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Separation of Protein Synthesis in Meningopneumonitis Agent from That in L Cells by Differential Susceptibility to Cycloheximide

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Cycloheximide had no effect on multiplication of the meningopneumonitis agent in L cells in concentrations which eliminated over 90% of the protein synthesis in the host cells. Infected L cells treated with cycloheximide, however, incorporated labeled amino acids into the trichloroacetic acid-insoluble fraction. This incorporation was attributed to the biosynthetic activity of the meningopneumonitis agent. Synthesis of meningopneumonitis protein was abolished by chloramphenicol and chlortetracycline, inhibitors of bacterial protein synthesis, at concentrations which did not inhibit protein synthesis in L cells. Protein synthesis in the meningopneumonitis agent was sustained at a high rate when the host cells remained viable and declined as the L cells died. Overall host protein synthesis was not inhibited by multiplication of the meningopneumonitis agent.

The obligate intracellular parasites of the psittacosis group (*Chlamydia* sp.) exist in two forms (1, 10). The mature extracellular chlamydial cells are small, rugged, and highly infectious, whereas the intracellular reproductive forms are large, fragile, and noninfectious. The most rapid synthesis of protein may be expected to occur in the large, multiplying chlamydial cells. Schechter (14) confirmed this expectation by following the course of protein synthesis in L cells infected with the meningopneumonitis agent by labeling with radioactive lysine at different times after infection and then separating the infected L cells into host and parasite fractions. The rate of protein synthesis in the meningopneumonitis agent reached a peak when the parasite population consisted chiefly of the large, multiplying cell type. However, this method of measuring protein synthesis in the meningopneumonitis agent growing in its L-cell host was not entirely satisfactory, because many of the fragile intracellular meningopneumonitis cells were destroyed during fractionation of the infected L cells. The experiments described in this paper show that cycloheximide, a specific inhibitor of protein synthesis in eucaryotic cells (3), has no effect on multiplication of the meningopneumonitis agent in concentrations which inhibit protein synthesis in L cells. Infected L cells treated with cycloheximide were used to follow meningopneumonitis-specific protein synthesis throughout the course of infection without separating the parasite from its L-cell host.

MATERIALS AND METHODS

Growth of L cells. The 5b subline of strain L cells used in previous investigations in this laboratory (7, 14, 21) was employed in all experiments. L cells were propagated in suspension culture in medium 199 (8; Grand Island Biological Co., Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum (Grand Island Biological Co.) and 100 μg of streptomycin per ml of medium. Counts of viable cells in suspension cultures were made by the trypan blue exclusion test (6). Control cultures of uninfected cells were taken from the same stock culture as cells that were infected, treated identically as the infected cells, and suspended to the same final volume and cell density with the same batch of growth medium. Cell densities ranged from 8×10^5 to 1×10^6 cells/ml.

Growth of meningopneumonitis agent in L cells. The Cal 10 strain of the meningopneumonitis agent, adapted to growth in L cells by more than 100 serial passages, was grown as previously described (14). The percentage of infected cells was determined by microscopic examination of L cells stained 20 hr after infection (14). Multiplicities of infection used in these experiments were approximately 20 chick embryo yolk sac LD_{50} per L cell, and the fraction of infected cells ranged from 90 to 100%. Most infected cells contained more than one meningopneumonitis inclusion.

Measurement of incorporation of labeled amino acids into protein. Radioactively labeled amino acids were added to uninfected and infected L-cell cultures under the conditions described in the legends to Fig. 2 to 6.

