JOURNAL OF BACTERIOLOGY, Oct., 1966
Vol. 92, No. 4

Printed in U.S.A.

Autoradiographic Study of Deoxyribonucleic Acid Synthesis in L Cells Infected with the Agent of Meningopneumonitis

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Received for publication 29 April 1966

ABSTRACT

MOORE, DOROTHY E. (University of Chicago, Chicago, Ill.), AND JAMES W. MOULDER. Autoradiographic study of deoxyribonucleic acid synthesis in L cells infected with the agent of meningopneumonitis. J. Bacteriol. 92:1128–1132. 1966.—L cells infected with the agent of meningopneumonitis were labeled with H^3-cytidine at 5-hr intervals after infection, and cell samples were fixed every 5 hr after labeling. These preparations were then digested with ribonuclease, stained by the Feulgen procedure, and examined by autoradiography. Labeled meningopneumonitis inclusions were first seen 15 hr after infection. Deoxyribonucleic acid (DNA) was synthesized in both L-cell nuclei and meningopneumonitis agent for as long as 40 hr after infection. Nuclear DNA synthesis was unaffected until 25 hr after infection, at which time synthesis of agent DNA reached its peak. After 25 hr, both meningopneumonitis and L cell DNA synthesis declined.

Members of the psittacosis group of microorganisms contain both DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) (13, 18, 19, 20) and multiply by binary fission in the cytoplasm of their host cells (1, 2, 6). Synthesis of parasite DNA in the cytoplasm has been distinguished from synthesis of host DNA in the nucleus by labeling infected cells with P^{32}-orthophosphate or H^3-cytidine and preparing autoradiographs (4, 17) or separating the infected cells into nuclear and cytoplasmic portions (13, 14). H^3-cytidine was used instead of the more conventional H^3-thymidine label, because thymidine is not incorporated into the DNA of psittacosis agents (4, 13, 16, 20).

Nucleic acid metabolism in L cells infected with the agent of meningopneumonitis, a typical member of the psittacosis group, has been studied in this laboratory by separating isotopically labeled infected cells into nuclear, cytoplasmic, and meningopneumonitis fractions (13, 14). The purpose of this investigation was to see if the picture of DNA metabolism in meningopneumonitis-infected L cells revealed by autoradiographic examination of intact cells was the same as that obtained by cell fractionation.

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MATERIALS AND METHODS

Growth of the meningopneumonitis agent in L cells. The 5b clone of L cells (5, 12) used in other investigations in this laboratory (13, 20) was grown in monolayer and suspension cultures as previously described (13). Suspensions of L cells were infected (13) with L cell-adapted meningopneumonitis agent (I. I. E. Tribby, M.S. Thesis, Univ. Chicago, Chicago, Ill.) at a multiplicity of 20 yolk sac LD_{50} per L cell and diluted to a concentration of 4×10^5 L cells per milliliter in medium 199 (10) containing 10% newborn calf serum (Grand Island Biological Co., Grand Island, N.Y.). Examination of Giemsa-stained preparations made 24 hr after infection showed that 83 to 99% of the L cells contained one or more meningopneumonitis inclusions.

Labeling with H^3-cytidine. For studying DNA metabolism in monolayer cultures, 1-ml volumes of the infected L cell suspension were added to Leighton tubes containing flying cover slips and were incubated at 37 C in an atmosphere of 5% CO_2 and 95% air. Uninfected monolayers were prepared in the same way from uninfected L cell suspensions. H^3-cytidine (Schwarz Bio Research Inc., Orangeburg, N.Y.) with a specific activity of 1 c/mmole was added to the monolayer cultures immediately after infection or at 5-hr intervals to a final concentration of 1 μc/ml. Samples were fixed at 5-hr intervals after labeling by washing the cover slips twice in 0.85% NaCl, once in cold absolute methyl alcohol, holding for 1 min in cold absolute methyl alcohol, and drying in air.

