INFECTION AND IMMUNITY, Feb. 1978, p. 607-612
0019-9567/78/0019-0607$02.00/0

Vol. 19, No. 2
Printed in U.S.A.

# Kinetics of Phagocytosis of *Chlamydia psittaci* by Mouse Fibroblasts (L Cells): Separation of the Attachment and Ingestion Stages

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Received for publication 8 August 1978

The kinetics of phagocytosis of *Chlamydia psittaci* (6BC) by monolayers of mouse fibroblasts (L cells) was studied with an assay that distinguished between the attachment and ingestion phases of phagocytosis. At multiplicities of 10 and 100 50% infectious doses ($ID_{50}$) per L cell, virtually all of the inoculated *C. psittaci* had been attached and ingested after 60 min at 37°C. At multiplicities of 500 to 5,000 $ID_{50}$ per L cell, the initial rates of attachment and ingestion of *C. psittaci* to L cells increased with the multiplicity of infection, but phagocytosis stopped even though many chlamydial cells remained free in suspension and readily available for attachment to the host-cell monolayers. Phagocytosis probably ceased because the L cells were injured when they took up large numbers of chlamydial cells. This injury prevented direct determination of the number of potential binding sites for *C. psittaci* on each L cell. However, this number is large enough to make the rates of chlamydial attachment and ingestion predominantly dependent on the multiplicity of infection.

In the accompanying paper (3), it was hypothesized that members of the genus *Chlamydia* enter nonprofessional phagocytes with unusual efficiency because they carry on their surfaces ligands with high affinity for normal, ubiquitously distributed structures on the surface of host cells. This mode of chlamydial entry was called parasite-specified phagocytosis to distinguish it from the host-specified immunological and non-immunological phagocytosis carried out by professional phagocytes. Since phagocytosis by professional phagocytes has been shown to occur in distinct stages of attachment and ingestion (10, 11), a method for separating the attachment and ingestion phases in the phagocytosis of *Chlamydia psittaci* (6BC) by mouse fibroblasts (L cells) was devised and used to study the kinetics of the process.

## MATERIALS AND METHODS

**Growth of L cells and *C. psittaci*.** Methods for propagating L-cell monolayers and growing, harvesting, titrating, partially purifying, and preparing radioisotopically labeled suspensions of *Chlamydia psittaci* (6BC) have already been described (2, 3, 6).

**Measurement of the phagocytosis of $^{14}$C-labeled *C. psittaci* by L-cell monolayers: separation of attachment and ingestion stages.** L cells were suspended in medium 199 containing 0.1% sodium bicarbonate, 200 μg of streptomycin sulfate per ml, and 10% heat-inactivated fetal calf serum (FCS) at a density of $10^6$ cells per ml. Four-milliliter portions were added to plastic flasks (25-$cm^2$ surface area) that had been previously equilibrated in an atmosphere of 5% $CO_2$ and 95% air, and the flasks were incubated at 37°C for 4 h. After incubation, the monolayers were observed with a phase-contrast microscope to be sure that the cells had attached and spread confluently on the substrate as expected. Labeled chlamydiae were diluted in sucrose-phosphate buffer (1) containing 2% FCS and 2 μg of cycloheximide per ml so that appropriate infectious doses would be contained in volumes of 0.6 to 0.8 ml. Cycloheximide inhibited regeneration of the L-cell sites that bind chlamydiae (2) and was without effect on their uptake during the relatively brief periods of incubation employed (3).

The medium was decanted from the monolayers and replaced with the chlamydial inoculum. For zero-time points, the inoculum was decanted immediately. The other flasks were incubated at 37°C for the required times with constant shaking at 125 strokes/min. After incubation, the inoculum was decanted and the monolayers were washed three times with 4 ml of ice-cold growth medium and three times with 4 ml of ice-cold phosphate-buffered saline (4). The washings were combined with the decanted inoculum, and 0.5 ml of 0.08% trypsin (Difco, 1:200) was added to each flask. The excess trypsin was decanted and added to the combined washings. After each monolayer had been at 25°C for 10 min, the cell layer was detached from the substrate by agitation with 2.5 ml of medium, and the L-cell suspension was transferred to a conical centrifuge tube. The flasks were washed once with 2.5 ml of medium, and the washings were combined with the cells already in the centrifuge tubes. The L-

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cell suspensions were then centrifuged for 5 min at 300 × *g* and separated into sedimented and supernatant fractions.

