# Toxicity of Low and Moderate Multiplicities of *Chlamydia psittaci* for Mouse Fibroblasts (L Cells)

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When mouse fibroblasts (L cells) were infected in suspension or in monolayer with 10 to 100 50% infectious doses ($ID_{50}$) of *Chlamydia psittaci* (6BC) per host cell, they showed signs of damage 24 to 48 h later. Host-cell injuries were termed multiplication dependent when both the ingestion and subsequent reproduction of *C. psittaci* were required; when only ingestion but not replication was needed, the injuries were considered to be multiplication independent. The time that the injury was first apparent, as well as its final magnitude, was proportional to the multiplicity of infection. When L cells ingested infectious or ultraviolet-inactivated *C. psittaci,* damage was manifested by failure to exclude trypan blue, by leakage of lactic dehydrogenase, by inhibition of reproduction as measured by ability to form colonies, by inhibition of protein and deoxyribonucleic acid synthesis, and eventually by cell disintegration. Infectious, but not ultraviolet-killed, chlamydiae stimulated host-cell glycolysis. Heat-killed chlamydiae were without measurable toxicity. The time of appearance of host-cell injury was always earlier, and its terminal magnitude always greater, with infectious inocula than with ultraviolet-inactivated ones. The multiplication-independent toxicity of ultraviolet-killed *C. psittaci* disappeared with inocula of less than 10 $ID_{50}$ per L cell, but an inoculum of only a single $ID_{50}$ of infectious chlamydiae per host cell injured most of the cells it infected, as evidenced by increased trypan blue staining and decreased efficiency of colony formation. The toxicity of multiplicities of infection between 10 and 100 $ID_{50}$ of infectious *C. psittaci* per host cell was the sum of both multiplication-dependent and -independent components. The effects of chloramphenicol and isoleucine deficiency on the ability of *C. psittaci* to injure L cells suggested that some synthesis of protein by both parasite and host may be essential for expression of multiplication-independent chlamydial toxicity. The failure of infectious chlamydiae to stimulate host-cell glycolysis in the presence of cycloheximide suggested that this multiplication-dependent consequence of chlamydial infection was also dependent on protein synthesis by the host.

When mouse fibroblasts (L cells) were infected with 200 to 1,000 50% infectious units ($ID_{50}$) per L cell of *Chlamydia psittaci* 6BC, they showed many different signs of injury within 1 h and were all dead by 20 h (19). Ingestion of chlamydial cells, but not their subsequent multiplication, was required for production of immediate toxicity, which appeared to result from injury to the plasma membrane of the host cell during its ingestion of *C. psittaci.* This paper describes the toxicity of lesser multiplicities (1 to 100 $ID_{50}$ per host cell) of *C. psittaci* for L cells. Injury of host cells at these multiplicities of infection also depended on ingestion of the chlamydial cells. However, the origins of the host-cell damage were more complex. In proportions that depended on multiplicity of infection and the state of both the host

and the parasite, L cells were injured in ways that were both independent of and dependent on multiplication of the ingested *C. psittaci.*

## MATERIALS AND METHODS

**Growth of L cells.** The 5b clone (28) of Earle L cells was grown in suspension and in monolayer as described by Hatch (11). Cultures were kept at 37°C in an atmosphere of 5% $CO_2$–95% air in medium 199 (17) containing 0.1% sodium bicarbonate, 200 µg of streptomycin sulfate per ml, and heat-inactivated fetal calf serum, 5% for suspensions and 10% for monolayers. Identical results were obtained in modified Eagle medium with nonessential amino acids (7). Cell densities were measured with a Coulter cell counter model Zb (Coulter Electronics, Hialeah, Fla.). Negative cultures for mycoplasma were obtained by Flow Laboratories (Rockville, Md.) at the beginning and at the end of this work.

**Growth, titration, and inactivation of *C. psittaci*.** *C. psittaci* 6BC was propagated in and harvested from L cells as described by Hatch (11). All chlamydial inocula consisted of crude harvests (11).

Chlamydial infectivity was titrated by the procedure of Hatch (11), which consists of determining the volume of a chlamydial suspension that will infect half of a precisely enumerated number of L cells. When crude harvests were titrated in terms of both $ID_{50}$ and plaque-forming units (3), the theoretical ratio of 1 $ID_{50}$ unit (for a single host cell) for every 0.7 plaque-forming unit was approached closely. Electron microscopic examination of thin sections of pellets of crude harvests by the methods of Friis (9) revealed that about half of the *C. psittaci* cells were elementary bodies and the rest were reticulate bodies. At the multiplicities employed here, all or nearly all of the infectious elementary bodies had been ingested by L cells after 2 h at 37°C (G. I. Byrne and J. W. Moulder, submitted for publication).

Crude harvests were inactivated with heat (3 min at 60°C) and ultraviolet light (1,000 ergs per $mm^2$) by the directions of Byrne (4). They were also inactivated by exposure to 50 mM methylmethane sulfonate for 10 min at 37°C. As used in these studies, all three inactivating agents caused an approximate 1,000-fold drop in the infectivity of *C. psittaci* for L cells. Multiplicities of infection with inactivated chlamydiae will be expressed in terms of the pre-inactivation titers.

**Assessment of L-cell damage by *C. psittaci*.** The exclusion of trypan blue (21) was observed by mixing 1 volume of 0.5% trypan blue (NA 0508, Allied Chemical, Morristown, N.J.), dissolved in distilled water and filtered through a 0.45-$\mu$m filter, with 2 volumes of cell suspension and immediately counting the percentage of trypan blue-staining cells in a hemocytometer. At least 200 cells were examined.