At appropriate intervals, 0.2-ml samples were added to tubes containing 2 ml of 5% trichloroacetic acid, and the tubes were placed in a boiling water bath for 10 min. An additional 2 ml of 5% trichloroacetic acid was then added to each sample, and the precipitates were collected by suction on 0.45-mμ filters, type HA (Millipore Corp., Bedford, Mass.). The tubes were washed five times with 2-ml portions of 5% trichloroacetic acid, and the filters were further washed with two 5-ml amounts of 5% trichloroacetic acid and 5 ml of 80% ethyl alcohol. The dried filters were placed in 15 ml of a scintillation fluid consisting of 0.4% of 2,5-diphenyloxazole (Packard Instrument Co., Inc., Downers Grove, Ill.) and 0.005% of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (Packard Instrument Co., Inc.) in toluene, and were counted in a Packard model 3324 Tricarb liquid scintillation spectrometer at a gain setting of 7% and with an efficiency of 78% for the ^{14}C standard. The counting errors were less than 1%, except in samples of minimal isotopic content.

Antibiotics. Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio); chlortetracycline from Lederle Laboratory Division, American Cyanamid Co. (Pearl River, N.Y.); chloramphenicol succinate from Parke, Davis & Co. (Detroit, Mich.). Cycloheximide was the gift of A. J. Lemin, The Upjohn Co. (Kalamazoo, Mich.).

RESULTS

Effect of inhibitors of protein metabolism on multiplication of the meningopneumonitis agent. Three kinds of inhibitors were tested: (i) puromycin (12), that interferes with protein synthesis in all kinds of cells; (ii) chlortetracylcine (12) and chloramphenicol (12), that inhibit synthesis of protein in procaryotic cells; and (iii) cycloheximide (3), that inhibits protein synthesis in eucaryotic cells. The effects of these inhibitors on multiplication of the meningopneumonitis agent in L cells are given in Fig. 1 in terms of percentage of the uninhibited titer. No infectious meningopneumonitis agent was produced when puromycin was added earlier than 10 hr after infection. There was a progressive release from inhibition when it was added later than 10 hr after infection; complete release occurred earlier with lower concentrations of puromycin. No infectious meningopneumonitis agent was formed in L cells exposed to chloramphenicol or to chlortetracycline during the first 20 hr after infection. Cycloheximide, at a concentration of 2 μg/ml, had no effect on meningopneumonitis reproduction when added during the first 20 hr after infection. Other experiments showed that 2 μg of cycloheximide per ml was also without effect when added to L cells 2 hr before infection. There was a slight inhibition of multiplication when 5 μg of cycloheximide per ml was added early in the infection.

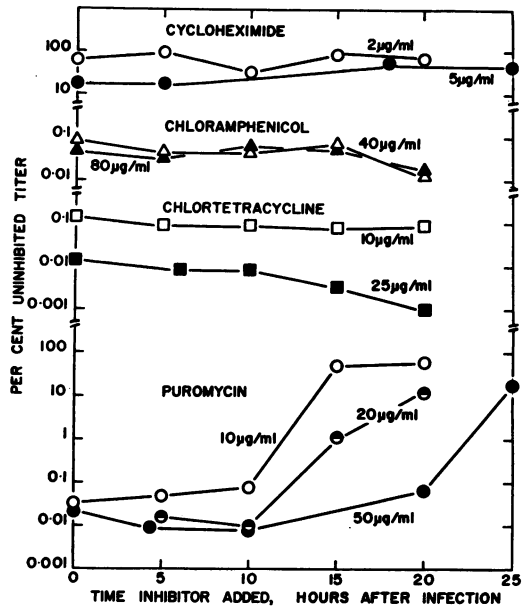

FIG. 1. *Effect of inhibitors of protein synthesis on multiplication of the meningopneumonitis agent in L cells. L cells were infected in suspension and incubated at 37 C. At the times indicated, duplicate 0.8-ml samples of infected cells were removed to Leighton tubes, 0.2 ml of a 5× concentration of the inhibitor in growth medium or 0.2 ml of growth medium alone was added, and incubation at 37 C was continued until 40 hr after infection. The supernatant fluid was then titrated for infectivity in chick embryo yolk sacs by the single-dilution method of Golub (4). Groups of 10 to 15 embryos, 6 to 7 days of age, were used for each titration. Each LD_{50} plotted is the mean of two independent observations. The uninhibited meningopneumonitis titer was 10^8 to 10^9 LD_{50} of supernatant fluid per ml.*

Cycloheximide inhibition of protein synthesis in L cells. Figure 2 shows that as little as 2 μg of cycloheximide per ml immediately abolished more than 90% of the protein synthesis in L-cell suspensions. A similar result has been reported by Ennis (2).