Suspension cultures were labeled with H^3-cytidine

in a similar fashion. At appropriate intervals, 1-ml samples were removed to Leighton tubes and, after allowing 90 min for the L cells to attach to the cover slips, were treated as described above.

Preparation of cells for autoradiography. The fixed cover slips were extracted in cold 5% trichloracetic acid for 1 hr, rinsed well in tap water, and cut in two. One half was incubated in 0.4 mg/ml of pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) dissolved in glass-distilled water and adjusted to *p*H 6.5 with 0.07 M Na_2HPO_4. The other half was incubated in 0.05 M tris(hydroxymethyl)-aminomethane buffer (*p*H 7.05). Both were incubated for 3 hr at 37 C. The cover-slip halves were then washed well in running water, dried in air, stained by the Feulgen method, mounted with Euparal (Flatters and Garnett, Ltd., Manchester, England) cell side up on glass slides, and dried overnight at 45 C.

When normal L cells were labeled with H^3-cytidine, treated with ribonuclease, and examined by autoradiography, cytoplasmic and nucleolar labeling was not observed. When HeLa cells were labeled with H^3-cytidine and treated with ribonuclease under the precise conditions employed here, further digestion with deoxyribonuclease removed all residual label (T. T. Crocker, *personal communication*). These observations indicate that the nucleic acid remaining after ribonuclease digestion was largely DNA.

Autoradiography. The general procedure described by Prescott (11) was followed. Mounted slides were placed back to back in staining jars immersed in a 45 C water bath in the darkroom. Melted photographic emulsion (Kodak Nuclear Track Beta-3, Eastman Kodak Co., Rochester, N.Y.) was poured into a Coplin jar in the water bath, and two slides were dipped simultaneously, separated, and allowed to dry. The coated slides were stored for 48 hr at room temperature in a light-tight box containing Drierite, and were then developed for 2 min with Dektol (Kodak), rinsed in distilled water, fixed with Kodak acid fixer for 2 min, and washed in running tap water for 20 min. After final mounting with Euparal and drying at 45 C, the slides were examined under oil immersion.

Grain counting. In each slide examined, the silver grains on each of 100 L cells were counted and categorized as being deposited on either the host cell nucleus or the meningopneumonitis inclusion. To test the reproducibility of grain counting, five different groups of 100 cells on a single slide were counted by the usual procedure. The percentage of labeled nuclei was counted to a standard error of less than 5%, whereas the percentage of nuclei more heavily labeled than their inclusions (and vice versa) was estimated to a standard error of less than 10%.

RESULTS

Because previous studies (13, 14) in this laboratory on the nucleic acid metabolism of meningopneumonitis-infected L cells had been done with suspension cultures, autoradiographs were made of cells labeled in suspension as well as in monolayer, the type of cell preparation usually used in autoradiography. Both types of cultures gave essentially the same results, but the grain counts from cells labeled in monolayer were more reproducible, probably because the cells labeled in suspension did not uniformly attach to the cover slips in the short time allowed. Therefore, only the results obtained with monolayers will be presented in detail.

Feulgen-positive cytoplasmic inclusions usually appeared in L cells 15 hr after infection, and in L cells labeled at zero hour (time of infection, measured from the end of the 1-hr absorption period) labeled meningopneumonitis inclusions also appeared at 15 hr (Fig. 1). In thin sections of infected cells examined with the electron microscope, active cell division of psittacosis group agents was first seen at 7 to 12 hr (2, 6, 8, 9). It thus appears that the meningopneumonitis inclusions became visibly labeled after only a few cell divisions had occurred. By 25 hr, the cytoplasm of infected cells was distended with large inclusions, and both nuclei and inclusions were heavily labeled (Fig. 2).

Monolayers of infected and uninfected L cells labeled continuously from 0 to 30 hr showed nearly identical rates of nuclear labeling. Some nuclei became labeled as early as 5 hr after addition of H^3-cytidine, and, by 20 hr, 70 to 80% of the cells had labeled nuclei (Fig. 3).