Since experiments with flasks containing no host cells showed that the inoculum did not remain attached to the plastic substrate after the appropriate number of washes, the entire chlamydial inoculum must have been, at any time after infection, either free in suspension, attached to the host cell surface, or inside the host cell. The chlamydiae found in the combined fractions composed of the decanted inoculum, the first six washes, and the excess decanted trypsin solution were defined as free chlamydiae. The concentration of free chlamydiae was assessed by determining the total acid-insoluble counts in the combined fraction just described and by titrating the infectivity of a portion of this sample in fresh L cells (2, 6). Since trypsin-sensitive binding sites on the host cell surface are required for phagocytosis of chlamydiae by L cells (2, 3), those chlamydiae that remained associated with the L cells during the initial washings but were rendered nonsedimentable at 300 × *g* after trypsinization of the monolayers were defined as externally attached parasites and were measured by the same methods used to determine the concentration of free chlamydiae. Ingested chlamydiae were represented by the $^{14}C$ counts that sedimented with the trypsinized L cells during centrifugation at 300 × *g* and were, therefore, not susceptible to removal from the host cell surface by tryptic digestion. That the chlamydiae classified as ingested in this assay were truly taken into L cells was shown by measuring ingestion of $^{14}C$-labeled *C. psittaci* both by the method just described and by determining the percentage of L cells infected (inclusion bearing) at low multiplicity. The good agreement between the two methods shows that the labeled chlamydiae that became associated with L cells and were not dissociated by trypsin actually entered host cells, multiplied, and formed visible inclusions. At higher multiplicities, the two methods were compared by measuring in the same sample the increase in $^{14}C$ label associated with L cells and the decrease in the *C. psittaci* infectivity remaining free and unattached to L cells (Fig. 1). The specific activity (number of $^{14}C$ counts per 50% infectious dose [$ID_{50}$]) of each labeled chlamydial preparation was measured each time it was used by titrating the infectivity and determining the number of counts in the ingested fraction after 2 h at 37°C for an inoculum (<10 $ID_{50}$/L cell) that was completely ingested by that time. All results were expressed as the number of $ID_{50}$ doses of *C. psittaci* attached or ingested per L cell in order to facilitate comparison between different experiments in which labeled chlamydial preparations of varying specific activity were used. One $ID_{50}$ unit (for one L cell) closely approaches the theoretical value of 0.7 of a plaque-forming unit (8). Friis (5) has shown by electron microscopic counts of freshly prepared *C. psittaci* suspensions that one plaque-forming unit is equal to approximately one elementary body.

**Scanning electron microscopy.** Methods for preparing L cells and chlamydiae for scanning electron microscopy were similar to those described by Kaplan et al. (7). L-cell monolayers were grown on round cover slips 12 mm in diameter in 60-mm-diameter petri dishes. The chlamydial inoculum was added in a volume of 1 ml of growth medium. After incubation at 37°C, the cover slips were washed three times with PBS and then fixed for 15 min with 2% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.45. The monolayers were dehydrated first through a graded series of ethanol solutions and then through a graded series of amyl acetate-ethanol solutions. The samples were rinsed several times with liquid $CO_2$ at the critical point in a Bomar drier. The dried samples were coated with gold-palladium vapor under a vacuum of $10^{-6}$ torr in an Edwards coater. Microscopy was performed with an Hitachi HFS-2 microscope.

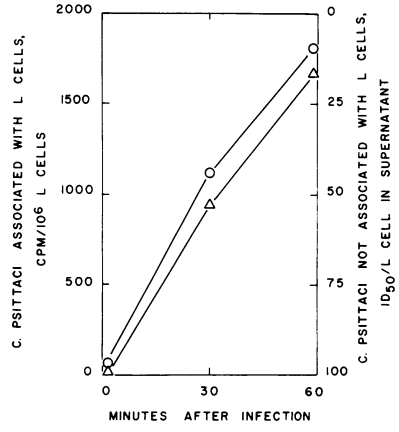


FIG. 1. *Relation between association of $^{14}C$ label with L cells and disappearance of free infectious chlamydiae when 100 $ID_{50}$ $^{14}C$-labeled C. psittaci per host cell was added to an L-cell monolayer. Symbols:* (○) *Attached plus ingested C. psittaci measured as described in the text;* (△) *infectious C. psittaci, free and unattached to L cells measured by $ID_{50}$ titration of the medium overlaying the L-cell monolayer. In the absence of host cells, C. psittaci did not lose infectivity under the conditions of these experiments.*

