rickettsial increment.

DISCUSSION

A comparison of the work of Weiss and Huang (1954) with feline pneumonitis virus and the present with *C. burnetii* revealed important differences in the factors favoring infection of chick embryo entodermal cells by the two organisms. The behavior of the host cells appeared to be the same in the two investigations, with the minor exception that some heterologous sera appeared to be detrimental to the cells in the present, but not in the previous experiments.

The most important condition facilitating infection of entodermal cells in the case of feline pneumonitis virus appeared to be a wide surface of exposure—in the case of *C. burnetii*, recent explantation of the tissues. This difference was responsible for the procedures selected for the cultivation of the two organisms: feline pneumonitis virus was added to the supernatant of well developed cultures, while *C. burnetii* infection was carried out immediately after explantation prior to embedding. The explants of any one *C. burnetii* group were inoculated all by the same inoculum, a procedure which eliminated the variable of separate inoculation of each culture. Because *C. burnetii* developed more slowly than feline pneumonitis virus and did not undergo a cycle of development, it was possible to delay the examination of the cultures until relatively large monolayers were formed. Infection by both the virus and rickettsia was apparently accomplished by only a fraction of the potentially infective particles. Virus infection could be enhanced by procedures such as shaking which increased the rate of contact between virus and host cells; enhancement of rickettsial infection was possibly caused by adding or maintaining host cell constituents (experiments V and VI).

The advantages of the FC method for the study of the factors influencing adsorption of *C. burnetii* onto host cells and growth are obvious and do not need to be discussed in detail. The FC method is similar to other tissue culture monolayer methods of virus quantitation (Dulbecco, 1952), except that observations are made microscopically. It allows more detailed observations of the foci of infection, but has the disadvantage that clones cannot readily be subcultured. In the experiments described in this paper a distinction between adsorption and growth of the rickettsiae has not been made, but the procedure can readily be modified to study the two phenomena separately.

SUMMARY

Monolayer cultures of entodermal cells derived from the avascular membrane of the 4-day-old chick embryo were used for the study of the growth of *Coxiella burnetii* and for the development of a quantitative method of assay. The organisms were readily recognized in stained cultures beginning on the fourth day after inoculation, developed in the cytoplasm of their host cells, slowly invaded neighboring cells, and, provided the concentration of the inoculum was small, formed discrete foci of infection between the fifth and tenth days. Enumeration of the foci of infection yielded consistent results and a titer for the inoculum which corresponded to the tissue culture ID_{50}. It was intermediate between the chick embryo LD_{50} and ID_{50}. Recent explantation of the cells, a high host-nutrient ratio, and the presence of host cell constituents, all tended to enhance infection. The focus count method of quantitation appeared to be well suited for a study of the factors that favored adsorption of the rickettsiae to host cells and that favored their growth.

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