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Infection Cycle of *Rickettsia rickettsii* in Chicken Embryo and L-929 Cells in Culture

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The infection cycle of *Rickettsia rickettsii*, studied in slide chamber cultures of chicken embryo and L-929 cells, was found to be complex and did not conform to a one-step growth cycle. Initial uptake kinetics resembled those established for *Rickettsia prowazekii*, but subsequent events showed very marked differences. Intracytoplasmic growth commenced exponentially without measurable lag. However, very soon after infection, intracytoplasmic rickettsiae began to escape from the host cell into the medium in large numbers, resulting in (i) failure of large numbers of rickettsiae to accumulate in the cytoplasm, (ii) sustained rapid division of the organisms in the cytoplasm, (iii) substantial accumulation of extracellular rickettsiae, and (iv) rapidly spreading infection in the culture, with most cells infected in 48 to 72 h. In the occasional cell, rickettsiae were found in the nucleus, where they multiplied to form compact masses. Thus, analysis of the growth characteristics of *R. rickettsii* must consider the entire culture as a unit in which the rickettsiae are distributed among three compartments in which they behave in different ways: (i) intranuclear, (ii) intracytoplasmic, and (iii) extracellular. The rickettsial traffic is bidirectional across the host cell plasma membrane and dominantly monodirectional across the nuclear membranes. The implications of this behavior with respect to location and range of receptors and substrates involved in membrane penetration are discussed. In older cultures, unique intracytoplasmic ring or doughnut colonies were common, indicating a change in the intracytoplasmic environment. The possible significance of the growth characteristics in cell culture to the characteristics of infection in humans and animals is discussed.

The early pioneering work of Wolbach and Schlesinger (19) and of Pinkerton and Hass (8) on the survival and growth of the prototype of the spotted fever group of rickettsiae, *Rickettsia rickettsii*, in plasma clot tissue explant cultures established the following important characteristics: (i) growth only within host cells, most often endothelial and fibroblastic cells; (ii) dispersed, sparse intracytoplasmic distribution; (iii) intranuclear growth, often in compact clumps; and (iv) morphology ranging from minute coccoidal to bacillary to filamentous. The tendency for intranuclear growth has subsequently been recognized as a spotted fever group characteristic. Over 30 years later, Schaechter et al. (10), applying modern cell culture methods and phase-contrast microscopy, demonstrated the following by direct continuous observation of *R. rickettsii*-infected living rat fibroblast (14pf) cultures: (i) division by transverse binary fission; (ii) dispersed, relatively sparse accumulation of rickettsiae in host cell cytoplasm; (iii) tendency for intracytoplasmic *R. rickettsii* to enter host cell processes and occasionally to escape from apparently undamaged host cells through the processes; (iv) occasional intranuclear growth; and (v) grossly apparent host cell damage after 5 or 6 days of incubation. Anderson et al. (1) showed by electron microscopy that *R. rickettsii* grew free in the cytoplasm and nucleus of host cells in culture and were not bound by an internal cell membrane. Kokorin (7), using *R. conori* in cell culture, stressed apparent intracellular motility of spotted fever group rickettsiae in early stages of infection and lack of motility in later stages and the development of filamentous forms under unfavorable conditions, and they suggested two phases of rickettsial development, vegetative (dividing, mobile) and resting (nonmobile). Burgdorfer et al. (4) mentioned the rapid spread of *R. rickettsii* in tissue culture monolayers and early destruction of host cells. Collectively, the observations outlined above, accumulated over a period of more than half a century, suggest that *R. rickettsii* ex-

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hibits some important and perhaps unique biological properties in its interaction with host cells.

Previous publications from these laboratories have described the infection cycle of *R. prowazekii* in chicken embryo cells in culture, have established methods and systems for the quantitative study of rickettsial uptake (penetration) and intracellular growth cycle, and have presented some quantitative information about host cell-rickettsia interactions with both virulent and attenuated strains of *R. prowazekii* (15–17). In the present study, a member of the spotted fever group of rickettsiae, *R. rickettsii*, was studied in similar detail in chicken embryo (CE) and L-929 cell slide chamber culture systems. Certain phenomena appear to contribute to a complex infection cycle that differs in important features from those displayed by the typhus group organism, *R. prowazekii*. Other publications will deal with the capsule or slime layer (D. J. Silverman, C. L. Wisseman, Jr., D. T. Brown, M. L. Cremer, and M. R. Jones, in preparation) and the ultrastructure of infected cells at different points in the infection cycle (D. J. Silverman, C. L. Wisseman, Jr., and M. R. Jones, in preparation).

MATERIALS AND METHODS

Rickettsiae. The seed used in most experiments was a 20% suspension in sucrose PG solution (3) of the yolk sacs of conventional embryonated hen eggs infected with the Sheila Smith strain of *R. rickettsii*, dispensed in 0.5- to 1.0-ml quantities to glass ampoules which were then flame-sealed, frozen in a dry ice-alcohol mixture, and stored at −70°C until use. This seed had a rickettsial body count (RLB) of 1.28 × 10^9/ml (11) and a plaque-forming unit (PFU) titer of 9.0 × 10^7/ml (13), giving an RLB/PFU ratio of 14.2. A similar seed of the Bitterroot strain of *R. rickettsii* was used in some initial studies. The infecting dose of this seed was determined on the basis of a preliminary dose-response type of uptake study (see below). A plaque-purified seed, amplified in the yolk sac of specific-pathogen-free eggs (SPAFAS) (50% yolk sac in brain heart infusion broth) of the Sheila Smith strain of *R. rickettsii*, with a titer of 8 × 10^7 PFU/ml and 1.34 × 10^9 RLB/ml (RLB/PFU = 16.8), was used in the study with L cells.

Cell culture methods. The general methods for preparing, incubating, and processing slide chamber cultures have been described (15–17). In the present study, secondary chicken embryo (CE) cells from embryonated specific-pathogen-free (SPAFAS) eggs and L-929 (NCTC 2071) cells (American Type Culture Collection no. CCL 1.1) were used as host cells. The medium was a low-glucose (1,000 mg/liter) formula of Dulbecco's modification of Eagle medium with Earle salts (12) diluted with an equal volume of glucose-containing Earle salts solution (GIBCO) and contained 10% fetal calf serum.

Uptake and growth methods. Uptake characteristics of *R. rickettsii* by CE cells were determined by the suspended-cell method described for *R. prowazekii* (C. L. Wisseman, Jr., and A. D. Waddell, in preparation).

For growth studies, the cells were infected either in slide chamber cultures (15, 17) or in suspension at 32°C for 30 to 60 min (Wisseman and Waddell, in preparation). In the latter instance, a sample of the infected suspension (zero time) was prepared in a Cytofuge (Shandon Elliot, Sewickley, Pa), stained, and counted as previously described (15, 16; Wisseman and Waddell, in preparation).

Either uninfected cells for infection in chamber or cells infected in suspension, about 10^5/ml in either instance, were distributed to eight-chambered culture slides (Lab-Tek Products, Naperville, Ill.), 0.3 ml/chamber. Incubation was at 32°C in a humid atmosphere of 5% CO_2 in air. At selected times, slides were processed, stained with Giménez stain (6), and counted as previously described (15, 17). Occasionally, slides were fixed in methanol and stained with Giemsa stain. Growth characteristics, including examination for lag phase, were plotted graphically and calculated with modifications (see below), as previously described for *R. prowazekii* (15, 16).