Synthesis of protein in meningopneumonitis-infected, cycloheximide-treated L cells. Two μg of cycloheximide per ml inhibited over 90% of the protein synthesis in L cells and yet had no effect on meningopneumonitis multiplication in these cells. Therefore, it might be predicted that, if, in the presence of cycloheximide, infected L cells would exhibit amino acid incorporation into protein, this incorporation could be ascribed solely to the biosynthetic activities of the meningopneumonitis agent.

One of the first experiments designed to test this prediction is described in the legend to Fig. 3. Cycloheximide was added to L cells 2 hr before

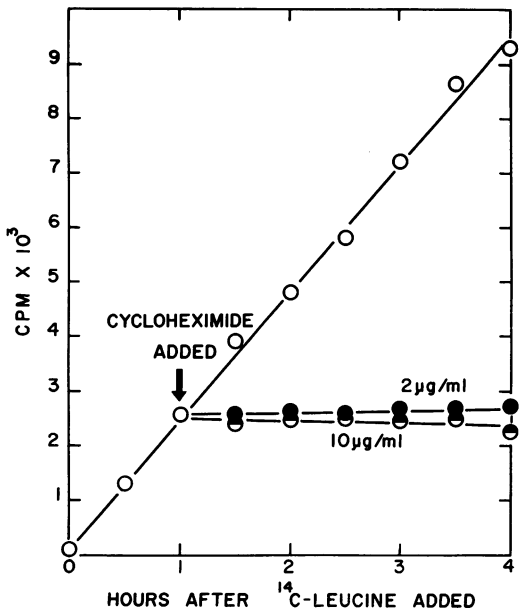

FIG. 2. *Cycloheximide inhibition of ^{14}C-leucine incorporation in L-cell protein. A 50-ml Erlenmeyer flask containing 17 ml of a logarithmically multiplying L-cell suspension was sealed with a silicone stopper and shaken in a water bath at 37 C. At zero time, prewarmed growth medium containing randomly labeled ^{14}C-L-leucine (Nuclear Chicago, Des Plaines, Ill.; specific activity, 7.9 µc/mmole) was added to a final concentration of 1 µc/ml. At 30-min intervals, three 0.2-ml samples were withdrawn for measurement of ^{14}C-leucine incorporation. At 60 min, three 4.9-ml samples were removed to 25-ml flasks. To one control flask 0.1 ml of growth medium was added; to each of the other two flasks, 0.1 ml of growth medium containing the appropriate concentration of cycloheximide was added. Incubation was continued at 37 C and samples were withdrawn for analysis at 30-min intervals for an additional 3 hr.*

infection and was present throughout the experiment. Radioactive amino acid incorporation into the hot trichloroacetic acid-insoluble fraction of meningopneumonitis-infected L cells was clearly evident in the labeling period 14 to 17 hr after infection, only a few hours after multiplication of the large intracellular forms began (5). Uptake reached a maximum at 24 to 27 hr and declined thereafter. Figure 3 also shows that 2 µg of cycloheximide per ml rapidly killed uninfected L cells after 20-hr exposure, and that infected L cells died more rapidly in the continuous presence of the antibiotic than in its absence.