## RESULTS

**Separation of the attachment and ingestion stages in the phagocytosis of *C. psittaci* by L cells.** Figure 2A shows the attachment and ingestion of *C. psittaci* at a multiplicity of infection of 10 $ID_{50}$ per L cell. The number of attached chlamydiae increased during the first 30 min after inoculation and then declined during the next 60 min. This was because, during the period 30 to 90 min after inoculation, previously attached chlamydiae were ingested faster than the remaining population of free *C. psittaci* became attached. By 90 min after inoculation of the monolayer, all of the initial population of *C. psittaci* had either been attached or ingested. Since 90% were not released from L cells by digestion with trypsin, they were considered to be ingested. With an inoculum of 100 $ID_{50}$ per L cell (Fig. 2B), the results were comparable to

those obtained with the 10-$ID_{50}$ inoculum. At 60 min after inoculation, most of the added *C. psittaci* had been attached and ingested. Some were still removed by trypsin (attached but not ingested), but very few were still free in suspension. With larger inocula (500 to 5,000 $ID_{50}$ per L cell; Fig. 2C-F), the number of chlamydiae ingested by each L cell increased as the chlamydial multiplicity was increased, but after 60 min of incubation, large fractions of the initial chlamydial population remained either externally attached or free in suspension. Free chlamydiae were not plotted in Fig. 2 because, within experimental error, the free values always equaled the difference between the chlamydiae added in the inoculum and the chlamydiae attached and ingested; that is, all the *C. psittaci* elementary bodies were accounted for.

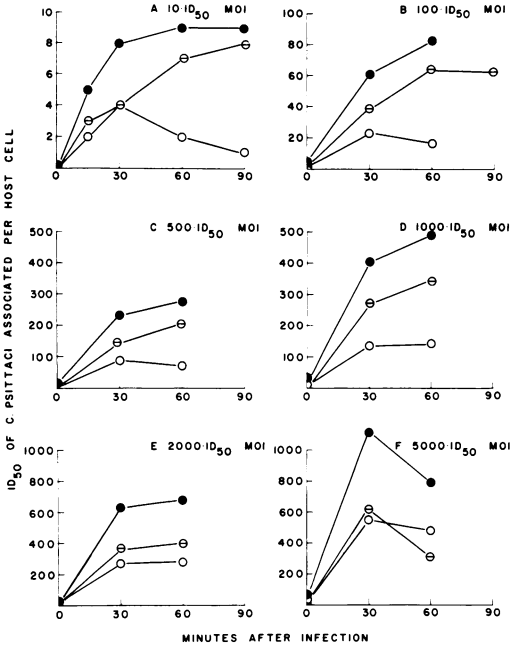


FIG. 2. *Phagocytosis of six different multiplicities of C. psittaci by L-cell monolayers: separation of attachment and ingestion stages. (A) Infection with 10 $ID_{50}$ of C. psittaci per L cell; (B) 100 $ID_{50}$; (C) 500 $ID_{50}$; (D) 1,000 $ID_{50}$; (E) 2,000 $ID_{50}$; (F) 5,000 $ID_{50}$. The experiment was conducted as described in the text. Symbols: (○) C. psittaci attached but not ingested; (⊖) C. psittaci ingested; (●) C. psittaci attached plus ingested.*

**Kinetics of attachment and ingestion of *C. psittaci* by L cells.** The kinetics of the phagocytosis of $^{14}C$-labeled *C. psittaci* by monolayers of L cells was established by comparing rates of attachment and ingestion over a 500-fold range of chlamydial multiplicity. Comparison was facilitated by replotting the data shown in Fig. 2 as three families of curves (Fig. 3). The first family (Fig. 3A) shows how the rate and extent of chlamydial attachment changed with increasing inoculum size. Chlamydiae that had attached to L cells at any given time after inoculation were taken to be the sum of the externally attached plus ingested parasites found at that particular time of measurement. The second family of curves (Fig. 3B) shows the rates of ingestion, and the third (Fig. 3C) shows the number of chlamydiae that had attached to L-cell surfaces but had not as yet been ingested. These curves demonstrate that the rate at which *C. psittaci* attached to L cells and the rate at which it was ingested increased with the multiplicity of infection over the entire range of inoculum size tested.