RESULTS

Uptake kinetics. Both rate and dose-response uptake studies were performed with *R. rickettsii* in the suspended CE cell system. The relationships revealed in the two systems with *R. rickettsii* (Fig. 1 and 2) were very similar to those previously established for the interaction of *R. prowazekii* with CE cells (16; Wisseman and Waddell, in preparation) and were readily reduced to straight lines with high correlation coefficients through the same data transformations. Thus, the average number of rickettsiae taken up per cell was a linear function of time for as long as 180 min, and the log *t*-probit plot was linear over the same period (Fig. 1). Likewise, in the dose-response experiment, both the log [PFU]-log *m* (uptake rate) and log [PFU]-probit plots were linear over the almost 50-fold range of concentration, which constituted the practical working range of the seed (Fig. 2). Moreover, the distribution among cells of rickettsiae taken up closely followed the Poisson distribution (data not shown), as was previously demonstrated with *R. prowazekii* (Wisseman and Waddell, in preparation). The PFU efficiency of uptake for *R. rickettsii* with CE cells, calculated from the dose-response data, was 11.8 ± 1.4; i.e., there were 11.8 chances of collision between a PFU and a CE cell for each RLB actually entering a cell.

Growth cycle. The growth cycle of *R. rickettsii* in CE and L cells did not follow the simple kinetics previously observed with *R. prowazekii* (15, 16), as was shown by both microscopic survey of cultures after different times of incu-

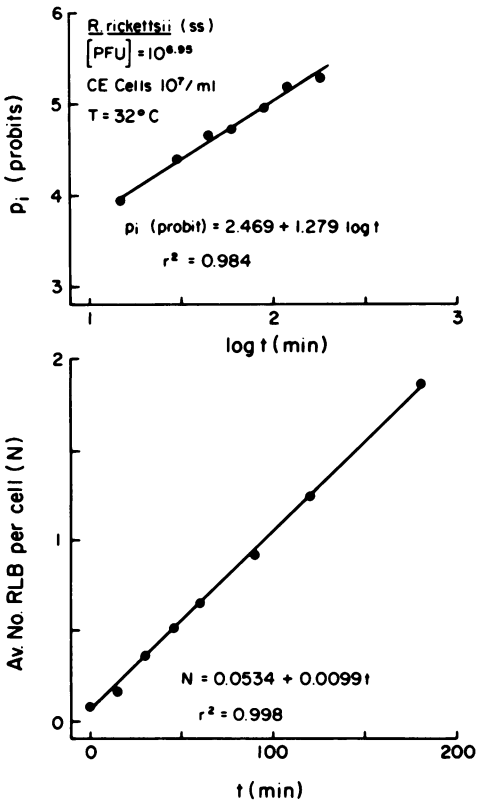

FIG. 1. *Time course of uptake of R. rickettsii (Sheila Smith strain) by CE cells in a suspended-cell system.*

bation (Fig. 3–7) and examination of the curves obtained by plotting percentage of cells infected (p_i), $\log_2$ average number of RLB per infected cell (N_i), and $\log_2$ average number of RLB per cell (N) against incubation time (Fig. 8–10).

Microscopic observations. Early in the growth cycle, beginning after about 10 h of incubation, an increase in the p_i was clearly measurable; by 21 to 24 h it was substantial; by 48 h most of the cells in the culture were infected; and by 72 h essentially all of the cells were infected. These observations imply rapid escape of rickettsiae from host cells from the earliest stages of infection, an impression reinforced by quantitative analysis (see below), and therefore the concept of a one-step growth cycle would appear to be untenable with this organism under these conditions.

The distribution of RLB in the cytoplasm was distinctive during the first 3 days (Fig. 4 and 5). Usually, single bacillary forms or dividing pairs were widely dispersed throughout the cytoplasm. Chains or microcolonies, seen frequently with *R. prowazekii* (15), were not observed. However, especially after modest numbers of RLB accumulated in the cytoplasm, there appeared to be a strong tendency for the organisms to concentrate along the host cell borders and in host cell cytoplasmic processes, in many instances giving the appearance of being on the verge of escaping (Fig. 3c,d; Fig. 4). Indeed, as incubation proceeded, more and more free, extracellular RLB were seen, despite the fact that processing of the slide cultures for examination involved removal of the medium and washing before fixation and staining, suggesting an accumulation of substantial numbers of extracellular rickettsiae during this period (Fig. 3d, Fig. 4). Even over an incubation period of 72 h, cells packed with cytoplasmic RLB were not encountered.

The number of irradiated host cells, whether

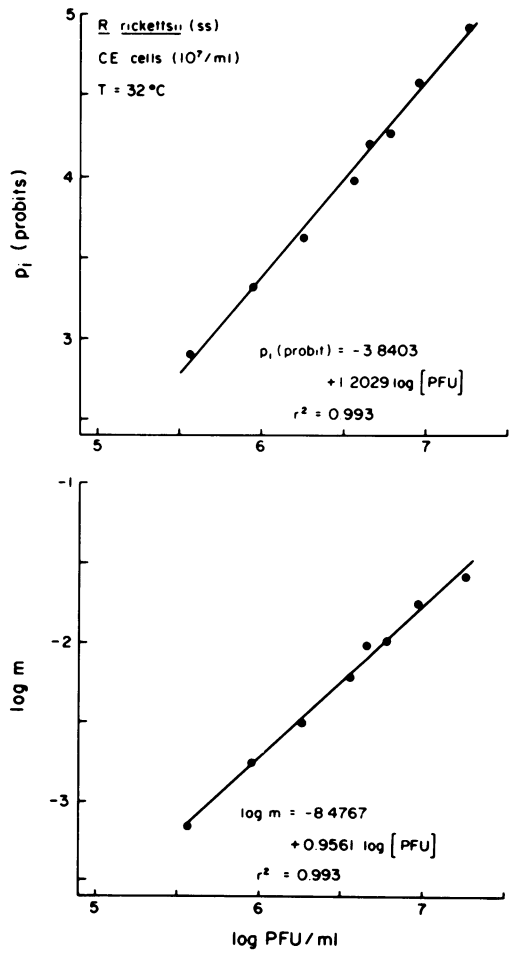

FIG. 2. *Relationship between R. rickettsii (Sheila Smith strain) concentration and uptake rate by CE cells in a suspended-cell system.*