Protein synthesis in L cells was determined as previously described (19). Synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was measured in a similar way by substituting [$^{14}$C]uridine and [$^3$H]thymidine for the L-$^{14}$C-labeled amino acid mixture used after protein synthesis. Isoleucine-depleted medium (11) was used for measuring incorporation of amino acids, but not for incorporation of nucleosides.

Glucose utilization was determined according to Sigma Technical Bulletin 635 (Sigma Chemical Co., St. Louis, Mo.) (8); lactate production was determined according to Sigma Technical Bulletin 826-UV (15); and extracellular release of lactic dehydrogenase was determined according to Sigma Technical Bulletin 340-UV (32).

The ability of infected L cells to form visible colonies on solid substrates was assayed as follows. An L-cell suspension fed 24 h previously was diluted to a density of 100 cells per ml in growth medium and added to 4 ml of medium in a plastic tissue culture flask (25 cm) previously equilibrated in 5% $CO_2$-95% air. After 10 days at 37°C, the L-cell colonies were washed three times with phosphate-buffered saline (6) and stained with Giemsa stain (11). After 10 min, the stain was drained off as completely as possible, and, without further treatment, the colonies were counted in a bacterial colony counter. A suspension of L cells with a count of 100 cells per ml in the Coulter counter produced 65 to 85 colonies per ml.

**Basic experimental plan.** Most experiments shared a common design. An L-cell suspension fed 24 h previously was centrifuged to sediment the L cells, which were then resuspended to a density of $10^6$ cells per ml in growth medium and dispensed in 25-ml volumes into 50-ml spinner bottles (14, 16) previously equilibrated in 5% $CO_2$-95% air. The chlamydial inoculum suspended in phosphate-buffered saline, any other additions also in phosphate-buffered saline, and enough growth medium to bring the culture to a volume of 50 ml and a cell count of 0.5 × $10^6$ per ml were then added. The spinner bottles were incubated at 37°C. Taking 2 h after addition of the *C. psittaci* inoculum as zero hour (the time needed for attachment and ingestion of the added chlamydiae [4]), samples were withdrawn 24, 48, 72, and sometimes 96 h later to measure the density of the L-cell suspension and its percentage of trypan blue-positive cells, as well as other indicators of L-cell damage or chlamydial multiplication that were desired in any particular experiment. More frequent observations did not add materially to the information obtained. When the inoculum was 50 $ID_{50}$ of infectious *C. psittaci* per L cell, host-cell damage was sometimes obvious as early as 18 h postinfection.

## RESULTS

The reaction of cultured cells with trypan blue is a long-established (21) and often uncritically accepted criterion of cell viability. "Dead" cells take up trypan blue and are stained; "live" cells exclude the dye and are not stained. It will be shown here that failure of an L cell infected with *C. psittaci* to exclude trypan blue is a sensitive and convenient indicator of host-cell injury, one that often but not always agrees well with other more laboriously obtained indexes of chlamydial toxicity. Some of the factors that determine how badly L cells are injured by low to moderate multiplicities of *C. psittaci* will first be illustrated with trypan blue staining as the sole indicator of L-cell damage. Chlamydial toxicity will then be examined further by using a number of other ways of assessing injury to host cells.

**Failure to exclude trypan blue as an index of host-cell injury.** At multiplicities of 10 to 100 $ID_{50}$ of *C. psittaci* per L cell, injury to the host cell may be separated into two components, one dependent upon and the other independent of parasite multiplication. This may be done by comparing the effect of infectious chlamydiae with that of chlamydiae that have been made incapable of multiplying without reducing their ingestibility. Although such inactivated chlamydiae were highly injurious to host cells, at any given multiplicity of infection, chlamydiae capable of multiplying after they were ingested were always more toxic than those that were ingested but did not multiply.

Figure 1 compares the percentage of trypan blue-positive host cells produced by exposing L cells to infectious and ultraviolet-inactivated *C. psittaci,* which are phagocytosed at equal rates (4). At a multiplicity of 10 $ID_{50}$ per host cell, the inactivated chlamydiae had a substantially smaller effect, but at 80 $ID_{50}$, they produced almost as many trypan blue-staining L cells as did infectious *C. psittaci.* Chlamydiae inactivated with methylmethane sulfonate are phagocytosed at undiminished rates (G. I. Byrne, personal communication), and, at a dosage of 100 $ID_{50}$ per L cell, they too were toxic (Fig. 2). Ultraviolet light and alkylating agent both made chlamydiae nonreproductive by inactivating DNA. It may be that any agent that inactivates without altering cell surfaces will produce toxic but nonreproducing chlamydial cells.

The existence of multiplication-dependent and -independent toxicity may also be shown by allowing L cells to ingest infectious chlamydiae and shortly thereafter blocking their multiplication with chloramphenicol (29). When the antibiotic was added to L-cell suspensions 2 h postinfection, chlamydial multiplication was stopped completely, but as measured by the increase in trypan blue-staining host cells, the multiplication-inhibited chlamydiae were still toxic (Fig. 3). As with ultraviolet-inactivated chlamydiae, the higher the multiplicity, the more apparent the host-cell damage. Rifampin also prevented chlamydial multiplication (29), but not host-cell injury.