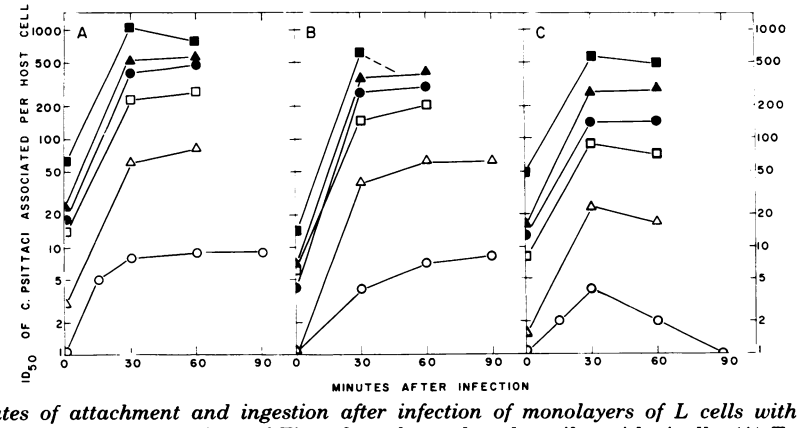


FIG. 3. *Rates of attachment and ingestion after infection of monolayers of L cells with six different multiplicities of C. psittaci. The data of Fig. 2 have been plotted semilogarithmically. (A) Total C. psittaci attached to L cells (sum of attached plus ingested); (B) C. psittaci ingested; (C) C. psittaci attached but not ingested. Symbols: (○) 10 $ID_{50}$ per L cell; (△) 100 $ID_{50}$; (□) 500 $ID_{50}$; (●) 1,000 $ID_{50}$; (▲) 2,000 $ID_{50}$; (■) 5,000 $ID_{50}$.*

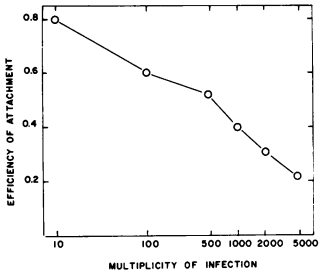


FIG. 4. *Efficiency of attachment of C. psittaci to L-cell monolayers at different multiplicities of infection ($ID_{50}$ per host cell). The efficiency of attachment is equal to the $ID_{50}$ of C. psittaci attached to each L cell (sum of attached plus ingested) 30 min after infection divided by the $ID_{50}$ of C. psittaci added for each L cell. For example, the efficiency of attachment at a multiplicity of 100 $ID_{50}$ per host cell was 62 (see Fig. 2B) divided by 100.*

However, these curves also show that the fraction of the chlamydial inoculum initially bound to L cells decreased with increasing multiplicity of infection. This trend is made clear in Fig. 4, in which the fraction of each inoculum that had been attached to L cells (attached plus ingested) 30 min after inoculation was plotted against the inoculum size. The curve indicates that as the multiplicity of infection became very large, the number of chlamydiae that had attached to L cells 30 min after inoculation was approaching a constant value.

**Morphology of L cells before and after inoculation with high multiplicities of *C. psittaci*.** When L cells were infected with *C. psittaci* at multiplicities greater than 100 $ID_{50}$ per host cell, scanning electron microscopy revealed gross morphological alterations only a few minutes after inoculation. Figure 5A shows an uninfected L cell, and Fig. 5B shows a comparable L cell inoculated 45 min previously with 2,000 $ID_{50}$ of *C. psittaci*.