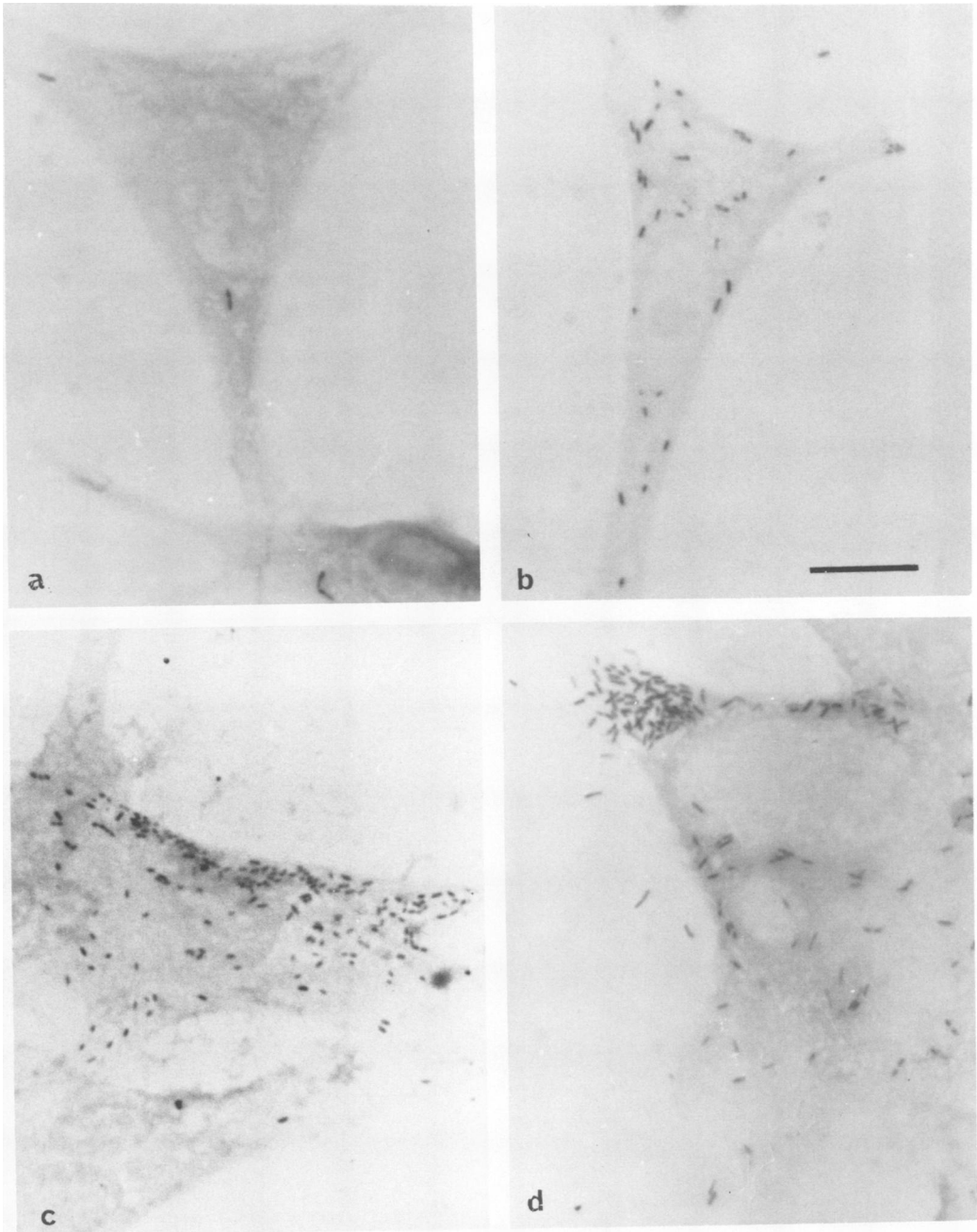

FIG. 3. *R. rickettsii (Sheila Smith strain) in irradiated CE cells in slide chamber cultures after different incubation periods: (a) 6 h; (b) 28 h; (c) 48 h; (d) 73 h. Note forms in binary fission even at 73 h, generally dispersed cytoplasmic distribution (b), and concentration of organisms along cell border (c) and in cell processes, with escape into extracellular space (d). Giménez stain. Bar = 10 μm.*

CE or L, did not decrease detectably by simple visual estimate over at least the first 72 h, and they retained reasonable morphological integrity. Admittedly, the Giménez stain is not well suited for detecting minor changes in morphology, and no tests for viability, such as trypan blue exclusion, were performed. However, there is no evidence that mass host cell destruc-

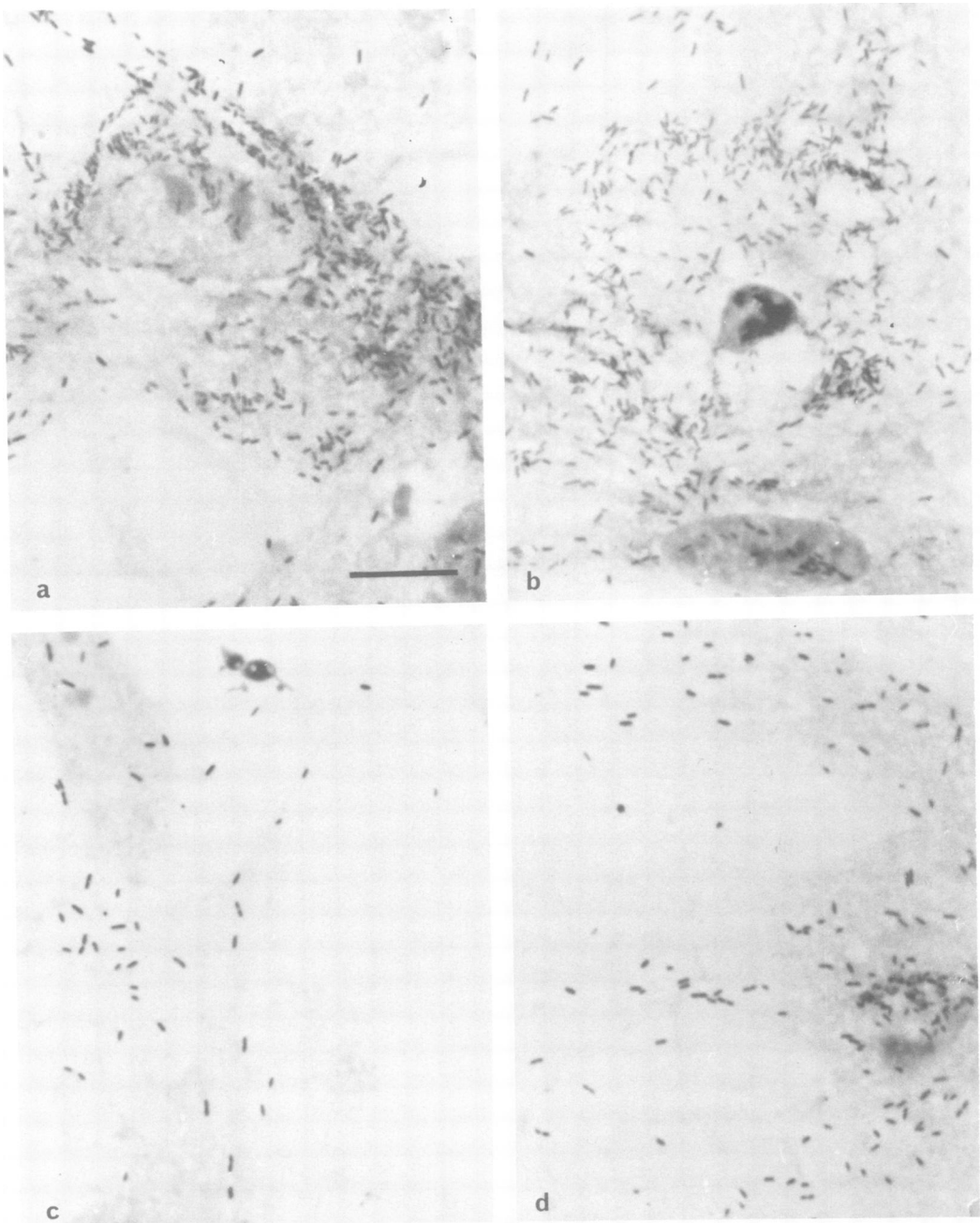

FIG. 4. *R. rickettsii (Sheila Smith strain) in irradiated CE cells in slide chamber cultures after 48 to 96 h of incubation. (a) 48 h; (b) 72 h; (c) 96 h; (d) 96 h. Note dispersed intracytoplasmic distribution of organisms, even when relatively large numbers are present (a, b), and tendency for organisms to permeate cell processes and to escape from cells. Giménez stain. Bar = 10 μm.*

tion is responsible for the release of rickettsiae into the extracellular environment. Instead, the morphological observations support the concept that *R. rickettsii* has a strong tendency to escape from the cytoplasm of infected cells into the extracellular environment and to enter other cells, at least in the first 3 to 4 days of incubation.