Multiplication-dependent chlamydial toxicity was not greatly affected by the multiplicity of infection. Five to 10 $ID_{50}$ of infectious *C. psittaci* per L cell generated trypan blue-positive host cells almost as fast as 50 to 100 $ID_{50}$ (Fig. 1 and



FIG. 2. *Trypan blue staining of L cells after exposure to infectious, methylmethane sulfonate-inactivated, and heat-killed C. psittaci. See text for procedural details. The infecting dose of C. psittaci was 100 $ID_{50}$ per L cell. Symbols:* O, *infectious C. psittaci;* ●, *methylmethane sulfonate-inactivated C. psittaci;* △, *heat-inactivated C. psittaci; and* ▲, *chlamydiae inactivated first with methylmethane sulfonate and then with heat.*



FIG. 3. *Trypan blue staining of L cells infected with C. psittaci in the presence and absence of chloramphenicol. Giemsa-stained monolayers were examined to be sure that the antibiotic had inhibited chlamydial reproduction. Symbols:* O, *no drug and* ●, *100 μg of chloramphenicol succinate per ml added 2 h after infection. (A) 5 $ID_{50}$ of C. psittaci per L cell. (B) 50 $ID_{50}$ per host cell.*

3). Multiplication-independent toxicity disappeared below the level of 5 to 10 $ID_{50}$, but even 1 and 2 $ID_{50}$ of infectious *C. psittaci* per L cell made most of the infected cells become trypan blue-positive within 72 h (Table 1).

Ingestion of chlamydial cells was obviously a prerequisite for multiplication-dependent toxicity, but the ingestion requirement for multiplication-independent injury was not self-evident. However, the toxicity of moderate chlamydial multiplicities that is manifested in the absence of parasite multiplication proved to be, like the
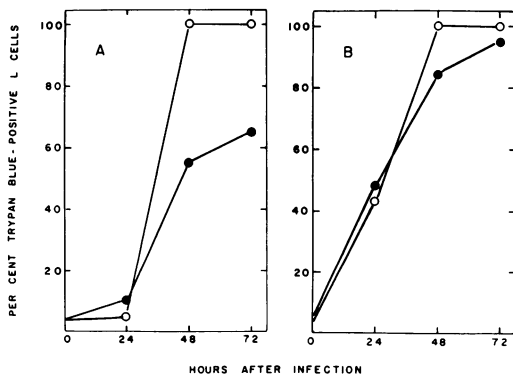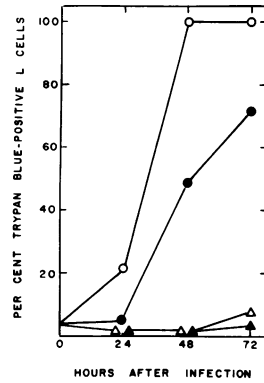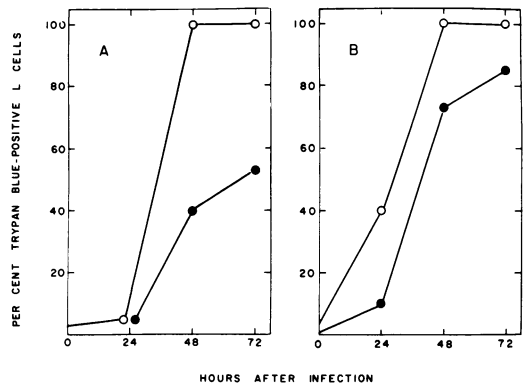


FIG. 1. *Trypan blue staining of L cells after exposure to infectious and ultraviolet-inactivated C. psittaci. The experiment was performed as described in the text. Symbols:* O, *infectious C. psittaci and* ●, *ultraviolet-inactivated C. psittaci. (A) 10 $ID_{50}$ of C. psittaci per L cell. (B) 80 $ID_{50}$ per L cell.*

immediate toxicity of high-multiplicity infection (19), dependent on ingestion of the chlamydial cells. Heat-inactivated *C. psittaci,* which is phagocytosed very inefficiently by L cells (4), did not cause any increase in the number of trypan blue-positive host cells (Fig. 2). When L cells were infected with 500 to 1,000 $ID_{50}$ of heat-inactivated chlamydiae as in an earlier study (19), they multiplied as if uninfected for many days and then finally succumbed to the progeny of the small number of infectious *C. psittaci* that survived the heat inactivation.

**Synthesis of protein by both parasite and host as a possible requirement for expression of multiplication-independent toxicity.** Although *C. psittaci* can injure host cells without multiplying, it appears likely that both chlamydiae and host cells must synthesize some protein if this multiplication-independent toxicity is to be expressed.

The ability of chloramphenicol-inhibited *C. psittaci* to make L cells stain with trypan blue was highly dependent on the time the antibiotic was added to infected L cells (Fig. 4). Delaying chloramphenicol addition from 0 h to 2 h after infection greatly increased the toxicity of the multiplication-inhibited chlamydiae, but further delay had little additional effect. These results show that, in the first 2 h after infection, *C. psittaci* carried out a chloramphenicol-sensitive reaction, probably synthesis of a protein, that
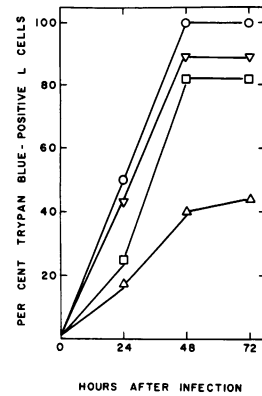


FIG. 4. *Effect of the addition of chloramphenicol at different times after infection with C. psittaci on trypan blue staining of L cells. The experiment was done as described in the text. Symbols: 50 $ID_{50}$ of C. psittaci per L cell with no added drug (O) and with 100 µg of chloramphenicol succinate added at 0 (△), 2 (□), and 4 (▽) h after infection.*

TABLE 1. *Trypan blue staining of L cells 72 h after infection with 1 and 2 $ID_{50}$ of C. psittaci per host cell[a]*

| *C. psittaci* inoculated ($ID_{50}$/host cell) | L cells with inclusions 24 h after infection | | Trypan blue-positive L cells 72 h after infection | |
|---|---|---|---|---|
| | Expected (%)[b] | Found (%) | Expected (%)[c] | Found (%) |
| 0 | | | | 4 |
| 1 | 50 | 50 | 33 | 31 |
| 2 | 75 | 74 | 60 | 59 |

[a] The experiment was performed as described in the text.