When the infected monolayers were labeled at successive 5-hr intervals after infection, their nuclei became labeled at the same rate as uninfected cells when H^3-cytidine was added at 5, 10, 15, or 20 hr (Fig. 3). However, beginning with the addition of label at 25 hr, a much smaller

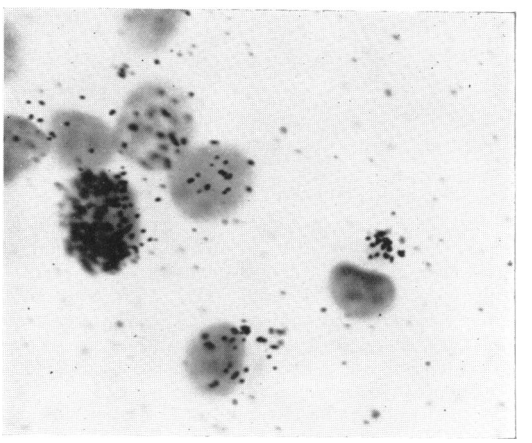

FIG. 1. *Autoradiograph of L cells infected with the meningopneumonitis agent, labeled in suspension with H^3-cytidine at zero hour, and fixed 15 hr later. One heavily labeled nucleus and a small labeled inclusion (arrow) may be seen.* × *1,500.*

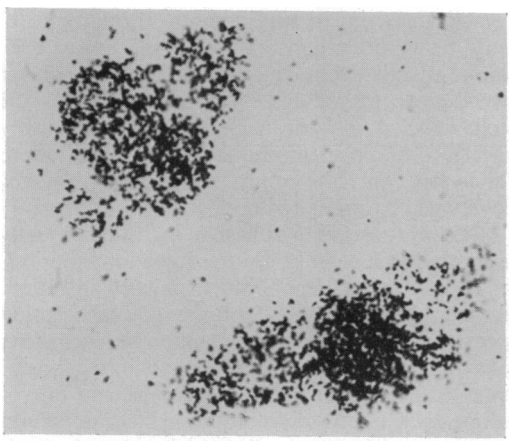

FIG. 2. *Autoradiograph of L cells infected with the meningopneumonitis agent, labeled in monolayer with H^3-cytidine at zero hour, and fixed at 25 hr. Nuclei and inclusions are heavily labeled. × 1,500.*

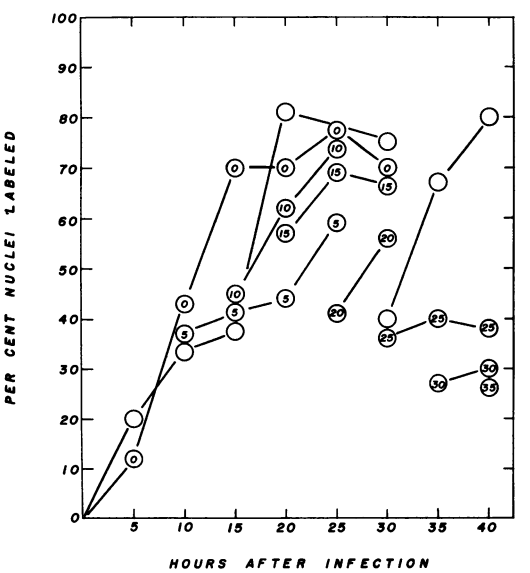

FIG. 3. *Percentage of labeled nuclei in an uninfected L cell monolayer labeled with H^3-cytidine at zero hour and in meningopneumonitis-infected monolayers labeled at 0, 5, 10, 15, 20, 25, 30, and 35 hr after infection. The number in the circle denotes the time of labeling of infected cultures. The circles corresponding to the uninfected culture are blank. The 30-hr uninfected value was abnormally low.*

proportion of infected L-cell nuclei became labeled, indicating that synthesis of DNA in host-cell nuclei was depressed in the later stages of the meningopneumonitis developmental cycle. Multiplication of the meningopneumonitis agent in L cells reaches a maximum at approximately 25 hr (7, 14).