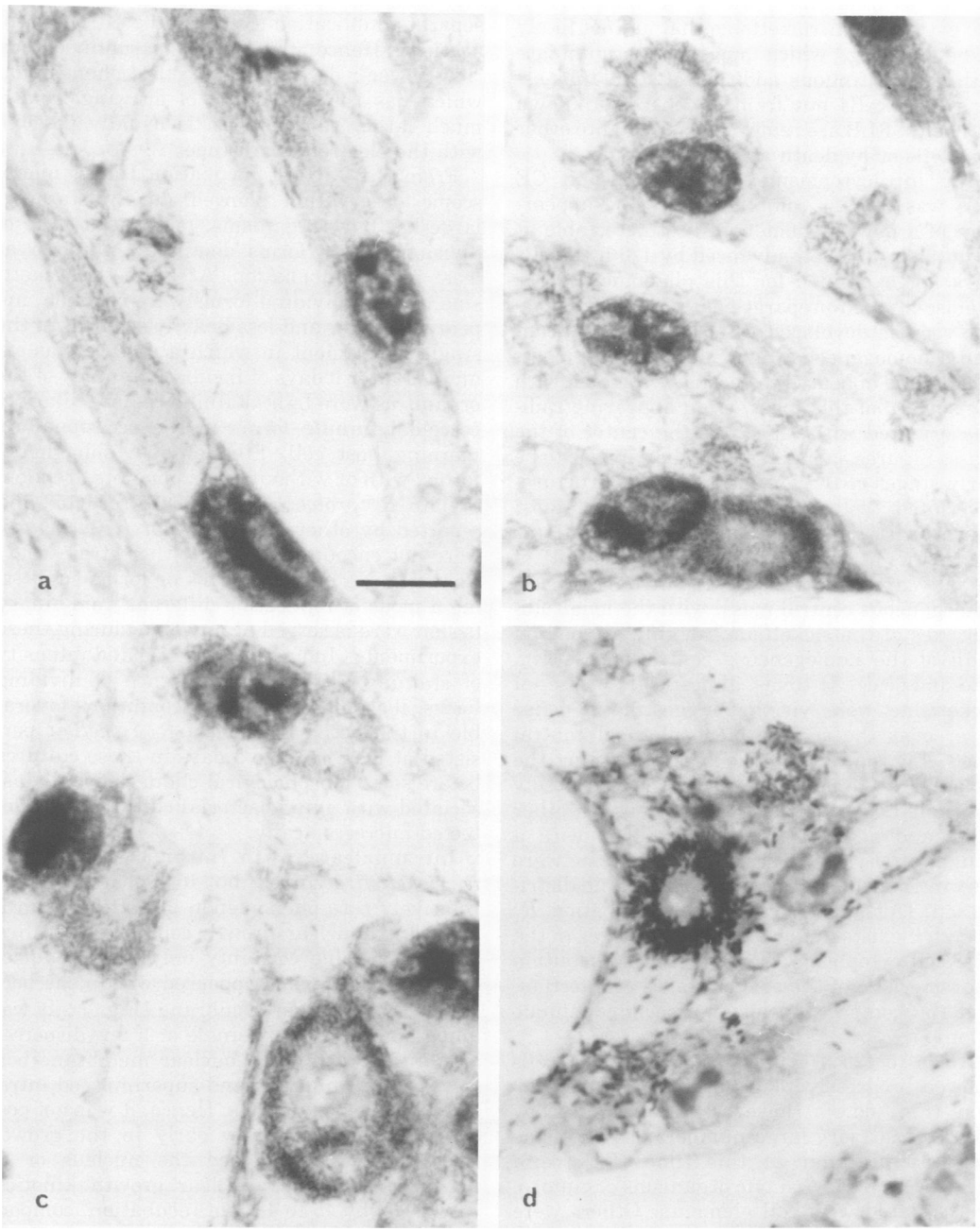

FIG. 5. *Intracytoplasmic colonies of R. rickettsii in CE cell cultures after 120 h of incubation. (a) Cytoplasmic "vacuole" containing homogeneous mass with a few associated rickettsiae. Giemsa stain. (b and c) Ring or "doughnut" intracytoplasmic rickettsial colonies surrounding central mass. Note tendency for radial arrangement of rickettsiae and close association of colonies with cell nuclei. Giemsa stain. (d) Typical, well-developed "doughnut" colony stained with Giménez stain. Note extensive vacuolization of host cell. Bar = 10 μm.*

Thus, after the first few hours of incubation, the in vitro system with *R. rickettsii* in CE or L cells appears to consist of several different rickettsial populations: (i) dispersed intracytoplasmic rickettsiae undergoing active growth and division but from which organisms are constantly escaping; (ii) intranuclear rickettsiae undergoing active growth and division and

tending to accumulate in compact masses; and (iii) extracellular rickettsiae that are not likely to be growing, which appear to accumulate with the continuous addition of RLB that escape from cells but from which an unknown number of RLB are removed by entry into other host cells or by death and degeneration.

The intracytoplasmic growth cycle in CE cells was further complicated by the appearance of a new phenomenon, first detectable at about 96 h and well advanced by 120 h (L cells were not examined for this phenomenon). In Giemsa-stained preparations, a large cytoplasmic vacuole developed which appeared to contain a homogeneous, light blue-staining mass. Rickettsiae appeared to become associated with the surface of this mass, often appearing radially oriented with respect to the center of the mass. Figures 5a and b show what appear to be early stages of the formation of this structure-associated "colony" of rickettsiae. The most characteristic form, probably a somewhat later stage, was a very dense ring or "doughnut" of radially arranged rickettsiae surrounding the homogeneous central mass, with the longitudinal axes of the rickettsiae roughly aligned on radii of the homogeneous central mass (Fig. 5c,d and 6c,d). At times, almost solid masses of rickettsiae were visible, suggesting a dense layer over the surface of a spherical central mass (Fig. 6a,b). The vacuoles containing the rickettsial colonies often appeared to be closely associated with the host cell nucleus. In other cells, and at times in the same cell with a doughnut of rickettsiae, other rickettsiae were also in their dispersed intracytoplasmic distribution. Thus, after 96 to 120 h of incubation, *R. rickettsii* exists in two distribution states in the host cell cytoplasm: the dispersed distribution characteristic of the earlier stages of infection, and the compact, inclusion-associated unique colony.

At 96 to 120 h of incubation, the host cells showed progressive degeneration and destruction (Fig. 6d,e). However, light microscopy showed that very large numbers of rickettsiae were extracellular at this time (Fig. 6e,f). Many were free, as single organisms or clumps, unassociated with cell elements. Others were associated with cell fragments, and intact extracellular doughnuts were occasionally seen. Thus, at the time the doughnuts were most prominent, host cells were undergoing extensive degenerative and destructive changes. Light microscopy amply demonstrated these major phenomena of rickettsial distribution and detected very gross cell changes. The evolution of these changes and their nature are better characterized by ultrastructural studies by electron microscopy, which are described in a separate publication (Silverman et al., in preparation). Hence, this report is confined to a "low-power" description of the phenomena, which has the advantage of drawing from a much larger sample than is usually possible with the electron microscope.

From the earliest incubation times, microscopic observation showed deeply staining, large bacillary organisms, many in a state of division. These forms dominated through at least 72 h of incubation. At 120 h the rickettsiae, though dividing forms were common, appeared smaller and less heavily stained. In the single experiment in which a culture was examined after 9 days of incubation, many of the organisms were light-staining coccobacillary to coccoidal minute forms in the occasional remaining host cell. Filamentous "sphaghetti" forms, with or without division points, frequent in late *R. prowazekii* cell cultures (15) and reported by others (7, 8, 19) for *R. rickettsii*, were not encountered with either strain of *R. rickettsii*. No unusual forms or forms suggesting a mode of replication different from binary fission were observed at any time during these experiments. Judging by the size and intensity of staining and by the presence of dividing forms, it would appear that conditions favorable to the active growth of *R. rickettsii* persisted at least for 3 to 5 days in these cultures before some morphological changes usually associated with aging bacterial cultures were detected microscopically.