In later experiments, the lethal effect of cycloheximide was avoided by adding the drug to infected cells only 1 hr before the labeling period. A single labeled amino acid, L-leucine, which is present in high concentrations in meningopneumonitis protein (11, 16), was substituted for the reconstituted protein hydrolysate used earlier. In the experiment shown in Fig. 4, cycloheximide-resistant protein synthesis in L cells infected with the meningopneumonitis agent was at its maximum 22 to 24 hr after infection. Thereafter, there was a rapid decrease in the rate of ^{14}C-leucine incorporation, paralleled by an equally rapid decrease in host-cell viability. In contrast, in the experiments summarized in Fig. 5 and 6, the infected L cells did not die, and cycloheximide-resistant ^{14}C-leucine incorporation continued at a high rate for a long time. Thus, there is a relation between the viability of the infected L cell

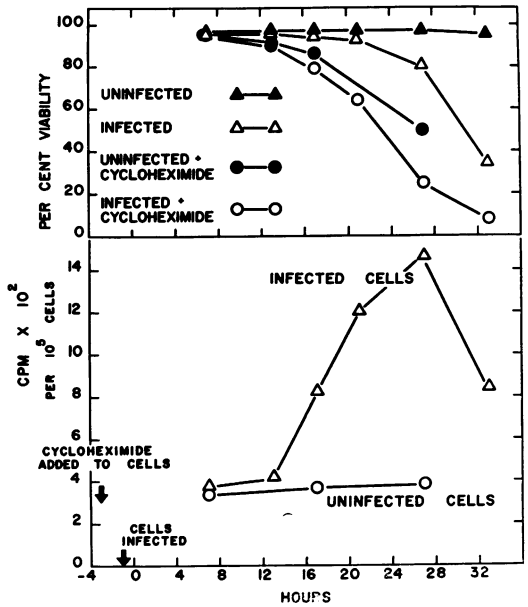

FIG. 3. *Incorporation of ^{14}C-labeled reconstituted protein hydrolysate into meningopneumonitis-infected L cells in the presence of cycloheximide. Cycloheximide (2 µg/ml, 7 × 10^{-6} M) was added to the L-cell suspension 2 hr before infection and was present throughout the experiment. At various times after infection, 4.5-ml portions of the infected cell suspension were placed in 25-ml flasks containing 5 µc of ^{14}C-labeled reconstituted protein hydrolysate (the gift of Schwarz Bio-Research, Inc., Orangeburg, N.Y.) in 0.5 ml of growth medium. After 3 hr at 37 C in a water-bath shaker, 0.2-ml samples were withdrawn in triplicate for measurement of amino acid incorporation into protein. Therefore, each point on the curves in the lower half of Fig. 3 represents the incorporation that occurred in the preceding 3-hr interval. The curves in the upper half of the figure show the loss of cell viability in the different L-cell suspensions. Viable cells were defined as those that excluded trypan blue (6), dead cells as those that did not.*

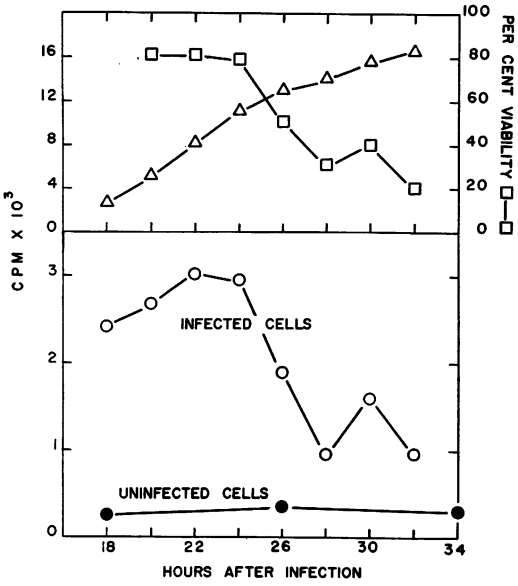

FIG. 4. *Incorporation of ^{14}C-leucine into infected L cells. At various times after infection with the meningopneumonitis agent, 4.4-ml samples of infected cells were added to 25-ml flasks containing 0.1 ml of a 50× concentration of cycloheximide (final concentration, 3 μg/ml or 1.05 × 10^{-5} M) or 0.1 ml of medium alone and were incubated at 37 C in a water-bath shaker. After 1 hr, 0.5 ml of prewarmed medium containing ^{14}C-leucine was added to give a final concentration of 1 μc/ml. Two hr later, triplicate 0.2-ml samples were removed for determination of ^{14}C-leucine incorporation. The lower half of Fig. 4 plots the ^{14}C-leucine incorporation observed in each 2-hr labeling period; the upper half shows cumulative ^{14}C-leucine uptake and L-cell viability. In plotting the data of this and succeeding figures, no attempt was made to equate counts per minute (cpm) with L-cell numbers, because the cell numbers remained constant throughout the experiments and were the same in comparable infected and uninfected samples.*