## DISCUSSION

When the phagocytosis of $^{14}C$-labeled *C. psittaci* by L-cell monolayers was measured by the trypsinization technique described in Materials and Methods, there was a clear-cut distinction between the chlamydiae that had been internalized and those that had become attached but were not as yet ingested. The externally attached *C. psittaci* were removed from the L-cell binding sites when these structures were functionally destroyed by trypsin (2, 3), but the ingested chlamydiae remained associated with L cells even after trypsinization.

After examination of a narrow range of chlamydial multiplicity with a method that measured only the sum of externally attached plus ingested chlamydiae, Friis (5) concluded that the rate of attachment of *C. psittaci* to L cells varied with the inoculum size. The experiments reported here confirm and extend his initial observations. At multiplicities of 10 to 100 $ID_{50}$ per L cell, phagocytosis continued until all or nearly all of the available chlamydiae had been attached or ingested. At each higher multiplicity (500 to 5,000 $ID_{50}$ per L cell), it appeared that saturation of the phagocytic capacity of the L cells had been achieved. Little or no attachment and ingestion took place after 30 min, even though large numbers of infectious chlamydial cells remained free and available for phagocytosis. However, raising the inoculum size always increased the initial rates of attachment and ingestion of *C. psittaci*, so it was plain that true saturation of the chlamydial binding sites on the L cells was never reached.

Reduction in the initial rates of attachment and ingestion would be expected as the concentration of free chlamydiae decreased and as the L-cell-associated fraction of the population increased because these rates were multiplicity dependent. However, the almost complete cessation of attachment and ingestion observed at high multiplicities is most reasonably explained in the following way. According to Vernon-Roberts (12), attachment of particles to macrophages depends only on the physical integrity of the plasma membrane, whereas ingestion depends on both membrane integrity and continued metabolic activity. If it is assumed that L-cell membrane integrity and metabolic activity are similarly important in chlamydial phagocytosis, then cessation of both attachment and ingestion by L cells shortly after contact with large numbers of chlamydiae suggests a simultaneous injury to both the plasma membrane itself and the metabolic machinery of the host cell. Direct evidence of damage to L-cell monolayers inoculated with high multiplicities of *C. psittaci* was obtained by scanning electron microscopy. This damage is consistent with the many physical and chemical signs of injury observed immediately after L cells were inoculated with comparable multiplicities of *C. psittaci* (9). The loss of $^{14}C$ counts from samples taken 60 min after inoculation with 5,000 $ID_{50}$ of *C. psittaci* per L cell (Fig. 2F) was yet another sign of host cell injury. Damaged cells detached from their substrate and were lost during washing.

Large numbers of *C. psittaci* per L cell did not interfere with phagocytosis by their mere external presence in the medium. Up to 30 min after inoculation, phagocytosis proceeded at rates that were related to chlamydial multiplicity. Attachment and ingestion stopped only after