Intranuclear growth. Intranuclear growth of *R. rickettsii* occurred, but it appeared to be a relatively rare phenomenon in both CE and L cells. Intranuclear growth could be identified with reasonable certainty only after compact masses of organisms appeared within the borders of the nuclear membrane (Fig. 7). It was impossible to classify single or a few dispersed organisms within the nuclear membrane border into intranuclear and superimposed intracytoplasmic populations. Hence, it was not possible to determine how early in the growth cycle rickettsiae entered the nucleus or to measure early intranuclear growth kinetics. However, by 45 to 48 h of incubation, compact intranuclear clumps of rickettsiae were rarely but readily identified. In contrast to the dispersed distribution of RLB in the cytoplasm, the intranuclear rickettsiae remained in compact masses and tended to increase in numbers, which at times almost completely filled the nucleus with a dense mass of organisms. Even when first seen, the masses were usually so dense that an accurate count of RLB was impossible, and the kinetics of intranuclear growth

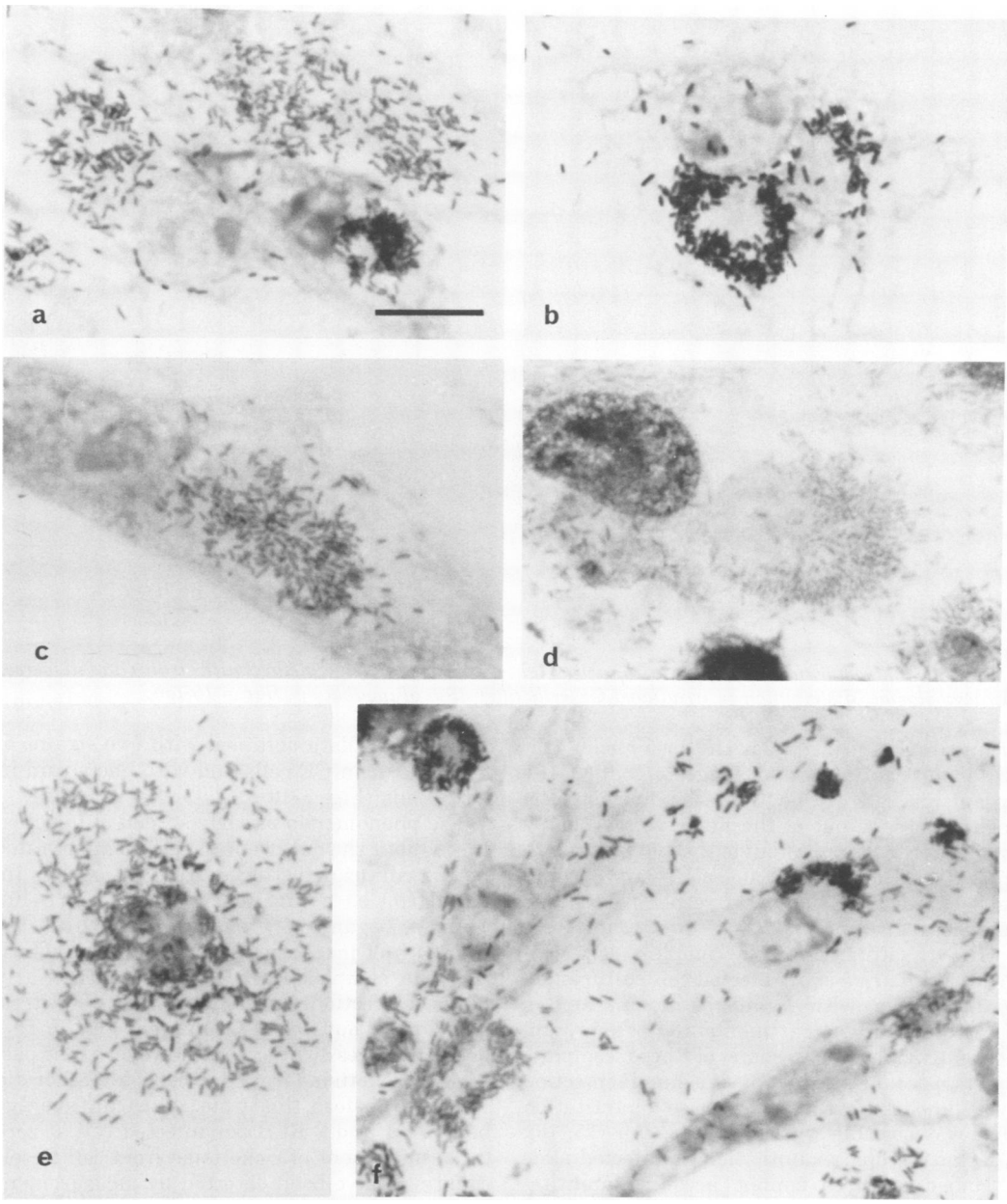

FIG. 6. *R. rickettsii-infected chicken embryo cells in culture after 120 h of incubation. (a and b) Cells with dense, nearly spherical intracytoplasmic colonies as well as less dense clusters. Giménez stain. (c) Intracytoplasmic vacuole containing homogeneous mass covered with rickettsiae. Giménez stain. (d) Radial arrangement of rickettsiae on homogeneous mass which appears partially separated from degenerating host cell. Giemsa stain. (e) Rickettsiae surrounding degenerating host cell nucleus. No cytoplasm visible. Giménez stain. (f) Various forms of extracellular rickettsiae: single free organisms, clumps, and free "doughnut" colonies. Giménez stain. Bar = 10 μm.*

cannot be described in quantitative terms from these observations.

The low frequency of intranuclear rickettsiae suggests that *R. rickettsii* does not enter the nucleus from the cytoplasm as readily as it enters through the plasma membrane into the cytoplasm from the extracellular environment. Moreover, the accumulation of large numbers

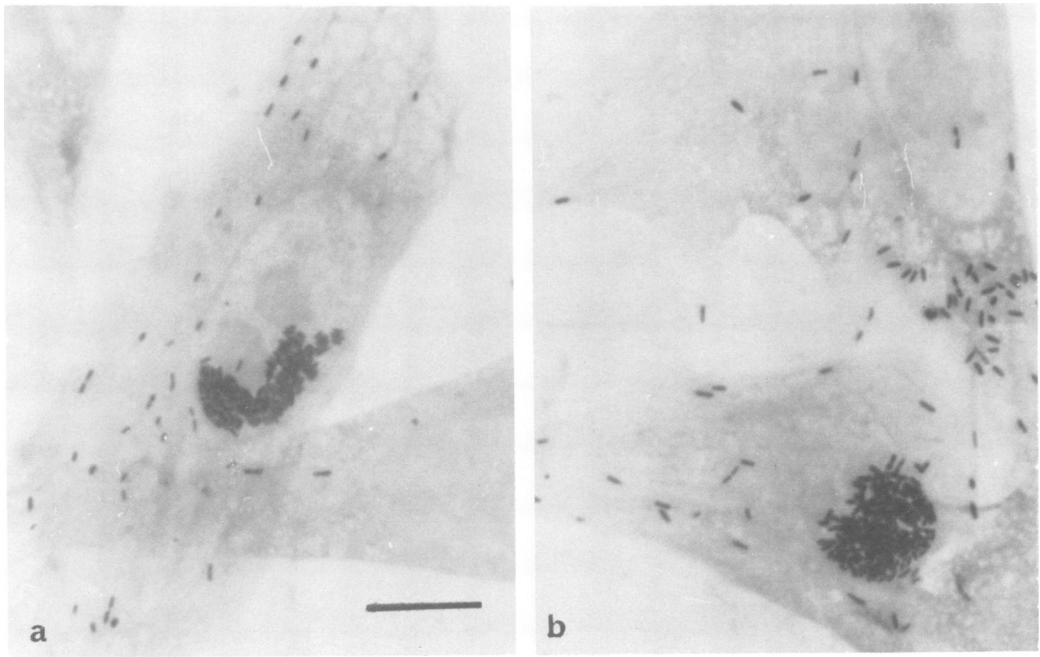

FIG. 7. *Contrast between compact intranuclear masses of R. rickettsii (Sheila Smith strain) and dispersed intracytoplasmic distribution in irradiated CE cells after 45 h of incubation. Bar = 10 μm.*