and its ability to sustain the cycloheximide-insensitive synthesis of protein. The cause of this variation in the rate of host-cell death in response to infection is unknown, but it may result from long-term, periodic changes within the L-cell population.

The data summarized in Fig. 5 may be used to estimate the effect of infection with the meningopneumonitis agent on the rate of host protein synthesis. At all labeling periods, the sum of the incorporation of ^{14}C-leucine into the protein of uninfected L cells and the incorporation into cycloheximide-treated, infected L cells equaled the incorporation into infected but untreated L cells. That is, infection with the meningopneumonitis agent did not inhibit host protein synthesis, and, provided the infected cells remained viable, the cycloheximide-resistant protein synthesis in infected cells took place in addition to the cycloheximide-sensitive protein synthesis normally occurring in the uninfected cells. This additive relationship may be seen most readily by examining the curves for cumulative incorporation into protein.

Inhibition of the cycloheximide-resistant protein synthesis of meningopneumonitis-infected L cells by chloramphenicol and chlortetracycline. The cycloheximide-resistant incorporation of ^{14}C-leucine was almost completely inhibited at all labeling periods when 80 μg of chloramphenicol per ml was added to infected L cells 1 hr before the addition of the label (Fig. 6). In other experiments, 25 μg of chlortetracylcine per ml produced a similar inhibition. At these concentrations, neither chloramphenicol nor chlortetracycline inhibited protein synthesis in uninfected, untreated L cells, as measured by incorporation of ^{14}C-leucine into hot trichloroacetic acid-insoluble fractions.

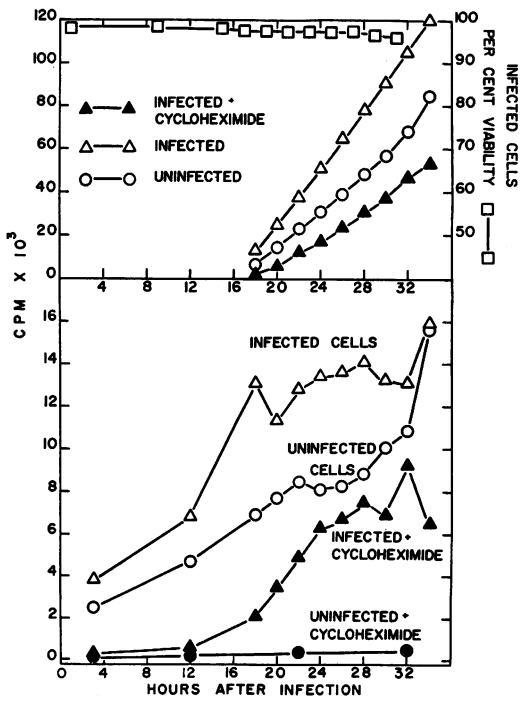

FIG. 5. *Incorporation of ^{14}C-leucine into infected and uninfected, cycloheximide-treated and untreated L cells. Cycloheximide was added 1 hr before ^{14}C-leucine and 2-hr labeling periods were used. The experiment was performed and the data were plotted as described in Fig. 4, except that the final concentration of cycloheximide was 5 μg/ml (1.75 × 10^{-5} M).*