[b] From the $ID_{50}$ titer of the inoculum, the volume required to infect 50% (1 $ID_{50}$) and 75% (2 $ID_{50}$) of the L-cell population was calculated as described by Hatch (11).

[c] The expected percentage of trypan blue-positive L cells was calculated on four assumptions. (i) All infected cells would not divide. (ii) All uninfected L cells would divide only once in 72 h when the medium was not changed (11). (iii) All originally infected L cells would be trypan blue-positive at 72 h. (iv) No L cells secondarily infected by the progeny of the first developmental cycle would be trypan blue-positive at 72 h. All assumptions are approximations of more complex realities.

was required for production of maximum host-cell damage. Lin (13) observed a comparable effect of the time of chloramphenicol addition on its ability to block the decline in thymidine kinase activity that follows infection of L cells with *C. psittaci.*

L cells in isoleucine-deficient medium 199 normally phagocytosed *C. psittaci,* but neither host nor parsite multiplied until the isoleucine deficiency was relieved and their rates of protein synthesis returned to normal (11). As judged by trypan blue staining, 10 $ID_{50}$ of *C. psittaci* per host cell did not injure L cells in isoleucine-deficent medium (Fig. 5A). When isoleucine was added 24 h after infection, the expected increase in trypan blue-positive cells occurred with a corresponding lag of 24 h. Therefore, even fully infectious chlamydiae did not damage the host cells by which they had been ingested in the absence of an amino acid required by both host and parasite. In two of four experiments with an inoculum of 50 $ID_{50}$ per host cell, the results were the same as indicated in Fig. 5A. In the other two (Fig. 5B), lack of isoleucine only partially blocked the appearance of trypan blue-positive L cells. We cannot explain why feeding isoleucine 24 h after infection appears to have transiently protected L cells against chlamydial injury. Figure 5C differs from 5B only in that the 50-$ID_{50}$ inoculum had been inactivated with ultraviolet light. When isoleucine was not limiting, the inactivated chlamydiae produced the expected percentage of cells that stained with trypan blue, but not only did they not injure isoleucine-deficient host cells, they also re-

mained without toxicity after isoleucine was added at 24 h. Therefore, the capacity to cause multiplication-independent damage was lost during a 24-h layover in isoleucine-deficient host cells, and only the multiplication-dependent capacity remained to be activated by addition of the missing amino acid.

The experiments with isoleucine-deficient host cells suggest that multiplication-independent chlamydial toxicity was not expressed when neither host nor parasite could synthesize protein. An obvious test of this would be to observe the toxicity of *C. psittaci* for L cells when protein synthesis by the parasite is blocked by chloramphenicol (29) and that of the host by cycloheximide (1). Unfortunately, this combination of drugs was in itself toxic to L cells, and the experiment could not be done. Cycloheximide alone slowed, but did not in the long run prevent, the appearance of trypan blue-positive L cells in populations infected with 10 $ID_{50}$ of infectious chlamydiae per host cell (Fig. 6). Thus, the complete suppression of chlamydial toxicity in isoleucine-deficient L cells cannot be attributed to inhibition of protein synthesis by the host alone. It must have also involved interference with chlamydial protein synthesis.

**Assessment of chlamydial damage to host cells by criteria other than trypan blue exclusion.** The *C. psittaci*-L cell systems used most frequently in these experiments were 10 and 50 $ID_{50}$ per host cell infection with infectious or ultraviolet-inactivated chlamydiae. Chloramphenicol-inhibited chlamydiae were not suitable for many experiments because the antibiotic interfered with the taking of measurements.

When the densities of L-cell suspensions were measured with the Coulter counter after infection with *C. psittaci*, the increases in host-cell numbers were those that would have resulted if every uninfected L cell had divided once in 48 h and about one-third to one-half of the infected cells had also divided once (Table 2). A total of 50 $ID_{50}$ per host cell inhibited the increase in L cells more than 10 $ID_{50}$, and ultraviolet-inactivated *C. psittaci* blocked host-cell division just as effectively as infectious organisms. Limited division of cells infected with *C. psittaci* was also observed by Officer and Brown (20).

The effect of infection with *C. psittaci* on the ability of L cells to multiply was also determined
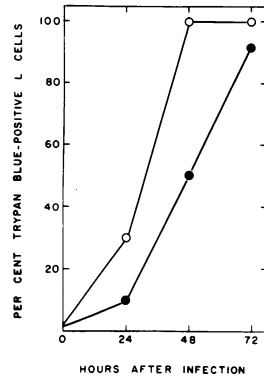