of intranuclear rickettsiae in those nuclei that are identifiably infected suggests that, if the organisms can escape from the nucleus, the rate of passage out of the nucleus is probably much slower than that out of the cytoplasm into the extracellular space, at least relative to the rates of multiplication in the respective cellular compartment.

Growth kinetics. The complex series of events in *R. rickettsii*-infected cell cultures described above, with organisms moving among three different compartments and possibly undergoing changes in some of these compartments, precludes reduction to a simple infection cycle and the relatively precise quantitation that was possible with *R. prowazekii* (15, 16). The microscopic examination of infected slide chamber cultures cannot account quantitatively for the extracellular organisms, nor can it ascertain infectivity or physiological state. Other methods are better adapted to this. Nevertheless, by plotting the data obtained by counting intracytoplasmic rickettsiae in the early stages of the infection, objective and quantitative demonstration of some of the phenomena described above, detection of certain phenomena not apparent by qualitative microscopic examination, and approximation of generation time and certain other growth characteristics are possible. Hence, we present the quantitative data obtained with two strains of *R. rickettsii* in CE cells and with one strain in L-929 cells (Fig. 8–10).

The phenomenon of early spread of infection throughout the culture is clearly demonstrated by the curves depicting percentage of cells infected (p_i) as a function of time (Fig. 8–10). Thus, p_i began to increase rapidly within the first 24 h of incubation. In the study in which multiple samples were counted in the early hours of infection, this increase was first detectable after about 10 h of incubation (Fig. 8). This does not necessarily mean, however, that passage of rickettsiae from one cell to another did not occur before this time. With an average between 1 and 2 RLB per infected cell at zero time, movement of rickettsiae from cell to cell could not have been detected by measurement of p_i until the number of rickettsiae had increased, i.e., about 10 h after infection.

The increase in p_i, once begun, was very rapid. The p_i at zero time was intentionally adjusted to about 20% to permit clear demonstration of this phenomenon. Under these conditions, almost all cells in the culture were infected by 48 h. Spread was not necessarily a continuous, uniform process (Fig. 10). Initially, the apparent transient cessation of the spread of infection between about 24 and 48 h (Fig. 10) was considered to be an experimental artifact,

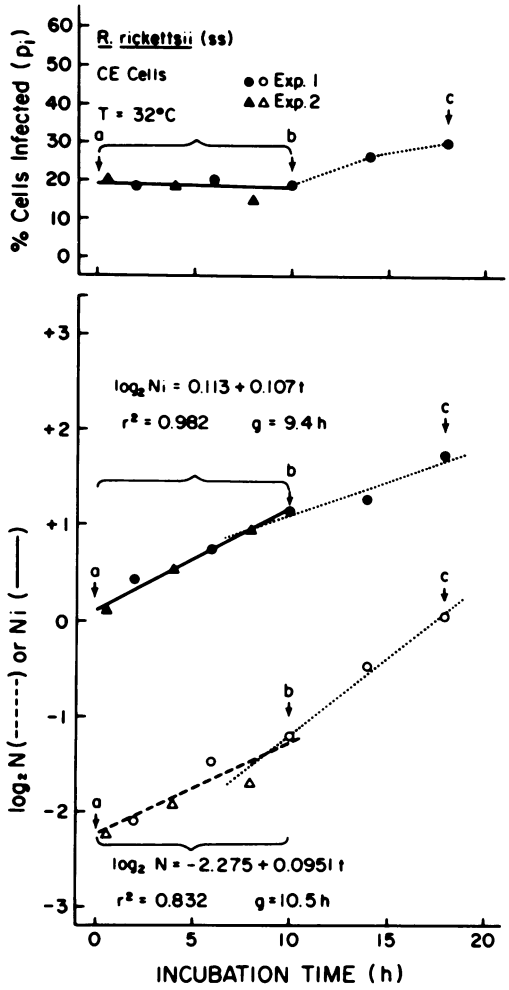

FIG. 8. *Curve of early growth cycle of Sheila Smith strain of R. rickettsii in CE cells. Note early beginning increase in percentage of cells infected and absence of lag phase.*

but subsequent experience with certain other spotted fever group rickettsiae (C. L. Wisseman, Jr., I. Steiman, and M. R. Jones, unpublished observations) indicates that it may be common phenomenon that coincides with an aberration in the growth curve. Thus, spread of infection throughout the culture may at times be a discontinuous process. Timing of samples seems to be critical in detecting this aberration. The explanation for this phenomenon is not apparent at this time. Nevertheless, these quantitative studies clearly establish the fact that *R. rickettsii*, unlike *R. prowazekii* (15), has the capacity to escape from one host cell and enter another from the early stages of the infection cycle and does not depend solely upon

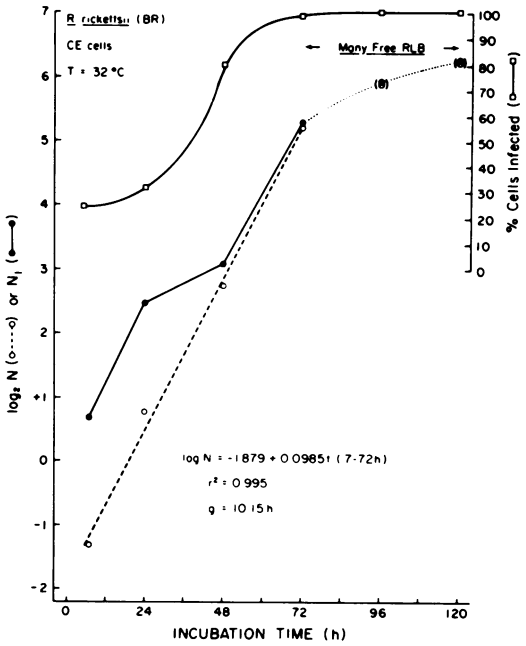

FIG. 9. *Growth curve of Bitterroot strain of R. rickettsii in CE cells.*

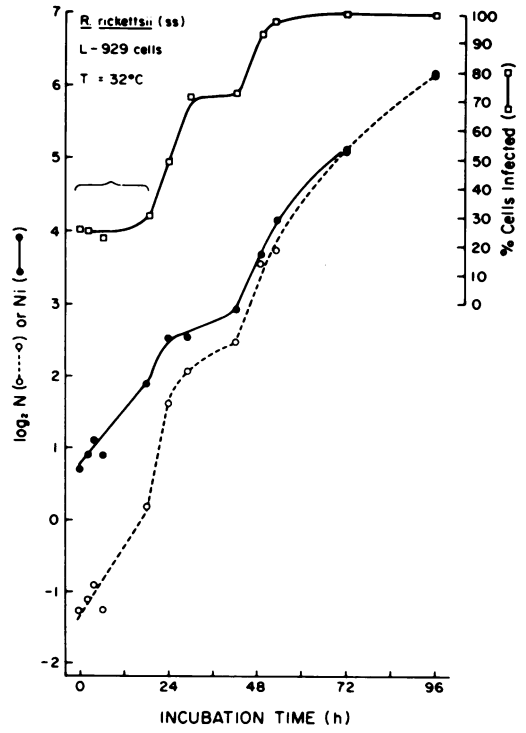

FIG. 10. *Growth curve of Sheila Smith strain of R. rickettsii in L-929 cells.*

destruction of the host cell for its release.