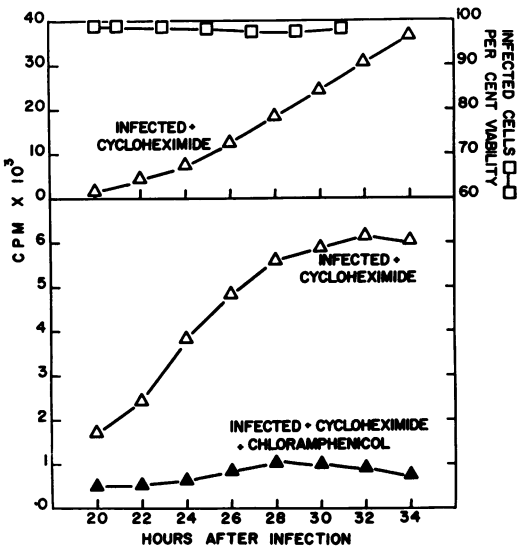

FIG. 6. *Incorporation of ^{14}C-leucine into infected cells treated with cycloheximide (5 μg/ml) or with cycloheximide plus chloramphenicol (80 μg/ml or 2.4 × 10^{-4} M). Both inhibitors were added 1 hr before ^{14}C-leucine. Consecutive 2-hr labeling periods were used. The experiment was carried out and the data were plotted as described in Fig. 4.*

DISCUSSION

The demonstration of a cycloheximide-resistant, chloramphenicol- and chlortetracycline-sensitive synthesis of protein in L cells infected with meningopneumonitis agent, together with Tamura's (17) finding that meningopneumonitis cells contain particles with the chemical and physical characteristics of procaryotic ribosomes, provides compelling evidence that this microorganism synthesizes its protein on ribosomes of the procaryotic type. It may therefore be assumed with reasonable certainty that the cycloheximide-insentitive incorporation of amino acids observed in infected L cells is a valid measure of the biosynthesis of protein by the meningopneumonitis agent.

Experiments of the type summarized in Fig. 5 show that host protein synthesis is not inhibited by infection with the meningopneumonitis agent. Schechter (14) tentatively reached a similar conclusion on the basis of techniques then available. However, the results of the experiments described here demonstrate that this organism can synthesize its protein and multiply normally in host cells synthesizing protein at less than 10% of the uninfected rate.

Synthesis of meningopneumonitis protein was maintained at a high rate when the L-cell host remained viable and declined when the L cell died, suggesting that a factor provided by the living host cell is required for synthesis of parasite protein. It has already been demonstrated that host synthesis of neither deoxyribonucleic acid (7) nor ribonucleic acid (18) is essential for multiplication of chlamydiae, and these experiments show that synthesis of host protein is also not required. Therefore, the factor provided by the living host cell must be something else. Moulder (9) has suggested that the chlamydiae are energy parasites which pirate the high-energy compounds of their host for their own biosynthetic needs, and there is evidence that adenosine triphosphate is one of these compounds (22). Since adenosine triphosphate is required for protein synthesis, it is possible that its concentration may become rate-limiting in dead or dying host cells.

The change in the susceptibility of meningopneumonitis multiplication to puromycin during the course of the developmental cycle—early susceptibility followed by late resistance—is a phenomenon observed with a number of drugs of widely different chemical structures and modes of action: *p*-fluorophenylalanine (19), 5'-fluorodeoxyuridine (20), 5'-fluorodeoxycytidine (15), aminopterin (13), actinomycin D (18), cytosine arabinoside (*unpublished results*), and puromycin aminonucleoside (*unpublished results*). The causes of the shift in sensitivity to these drugs remains unknown and may be different for each drug, although a single general explanation seems more logical.

Ennis (2) has reported that cycloheximide eventually causes an inhibition of deoxyribonucleic and ribonucleic acid synthesis in L cells. This suggests that the cycloheximide method for following the synthesis of meningopneumonitis protein can be extended to studying the synthesis of other macromolecules in chlamydiae without resort to cell fractionation. It is also possible that cycloheximide-treated host cells may be of value in studying macromolecular syntheses in other procaryotic intracellular parasites.

ACKNOWLEDGMENTS

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