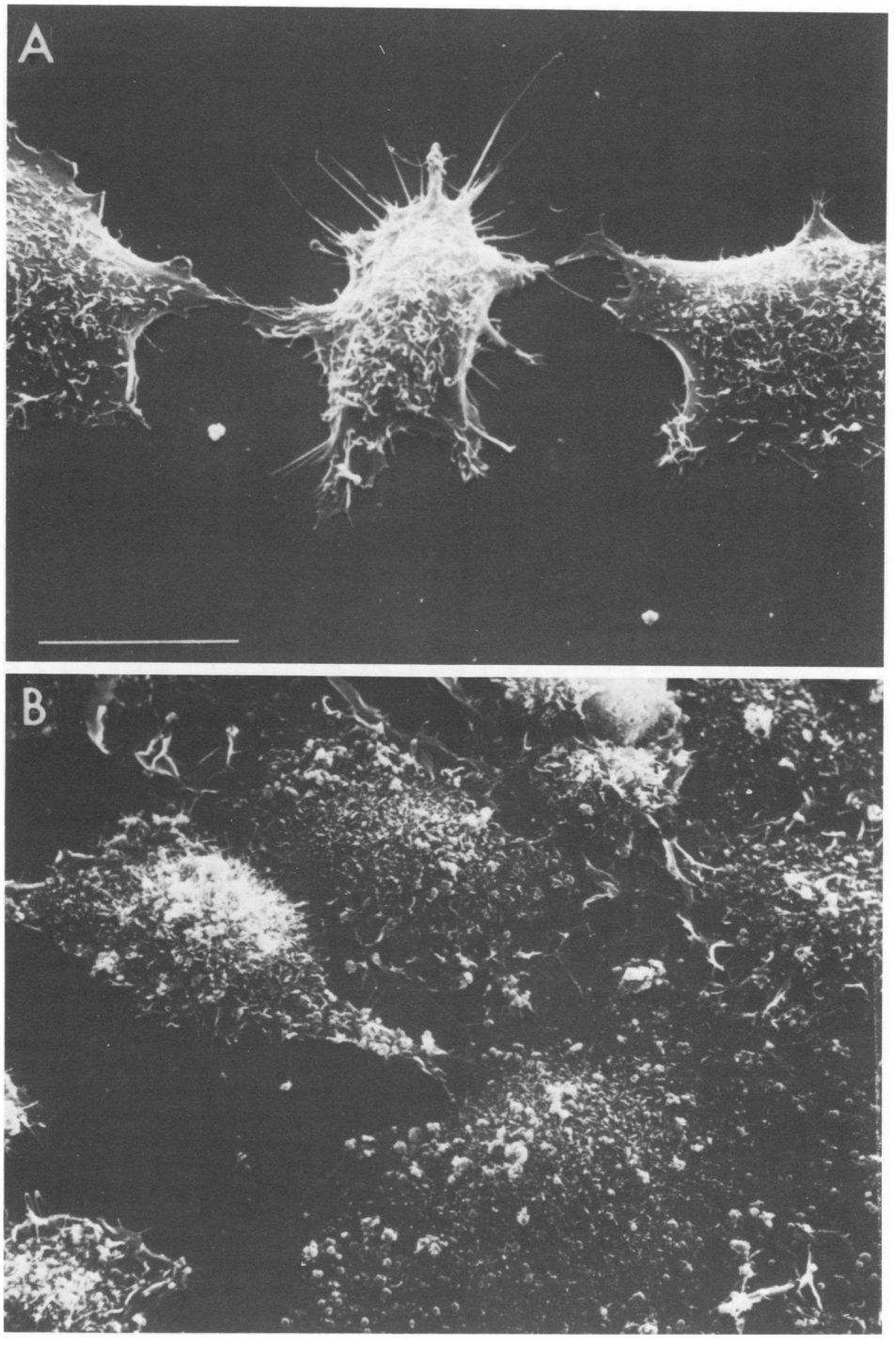


FIG. 5. *Scanning electron micrographs of uninfected and infected L cells. (A) Uninfected L-cell monolayer 3.75 h after plating out on substrate. (B) L-cell monolayer infected with 2,000 $ID_{50}$ of C. psittaci per host cell 3 h after plating and fixed for electron microscopy 45 min later. ×2,800. The bar in (A) represents 10 μm.*

damaging numbers of chlamydiae had been ingested by the L cells. These observations are in accord with the earlier conclusion that the immediate toxicity of *C. psittaci* for L cells resulted from the ingestion event itself (9).

Although the near-complete cessation of attachment and ingestion observed 30 min after inoculation of *C. psittaci* was not due to depletion of available binding sites on the L-cell surface, saturation of these sites should have been theoretically possible unless limitless areas of the L-cell surface were available for binding *C. psittaci*. The interference with phagocytosis produced by large inocula precluded direct measurement of the number of chlamydial binding sites on each L cell. That number, however, must be considerably larger than the toxic tolerance of the L cell. Therefore, under most conditions of chlamydiae-host cell interaction, the rates of attachment and ingestion will be determined largely by the multiplicity of infection. Because the phagocytosis of chlamydiae may in itself be injurious to host cells, the ingestion of a critical number of chlamydial cells may alter the rates at which additional parasites are attached and ingested.

## ACKNOWLEDGMENTS

I thank James W. Moulder for help and encouragement in this study. The scanning electron micrographs were taken in the Microscope User's Laboratory of the University of Chicago, which is supported by a grant from the Biotechnical Resources Branch of the National Institutes of Health.

This investigation was supported by Public Health Service research grants AI-1594 and AI-13175 from the National Institute of Allergy and Infectious Diseases and by Public Health Service training grant GM-603 from the National Institute of General Medical Sciences.

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