As might be expected, the growth curve, i.e., the plot of $\log_2 N_i$ (average number of RLB per infected cell) against time, was complex with both types of host cell. In one set of experiments with the Sheila Smith strain, in which multiple samples were taken in the early hours after infection (Fig. 8), no evidence for a lag phase was obtained. The growth plot was reasonably linear in the first few hours of incubation, after which it deviated significantly from linearity and finally assumed a steadily declining slope. Counts were not reliable after 72 h, when N_i had attained only about 30 to 50 RLB/infected cell, and the curves do not portray the changes after doughnut formation was prominent.

On the assumption that many of the rickettsiae that escaped from infected cells entered other cells, especially during the early part of the period of increase in p_i, it is possible that a curve in which $\log_2 N$ (average number of RLB per cell) is plotted against time would be a better approximation of the rate of increase in numbers of RLB and hence permit a closer approximation of the generation time, although obviously this does not account for the rickettsiae remaining extracellularly. Replotting data from earlier *R. prowazekii* studies (15, 16) showed that $\log_2 N_i$ and $\log_2 N$ plots yielded parallel regression lines and similar generation times. When this was done with *R. rickettsii*, the curve tended to approach a linear form for a somewhat longer period of time, but it too was complex and soon began to level off and, as p_i approached 100%, the $\log_2 N_i$ and $\log_2 N$ curves merged into a single curved line. Generation times calculated from the very early part of the growth curve, before the rapid increase in p_i, yielded values of about 10 to 10.5 h for CE cells and 12.5 for L cells. True generation times are probably shorter, which brings the value for *R. rickettsii* close to the range observed for *R. prowazekii* under similar conditions.

DISCUSSION

The course of infection of *R. rickettsii* in CE or L-929 cell cultures was predictable and reproducible, but it was complex and was not readily amenable to the calculation of some of the simple growth characteristics. Nevertheless, both qualitative and quantitative analysis of the growth patterns revealed important features of the rickettsial growth and interaction with host cells.

In the early stages of the growth cycle of *R. rickettsii*, the organisms escaped from the cytoplasm into the medium without causing grossly detectable damage to the host cell, and some of these organisms infected other host cells, causing a rapidly spreading infection throughout the entire cell culture without accumulating large numbers of microorganisms in the cytoplasm of host cells. Moreover, organisms penetrated the nucleus in a fraction of the infected cells and accumulated there in substantial numbers. Thus, a comprehensive description of growth kinetics requires consideration of the entire culture in which rickettsiae are distributed among three compartments in the culture: (i) intranuclear, (ii) intracytoplasmic, and (iii) extracellular. In each of these compartments, the microorganisms may behave differently from those in other compartments and may vary in a given compartment at different times during the infection cycle. However, at least in the early stages, *R. rickettsii* infection in the host cell cytoplasm appears to behave for a time somewhat like a chemostat, in which rickettsiae are constantly being removed and hence maintain prolonged active growth at low net population density. This differs substantially from the intracellular growth pattern of *R. prowazekii* in which each infected cell behaves like an independent fluid miniculture in which organisms pass through the classical bacterial growth phases and remain confined to the host cell until it finally breaks down (15).

Analysis of the growth patterns, both in individual cells and in the culture as a whole, suggests that the kind and extent of interaction of *R. rickettsii* with the various host cell membranes may be the single most important property of this organism that determines its unique growth pattern. Thus, one of the striking properties of *R. rickettsii* appears to be its capacity for bidirectional transit through the plasma membrane. Passage of *R. rickettsii* through the plasma membrane from extracellular compartment into cytoplasm has been studied in quantitative terms in the form of uptake kinetics, which are very similar to those of *R. prowazekii* (16; Wisseman and Waddell, in preparation). It is of interest that, in the dose-response type of uptake experiments, the slopes of the curve depicting log m-log [PFU], with comparable yolk sac seeds, were 0.956 and 0.980 for *R. rickettsii* and *R. prowazekii*, respectively, indicating very similar dependencies of rate of uptake on viable rickettsial concentration. The parallel displacement of the curves for the two organisms can be used to measure relative efficiency of uptake when it becomes possible to determine the absolute number of viable organisms in rickettsial suspensions.

Passage of *R. rickettsii* through the plasma membrane in the opposite direction, i.e., from cytoplasm to the extracellular compartment,

without detectable gross damage to the host cell, was a most striking and important feature of the infection. Schaechter et al. (10) first qualitatively demonstrated the phenomenon by observing under phase-contrast microscopy the actual escape of *R. rickettsii* from cytoplasmic processes of living infected cells. In the present study of stained cultures, organisms often appeared to be on the verge of escaping from cells in the absence of gross host cell damage, and there were increasing numbers of extracellular rickettsiae. Indeed, in studies in progress it has been possible to harvest substantial numbers of rickettsiae from the cell-free culture medium drawn from infected CE cell cultures, a possible practical application of the transient chemostat-like phenomenon. The magnitude of this phenomenon was illustrated by the quantitative measure of the spread of infection throughout the culture. Thus, the tendency to escape from one host cell and to enter another host cell (i) was measurable after only 10 to 12 h of incubation and (ii) was rapid and extensive, with rickettsiae spreading to all of the host cells in 48 to 72 h. Additional support for the active and large-scale escape of rickettsiae from the cytoplasm lies in the failure of cells infected with *R. rickettsii* to accumulate large numbers of intracytoplasmic rickettsiae, despite morphological evidence of continued division, a prominent phenomenon in the present study that is recognizable in the drawings of Wolbach and Schlesinger (19), was specifically noted and contrasted with typhus rickettsiae by Pinkerton and Hass (8), and was described by Schaechter et al. (10). What is observed in the cytoplasm at any given time in the fixed and stained preparations appears to be the net result or balance among the dynamic rickettsial processes of (i) entry into the cell, (ii) rickettsial multiplication, and (iii) escape from the cell, which in turn are influenced by changes in the host cell cytoplasm. The rate of escape of rickettsiae relative to the generation time (see below), at least in the first 2 to 4 days, is sufficiently great to prevent rapid buildup of intracytoplasmic organisms. These findings stand in sharp contrast to those with *R. prowazekii* in the same host cell system, in which organisms accumulated in the cytoplasm of infected cells and spread of infection in the culture did not occur until the host cells broke down and permitted escape of the rickettsiae (15).

The intranuclear growth and accumulation of *R. rickettsii* in a minority of infected cells suggests that this organism has at least some unidirectional capacity to penetrate through the nuclear membranes, but that its capacity to penetrate the membranes in the opposite direction, if indeed it exists, is very small relative to the intranuclear growth rate. Current methods do not permit us to assess the capacity of *R. rickettsii* to pass through the nuclear membrane from the inside out. *R. prowazekii*, on the other hand, does not possess measurable capacity to penetrate through the nuclear membranes.