FIG. 6. *Trypan blue staining of L cells infected with C. psittaci in the presence and absence of cycloheximide. Symbols: 10 $ID_{50}$ per host cell of C. psittaci without (O) and with (●) 2 μg of cycloheximide per ml added 2 h after infection. Cycloheximide alone did not increase the number of trypan blue-positive L cells.*
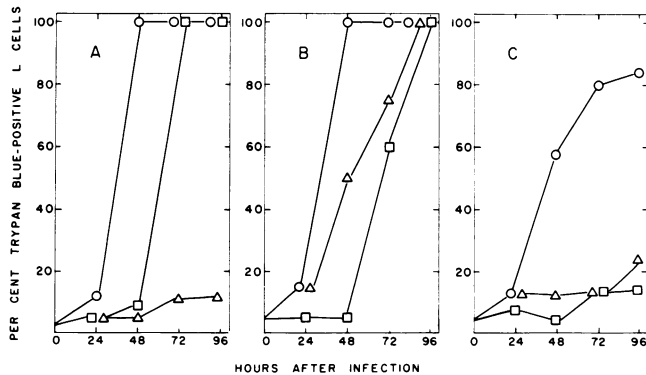


FIG. 5. *Trypan blue staining of L cells exposed to infectious and ultraviolet-inactivated C. psittaci in fresh and in isoleucine-depleted growth medium. Experimental details are described in the text. Isoleucine-deficient medium 199 (with 5% heat-inactivated fetal calf serum) was produced by inoculating it with $10^6$ L cells per ml and allowing them to double once in the next 24 h (11). Symbols: O, fresh medium throughout the experiment; △, isoleucine-deficient medium at all times; □, isoleucine-deficient medium 0 to 24 h after infection, completely replaced at 24 h with fresh medium. (A) 10 $ID_{50}$ of infectious C. psittaci per L cell. (B) 50 $ID_{50}$ of infectious chlamydiae per host cell. (C) 50 $ID_{50}$ of ultraviolet-inactivated C. psittaci per L cell.*

by diluting out the L-cell suspensions immediately after infection, plating out on a solid substrate at a density of four cells per $cm^2$, and counting the number of L-cell colonies visible 10 days later (Table 3). Inocula of 1 and 2 $ID_{50}$ per L cell of infectious chlamydiae significantly reduced colony formation, but not as much as predicted by the trypan blue-staining experiment (Table 1), and 10 and 50 $ID_{50}$ almost completely suppressed it. Heated chlamydiae caused no reduction in colony count. The effect of ultraviolet-inactived C. psittaci on colony formation was dependent on the time the infected cells were diluted out for plating. If the L cells were diluted and plated immediately after infection, ultraviolet-inactivated C. psittaci failed to reduce the number of colonies formed. However, if the L cells infected with the inactivated chlamydiae were held in spinner bottles at a density of $0.5 \times 10^6$ cells per ml for 24 h after infection and then diluted and plated, colony formation was inhibited almost as much as if the chlamydiae had been infectious. This curious phenomenon is being studied further.

The effect of infection with C. psittaci on the appearance of L cells in monolayer was next determined after it had been established that both infectious and ultraviolet-inactivated chlamydiae produced trypan blue-staining L cells at roughly the same rate in suspension and in monolayer. Figure 7 shows the appearance under phase-contrast microscopy of monolayers 2 and 5 days after infection with 10 and 50 $ID_{50}$ per host cell of infectious, heated, and ultraviolet-inactivated C. psittaci. Monolayers of L cells exposed to heated chlamydiae (Fig. 7A to D) were indistinguishable from uninfected ones (not shown). When the inoculum was infectious C. psittaci, the L cells were packed with inclusions at 2 days and were completely destroyed at 5 days (Fig. 7E to H). Monolayers infected with ultraviolet-inactivated chlamydiae showed moderate cytopathic effects at 2 days and consisted

solely of grossly abnormal cells at 5 days (Fig. 7I to L). Normal-appearing cells could not be found, but most of the original L-cell population was still attached to its substrate. Monolayers of L cells exposed to infectious C. psittaci and then treated with chloramphenicol 2 h after infection showed changes very similar to those seen in L cells infected with ultraviolet-inactivated organisms (not shown).

In the experiments just described, the extent of the damage done to L cells by different multiplicities of infectious and inactivated C. psittaci roughly correlated with the extent of trypan blue staining under comparable circumstances, although point-for-point comparisons were not possible. However, direct comparisons could be made when the rates of protein, RNA, and DNA synthesis and the percentages of trypan blue-

TABLE 3. Ability of single L cells to form colonies after exposure to infectious, heat-inactivated, and ultraviolet-inactivated C. psittaci[a]

| Inoculum | | L-cell colonies formed (% of colonies formed by uninfected population)[b] |
|---|---|---|
| $ID_{50}$/L cell | Inactivating agent | |
| None | | 100 |
| None[c] | | 96 |
| 1 | None | 70 |
| 2 | None | 54 |
| 10 | None | 4 |
| 50 | None | 1 |
| 50 | Ultraviolet light | 102 |
| 50[c] | Ultraviolet light | 6 |

[a] The experiment was done as described in the text.
[b] When 100 uninfected L cells were plated out, 70 formed visible colonies in 10 days.
[c] These L-cell suspensions were held for 24 h after infection (or mock infection) at a density of $0.5 \times 10^6$ cells per ml before being plated out at a density of 4 cells per ml. All other suspensions were so diluted at zero hour after infection.