A useful way of comparing the rate of DNA synthesis in nuclei and cytoplasmic inclusions is illustrated in Fig. 4. On the basis of quantitative grain counts, each infected cell was classified into one of three categories: (i) nucleus more heavily labeled than inclusion, (ii) nucleus and inclusion more or less equally labeled, and (iii) inclusion more heavily labeled than nucleus (predominant agent label). In monolayers labeled with H^3-cytidine at successive 5-hr intervals after infection and fixed every 5 hr after labeling, the proportion of infected L cells with predominant agent label increased linearly in the 0 to 20-hr labeling periods; that is, the longer the addition of H^3-cytidine was delayed, the greater was the rate of its incorporation into meningopneumonitis DNA as compared to L cell DNA. The meningopneumonitis agent most effectively incorporated the label added at 20 hr; 10 hr later, over 95% of infected cells had predominant agent label. When the H^3-cytidine was added at 25 hr and later, there was a sharp decline in the proportion of host cells with predominant agent label. Since this is the period in which a decline in number of labeled host-cell nuclei also occurred (Fig. 3 and 4), it appears that the rate of DNA synthesis in both host and parasite was depressed late in the meningopneumonitis developmental

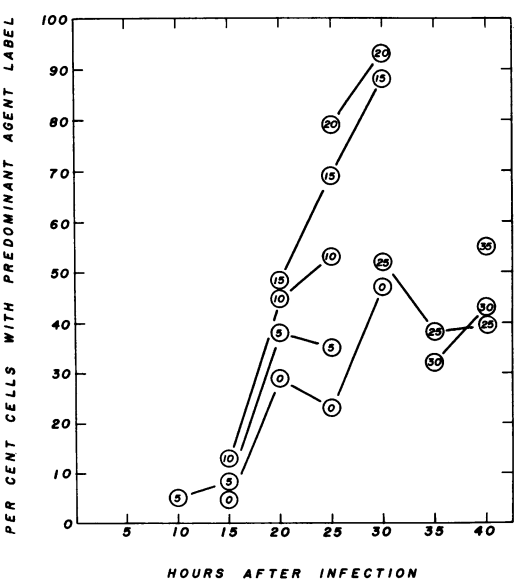

FIG. 4. *Percentage of cells with predominant agent label in meningopneumonitis-infected L cell monolayers labeled with H^3-cytidine 5 to 35 hr after infection. The number in the circle indicates the hour of labeling.*

cycle. However, both L cells and meningopneumonitis agent continued to incorporate H^3-cytidine into their DNA for as long as 40 hr after infection.

Meningopneumonitis-infected L cells from the same set of samples used to obtain the data shown in Fig. 3 and 4 were examined to see if the proportion of labeled inclusions in host cells with labeled nuclei was higher than in host cells with unlabeled nuclei. The proportion of labeled inclusions in the two groups was the same, indicating that synthesis of DNA in the host nuclei was not essential for synthesis of DNA in the meningopneumonitis inclusions. Similar conclusions have been reached in other types of experiments (3, 15). At 15 hr, only about 40% of the Feulgen-positive cytoplasmic inclusions had silver grains deposited over them; by 25 hr, more than 95% were labeled. This difference is probably at least in part related to the difficulty of detecting significant grain deposition over structures as small as the early meningopneumonitis inclusions.

DISCUSSION

After infection of L cells with the meningopneumonitis agent, there was a period of 10 to 15 hr during which the organism was not accessible to autoradiographic observation. At 15 hr, synthesis of DNA by the meningopneumonitis agent in cytoplasmic inclusions became apparent, increased in rate until about 25 hr, and continued until 40 hr after infection, but at a lessened rate. Synthesis of DNA in L-cell nuclei occurred throughout the infection, but it also decreased after 25 hr. The general picture emerging from these observations is that the L cell infected with the meningopneumonitis agent contains two independent sites of DNA synthesis, the normal nuclear site, and the abnormal cytoplasmic one. As long as the cytoplasmic center of DNA production is relatively small, nuclear synthesis is undisturbed, but when the cytoplasmic site begins to approach the nuclear site in rate of DNA synthesis, DNA production in both locations slackens off. It is likely that some intermediate common to both centers of DNA synthesis becomes limiting.