Although the precise mechanisms of passage of rickettsiae through membranes is unknown, it is reasonable to assume that it requires attachment of the organism to sites on the membrane, then some energy-requiring action of the rickettsia on the membrane to produce a defect, and, finally, possibly some action of the host cell (9, 14, 16; L. Warfel et al., in preparation). According to this model, it would appear that both receptors and substrates for *R. rickettsii* are located on both surfaces of the plasma membrane and, to a lesser extent, on the outer surface of the nuclear membrane, whereas the combination of receptors and substrate for *R. prowazekii* is located only on the outer surface of the plasma membrane. This would suggest either (i) that the receptors or substrate or both for *R. rickettsii* differ from those for *R. prowazekii* or (ii) that *R. rickettsii* possesses the capacity to interact with a broader range of receptor sites and/or substrates.

Schaechter et al. (10) described intracytoplasmic movement of *R. rickettsii* through cytoplasmic streaming. Kokorin (7), studying *R. conori*, noted by cinemicrophotography such movement in early stages of infection, but found that such movement ceased in later stages. He interpreted these observations to indicate that *R. conori* exists in two forms: (i) a vegetative, motile form and (ii) a nonmotile, resting form. However, Schaechter et al. (10) and Kokorin (7) noted host cell damage after 5 to 6 days of incubation. We also noted gross alteration in host cells after 4 to 5 days, with cell destruction easily detectable at 5 days. At this time, we noted the development of doughnut or ring colonies of rickettsiae surrounding a central mass and associated often with a visible vacuolar structure. Details of these changes are being studied by electron microscopy. However, it is possible that these structures and colonies and the cessation of movement observed by Kokorin may be due to cytotoxic changes in the host cell, with loss of cytoplasmic streaming and alteration of cytoplasmic structures, rather than due to a change in motility of the microorganism (Silverman et al., in preparation). It is our current opinion that during the infection cycle the cytoplasmic compartment of the host cell undergoes progressive degenerative changes and that changes in the apparent behavior and

distribution of the rickettsiae are largely due to changes in the host cell.

No lag phase was detected with the yolk sac seeds of *R. rickettsii* used in this study. However, in studies currently under way (Wisseman et al., unpublished data), seeds prepared by prolonged cultivation in cell culture did display a lag phase, just as was the case with similar seeds of *R. prowazekii* (16). Accurate measurement of generation time was impossible, but the approximations made from the early stages of growth suggest that it may be as long as *R. prowazekii* or perhaps shorter. The sparse intracytoplasmic organisms stained well for 3 to 4 days and dividing forms were common, suggesting that active growth and division was taking place for considerable periods of time. Only in surviving cells in old cultures did *R. rickettsii* show the diminution in staining intensity and in size that was commonly observed in the *R. prowazekii*-laden cells by 72 h of incubation.

The early and rapid spread of *R. rickettsii* through the cell culture may have its in vivo counterparts. Thus, the average incubation period of Rocky Mountain spotted fever in man is roughly 5 to 7 days, whereas that of louse-borne, epidemic typhus fever is 9 to 12 days. Moreover, *R. rickettsii* grows in, and damages, not only the endothelium of small blood vessels but also the muscular coat, whereas sites of proliferation and damage by *R. prowazekii* are limited to the endothelial layer (18). Chicken embryos inoculated by the yolk sac route die much more quickly with *R. rickettsii* than with *R. prowazekii*. It is tempting to speculate that the capacity of *R. rickettsii* to move rapidly from cell to cell in tissues may be partly responsible for these in vivo characteristics.

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LITERATURE CITED

1. **Anderson, D. R., H. E. Hopps, M. F. Barile, and B. C. Bernheim.** 1965. Comparison of the ultrastructure of several rickettsiae, ornithosis virus, and *Mycoplasma* in tissue culture. J. Bacteriol. **90**:1387–1404.
2. **Boese, J. L., C. L. Wisseman, Jr., W. T. Walsh, and P. Fiset.** 1973. Antibody and antibiotic action on *Rickettsia prowazeki* in body lice across the host-vector interface, with observations on strain virulence and retrieval mechanisms. Am. J. Epidemiol. **98**:262–282.
3. **Bovernick, M. R., J. C. Miller, and J. C. Snyder.** 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. **59**:509–522.
4. **Burgdorfer, W., D. J. Sexton, R. K. Gerlogg, R. L. Anacker, R. N. Philip, and L. A. Thomas.** 1975. *Rhipicephalus sanguineus*: vector of a new spotted fever group rickettsia in the United States. Infect. Immun. **12**:205–210.
5. **Cohn, Z. A., F. M. Bozeman, J. M. Campell, J. W. Humphries, and T. K. Sawyer.** 1959. Study on growth of rickettsiae. V. Penetration of *Rickettsia tsutsugamushi* into mammalian cells in vitro. J. Exp. Med. **109**:271–292.
6. **Giménez, D. F.** 1964. Staining rickettsiae in yolk sac cultures. Stain Technol. **39**:135–140.
7. **Kokorin, I. N.** 1968. Biological peculiarities of the development of rickettsiae. Acta Virol. **12**:31–35.
8. **Pinkerton, H., and G. M. Hass.** 1932. Spotted fever. I. Intranuclear rickettsiae in spotted fever studied in tissue culture. J. Exp. Med. **56**:151–156.
9. **Ramm, L. E., and H. H. Winkler.** 1976. Identification of chloresterol in the receptor sites for rickettsiae on sheep erythrocyte membranes. Infect. Immun. **13**:120–126.
10. **Schaechter, M., F. M. Bozeman, and J. E. Smadel.** 1957. Study on the growth of rickettsiae. II. Morphologic observations of living rickettsiae in tissue culture cells. Virology **3**:160–172.
11. **Silberman, R., and P. Fiset.** 1968. Method for counting rickettsiae and chlamydiae in purified suspensions. J. Bacteriol. **95**:259–261.
12. **Smith, D. J., G. Freeman, M. Vogt, and R. Dulbecco.** 1960. The nucleic acid of polyoma virus. Virology **12**:185–196.
13. **Wike, D. A., G. Tallent, M. G. Peacock, and R. A. Ormsbee.** 1972. Studies of the rickettsial plaque assay technique. Infect. Immun. **5**:715–722.
14. **Winkler, H. H., and L. E. Ramm.** 1975. Adsorption of typhus rickettsiae to ghosts of sheep erythrocytes. Infect. Immun. **11**:1244–1251.
15. **Wisseman, C. L., Jr., and A. D. Waddell.** 1975. In vitro studies of rickettsia-host cell interactions: intracellular growth cycle of virulent and attenuated *Rickettsia prowazeki* in chicken embryo cells in slide chamber cultures. Infect. Immun. **11**:1391–1401.
16. **Wisseman, C. L., Jr., A. D. Waddell, and D. J. Silverman.** 1976. In vitro studies on rickettsia-host cell interaction: lag phase in intracellular growth cycle as a function of stage of growth of infecting *Rickettsia prowazeki*, with preliminary observations on inhibition of rickettsial uptake by host cell fragments. Infect. Immun. **13**:1749–1760.
17. **Wisseman, C. L., Jr., A. D. Waddell, and W. T. Walsh.** 1974. *In vitro* studies of the action of antibiotics on *Rickettsia prowazeki* by two basic methods of cell culture. J. Infect. Dis. **130**:564–574.
18. **Wolbach, S. B.** 1948. The pathology of the rickettsial diseases of man, p. 118–125. *In* F. R. Moulton (ed.), Rickettsial diseases of man. American Association for the Advancement of Science, Washington, D.C.
19. **Wolbach, S. B., and M. J. Schlesinger.** 1923–24. The cultivation of the micro-organisms of Rocky Mountain spotted fever (*Dermocentroxenus rickettsi*) and of typhus (*Rickettsia prowazeki*) in tissue plasma cultures. J. Med. Res. **44**:231–256.