TABLE 2. Increase in cell numbers in suspensions of L cells infected with infectious and ultraviolet-inactivated C. psittaci[a]

| Time after infection (h) | L cells infected with: | | | | |
|---|---|---|---|---|---|
| | Uninfected | Infectious C. psittaci (10 $ID_{50}$/L cell) | Ultraviolet-inactivated C. psittaci (10 $ID_{50}$/L cell) | Infectious C. psittaci (50 $ID_{50}$/L cell) | Ultraviolet-inactivated C. psittaci (50 $ID_{50}$/L cell) |
| 0 | 0.5[b] | 0.5 | 0.5 | 0.5 | 0.5 |
| 24 | 0.66 ± 0.16 (14)[b] | 0.60 ± 0.14 (13) | 0.58 ± 0.11 (10) | 0.56 ± 0.07 (9) | 0.54 ± 0.11 (14) |
| 48 | 0.97 ± 0.29 (14) | 0.83 ± 0.19 (12) | 0.89 ± 0.20 (9) | 0.69 ± 0.11 (9) | 0.73 ± 0.15 (14) |
| 72 | 0.92 ± 0.28 (12) | —[c] | 0.84 ± 0.19 (9) | —[c] | 0.66 ± 0.18 (12) |

[a] The experiment was carried out as described in the text.
[b] Zero-hour counts ranging from $0.39 \times 10^6$ to $0.52 \times 10^6$ L cells per ml were normalized to $0.5 \times 10^6$ cells per ml. Subsequent counts were adjusted to the normalized zero-hour value and are given as millions of L cells per ml ± standard error. Number in parentheses represents number of independent L-cell counts.
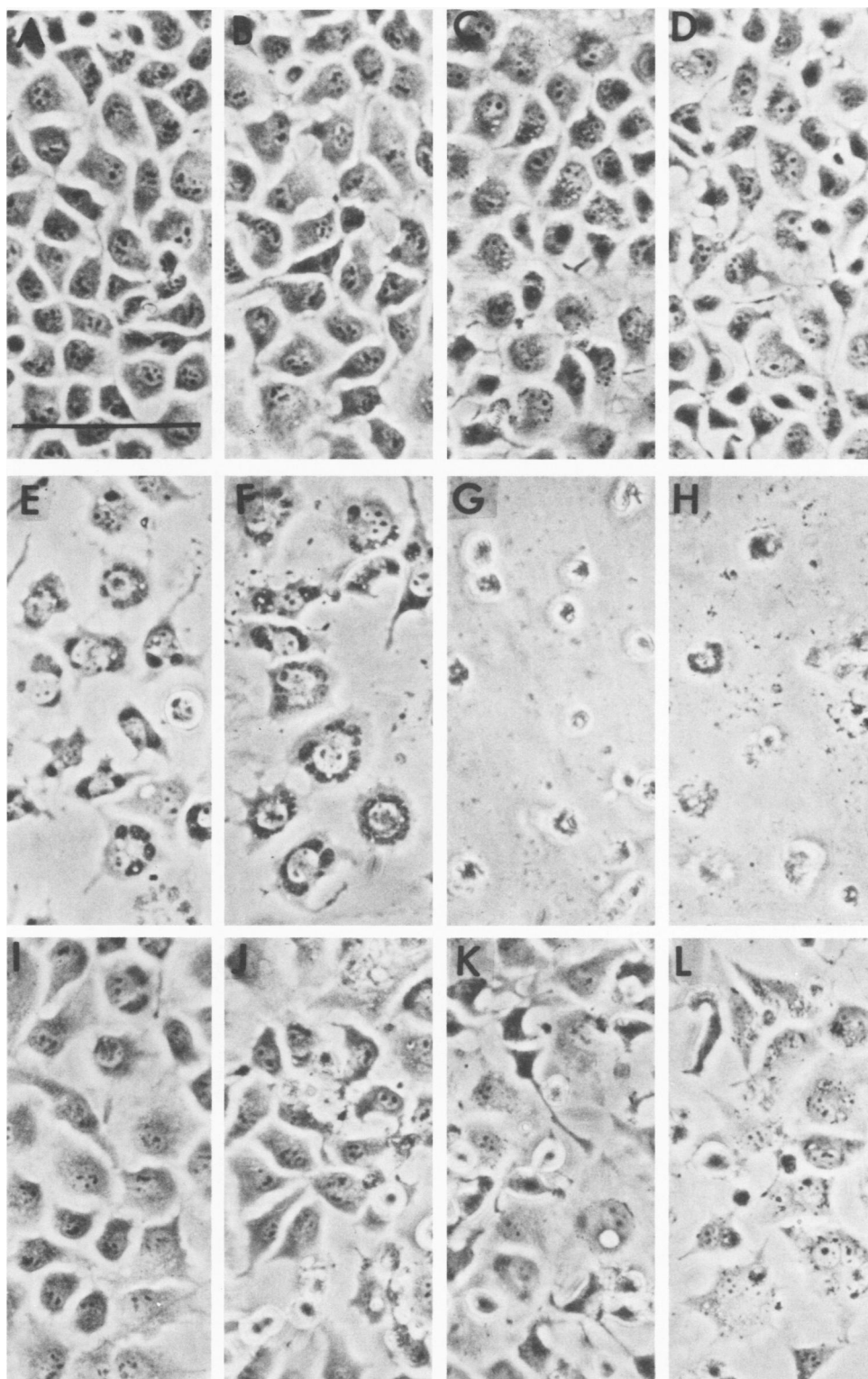[c] —, Cell counts were not made because extensive cell destruction had occurred by this time.

FIG. 7. *Effect of infectious, ultraviolet-inactivated, and heat-inactivated C. psittaci on monolayers of L cells. L cells were infected in suspension and plated out in plastic tissue culture flasks at a density of 10,000 cells per* $cm^2$. *(A) through (D) Heat-inactivated C. psittaci. (A) 10* $ID_{50}$ *per L cell at 2 days. (B) 50* $ID_{50}$ *at 2 days. (C) 10* $ID_{50}$ *at 5 days. (D) 50* $ID_{50}$ *at 5 days. (E) through (H) Infectious C. psittaci. (E) 10* $ID_{50}$ *per L cell at 2 days. (F) 50* $ID_{50}$ *at 2 days. (G) 10* $ID_{50}$ *at 5 days. (H) 50* $ID_{50}$ *at 5 days. (I) through (L) Ultraviolet-inactivated C. psittaci. (I) 10* $ID_{50}$ *at 2 days. (J) 50* $ID_{50}$ *at 2 days. (K) 10* $ID_{50}$ *at 5 days. (L) 50* $ID_{50}$ *at 5 days. Photomicrographs were taken with phase contrast at a magnification of* $\times260$ *on Polaroid film no. 107. Bar, 100* $\mu m$.

positive L cells were determined 24 and 48 h after infection with infectious and ultraviolet-inactivated chlamydiae (Table 4). Synthesis of DNA was severely inhibited by 24 h, synthesis of protein was strongly inhibited at 48 h but not at 24 h, and synthesis of RNA was hardly inhibited at all. Inhibition of macromolecule synthesis and the percentage of trypan blue-positive cells gave discordant estimates of host-cell damage in at least three ways. First, ultraviolet-inactivated chlamydiae were just as effective in inhibiting protein and DNA synthesis as infectious ones, although they produced substantially fewer L cells that stained with trypan blue. Second, DNA synthesis was greatly depressed by 24 h after infection, yet trypan blue-positive cells had just begun to appear in the infected

L-cell populations. Third, RNA synthesis continued unabated in L-cell populations that were almost all trypan blue positive. Under these conditions, there appears to be little relationship between ability to synthesize macromolecules and ability to exclude trypan blue.

When *C. psittaci* multiplies inside of L cells, it causes them to consume more glucose and accumulate more lactate, probably because the parasites in some unknown way impair the operation of oxidative pathways in their hosts (10, 18). The ability of chlamydiae to stimulate host-cell glycolysis is almost exclusively a multiplication-dependent property of *C. psittaci* (Table 5). Chloramphenicol-inhibited chlamydiae only slightly increased lactate accumulation, and ultraviolet-inactivated organisms did not increase

TABLE 4. *Synthesis of macromolecules in L cells infected with infectious and ultraviolet-inactivated C. psittaci[a]*

| Labeled intermediate added | Time after infection (h) | C. psittaci added | | Trypan blue-positive L cells (%) | Label incorporated into acid-insoluble form in 15 min (cpm/$10^5$ L cells) |
| | | $ID_{50}$/L cell | Inactivated with ultraviolet light | | |
|---|---|---|---|---|---|
| [$^{14}$C]isoleucine[b] | 24 | 0 | | 2 | 2,540 |
| | | 10 | No | 4 | 2,420 |
| | | 10 | Yes | 3 | 2,600 |
| | | 50 | Yes | 15 | 1,630 |
| | 48 | 0 | | 3 | 2,840 |
| | | 10 | No | 86 | 142 |
| | | 10 | Yes | 32 | 100 |
| | | 50 | Yes | 91 | 49 |
| [$^3$H]uridine[c] | 24 | 0 | | 3 | 1,900 |
| | | 10 | No | 1 | 1,550 |
| | | 10 | Yes | 2 | 1,820 |
| | | 50 | Yes | 10 | 1,610 |
| | 48 | 0 | | 1 | 2,770 |
| | | 10 | No | 88 | 2,420 |
| | | 10 | Yes | 31 | 2,750 |
| | | 50 | Yes | 60 | 2,380 |
| [$^3$H]thymidine[d] | 24 | 0 | | 2 | 10,420 |
| | | 10 | No | 3 | 4,420 |
| | | 10 | Yes | 5 | 2,080 |
| | | 50 | Yes | 16 | 1,560 |
| | 48 | 0 | | 5 | 496[e] |
| | | 10 | No | 95 | 86 |
| | | 10 | Yes | 24 | 310 |
| | | 50 | Yes | 51 | 759 |

[a] The experiment was conducted as described in the text. The experiments with the different labeled intermediates were done with different populations of L cells and *C. psittaci*.

[b] Protein synthesis was estimated in terms of isoleucine incorporation.

[c] RNA synthesis was estimated from incorporation of uridine. DNA synthesis from uridine was negligible during the short labeling period.

[d] DNA synthesis was estimated from the thymidine incorporation.

[e] L cells transferred to fresh growth medium went through only a single period of DNA synthesis, which had been completed well before the addition of [$^3$H]thymidine at 48 h.

TABLE 5. *Glucose utilization, lactate production, and extracellular release of lactic dehydrogenase in L cells after exposure to C. psittaci under a variety of conditions[a]*

| Interval of measurement (h after infection) | Measurement | No *C. psittaci* | Infectious *C. psittaci* | | | | Ultraviolet-inactivated *C. psittaci* (50 ID₅₀/L cell) |
|---|---|---|---|---|---|---|---|
| | | | 10 ID₅₀/L cell | | 50 ID₅₀/L cell | | |
| | | | No further additions | Cyclohexi-mide[b] | No further additions | Chloram-phenicol[c] | |
| 0–24 | Trypan blue-positive L cells (%) | 4 | 17 | 11 | 60 | 45 | 54 |
| | Glucose used (nmol/10⁵ L cells) | 386 | 468 | 236 | 495 | 318 | 215 |
| | Lactate formed (nmol/10⁵ L cells) | 336 | 800 | 276 | 945 | 435 | 349 |
| | Lactic dehydrogenase released (IU/10⁵ L cells)[d] | 4 | 5 | 4 | 39 | 15 | 18 |
| 0–48 | Trypan blue-positive L cells (%) | 7 | <95 | <95 | <95 | 79 | <95 |
| | Glucose used (nmol/10⁵ L cells) | 506 | 770 | 374 | 561 | 592 | 374 |
| | Lactate formed (nmol/10⁵ L cells) | 348 | 1,160 | 432 | 1,060 | 751 | 536 |
| | Lactic dehydrogenase released (IU/10⁵ L cells) | 10 | 23 | 19 | 39 | 27 | 17 |

[a] See text for description of experiment.

[b] Cycloheximide was added at zero hour after infection to a final concentration of 2 µg/ml.

[c] Chloramphenicol succinate was added 2 h after infection to a final concentration of 100 µg/ml.

[d] Lactic dehydrogenase was assayed in supernatant fluids obtained by centrifuging L-cell suspensions for 10 min at 500 × *g*.

it at all. Cycloheximide almost completely suppressed the glycolysis-stimulating action of infectious *C. psittaci*. This is further evidence that the L cell synthesizes proteins that contribute to the injuries it suffers during infections with chlamydiae. The alterations in glucose metabolism after infection with *C. psittaci* appear to have had no direct relation to the appearance of trypan blue-staining L cells.

The possibility that chlamydiae damage the plasma membranes of the cells they infect was tested by looking for leakage of intracellular constituents from infected L cells. Lactate dehydrogenase, a cytosol enzyme, was assayed in the extracellular phase of suspensions of L cells, uninfected and infected with multiplying and nonmultiplying *C. psittaci* (Table 5). At 24 h after infection, the amount of enzyme that had been released into the extracellular milieu roughly paralleled the percentage of trypan blue-positive host cells present at that time, but at 48 h, the correlation was not as good. Release of intracellular inorganic cations and anions also correlated well with trypan blue staining (G. T. Chang and J. W. Moulder, manuscript in preparation).

## DISCUSSION

Injury to host cells is a frequent consequence of the ingestion of both *C. psittaci* and *C. trachomatis* (1, 2, 5, 9, 12, 13, 20, 24, 25, 26, 27, 30). Understanding of the damaging effect of the ingestion of chlamydial cells and of the factors controlling the nature and extent of the damage is a prerequisite to elucidation of the pathogenesis of chlamydial diseases. The demonstration here and in a previous paper (19) that nonmultiplying chlamydiae may injure the cells that have ingested them allowed a distinction to be made between chlamydial toxicity that is dependent on or independent of intracellular multiplication of the parasite. The relative contribution of the multiplication-dependent and -independent components of a given multiplicity of infection was estimated by comparing the toxicity of the same multiplicity of multiplying and nonmultiplying *C. psittaci* for different portions of the same L-cell population. The contribution of each type of toxicity to the total damage produced in L cells by fully infectious *C. psittaci* depended mainly on the multiplicity of infection. With inocula of less than 10 ID₅₀ per host cell, damage was almost entirely multiplication dependent; at 10 to 100 ID₅₀ per L cell, it was both multiplication dependent and independent; and at more than 200 ID₅₀ per host cell, it was almost entirely multiplication independent.

Low and moderate multiplicities of *C. psittaci*, like the high multiplicities studied earlier (19), were toxic for L cells only when they were ingested. The multiplication-independent toxicity of high chlamydial multiplicities was ascribed to injury of the plasma membrane of the host cell when it ingested large numbers of chlamydial cells and was manifested in the absence of protein synthesis by either *C. psittaci* or L cells (19). However, the genesis of the multiplication-independent toxicity of moderate chlamydial multiplicities must be more complex. Evidence presented in this paper strongly suggests that at least some protein synthesis by both host and parasite is essential for full expression of multiplication-independent toxicity by inocula of from 10 to 100 $ID_{50}$ of *C. psittaci* per host cell. In addition, at least one multiplication-dependent consequence of chlamydial infection, stimulation of glycolysis in the host cell, also appears to require protein synthesis by the host. How these hypothetical proteins might participate in producing host-cell injury remains to be determined.

Although the damage to host cells that did not depend on chlamydial reproduction became less and less important as the multiplicity of infection was reduced, as few as 10 $ID_{50}$ of *C. psittaci* per host cell, either ultraviolet inactivated or chloramphenicol inhibited, severely injured L cells as judged by several criteria. Ultraviolet-killed chlamydiae were especially effective in inhibiting synthesis of protein and DNA. It may be that a major portion of the inhibition of host protein and DNA synthesis observed after exposure of host cells to infectious *C. psittaci* (2, 5, 13, 23, 30) was produced by multiplication-independent mechanisms.

Cells probably lose their ability to remain unstained in the presence of trypan blue when the integrity of their plasma membranes is compromised in any one of several ways. Therefore, injuries closely related to loss of membrane function should correlate well with the results of the trypan blue exclusion test, and injuries more distantly related should correlate poorly. Recent reports (22, 31) have emphasized that cells judged to be "alive" by their exclusion of trypan blue may be "dead" when judged by other criteria of cell performance. The severe inhibition of DNA synthesis in trypan blue-negative L-cell populations 24 h after infection with *C. psittaci* is another example of such a lack of correlation. The reverse situation may also occur, as illustrated by the undiminished RNA synthesis in L cells that were almost all stained with trypan blue 48 h after infection. However, despite these occasional failures of the trypan blue exclusion test to give accurate reflections of host-cell dam-

age, it is a useful and convenient way to estimate the "viability" of host cells after infection with chlamydiae, provided that its limitations are always kept in mind.

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