These results are in agreement with the autoradiographic studies of Crocker et al. (4) on the growth of an ornithosis agent in HeLa cells. They observed the synthesis of ornithosis DNA throughout its developmental cycle and noted no inhibition of nuclear DNA synthesis for 15 to 20 hr after infection, but found a noticeable decline in the number of labeled nuclei thereafter. They explained the decrease in terms of competition between the ornithosis agent and the HeLa cell nucleus for DNA precursors. Tamura (17) also observed a drop in labeled L-cell nuclei 25 hr after infection with the meningopneumonitis agent.

This autoradiographic investigation of DNA synthesis in L cells infected with the meningopneumonitis agent has yielded results that are in harmony with those obtained by breaking up infected cells after brief exposure to H^3-cytidine at various times in the developmental cycle and obtaining nuclear, cytoplasmic, and agent fractions. By this procedure, Schechter (13) found that DNA synthesis in the meningopneumonitis agent was first detectable at 15 hr, reached a maximum at 25 hr, and continued at a lessened rate for the remainder of the developmental cycle. Incorporation of H^3-cytidine into L-cell nuclei was unaffected until 20 hr. After this time, it proceeded at only half the uninfected rate. The general agreement between the two methods suggests that both yield reliable descriptions of the course of DNA synthesis in meningopneumonitis-infected L cells.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grant AI-01594 and graduate training grant AI-238 from the National Institute of Allergy and Infectious Diseases, and by a grant from the Abbott Laboratories, North Chicago, Ill.

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. ARMSTRONG, J. A., AND S. E. REED. 1964. Nature and origin of initial bodies in lymphogranuloma venereum. Nature **201**:371–373.
3. CROCKER, T. T., AND J. M. EASTWOOD. 1963. Subcellular cultivation of a virus: Growth of ornithosis virus in nonnucleate cytoplasm. Virology **19**:23–31.
4. CROCKER, T. T., S. R. PELC, B. I. NIELSEN, J. M. EASTWOOD, AND J. BANKS. 1965. Population dynamics and deoxyribonucleic acid synthesis in HeLa cells infected with an ornithosis agent. J. Infect. Diseases **115**:105–122.
5. EARLE, W. R. 1943. Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cell. J. Natl. Cancer Inst. **4**:165–212.
6. HIGASHI, N. 1965. Electron microscope studies on the mode of reproduction of trachoma virus and psittacosis virus in cell cultures. Exptl. Mol. Pathol. **4**:24–39.
7. HIGASHI, N., A. TAMURA, AND M. IWANAGA. 1962. Developmental cycle and reproductive

mechanism of the meningopneumonitis virus. Ann. N.Y. Acad. Sci. **98**:100–121.

8. LITWIN, J. 1959. The growth cycle of the psittacosis group of microorganisms. J. Infect. Diseases **105**:129–160.
9. LITWIN, J., J. E. OFFICER, A. BROWN, AND J. W. MOULDER. 1961. A comparative study of the growth cycles of different members of the psittacosis group in different host cells. J. Infect. Diseases **109**:251–279.
10. MORTON, J. F., H. L. MORTON, AND R. C. PARKER. 1950. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exptl. Biol. Med. **73**:1–8.
11. PRESCOTT, D. M. 1964. Autoradiography with liquid emulsion, p. 365–370. *In* D. M. Prescott [ed.], Methods in cell physiology, vol. 1. Academic Press, Inc., New York.
12. SANFORD, K. K., W. R. EARLE, AND G. D. LIKELY. 1948. The growth in vitro of single isolated cells. J. Natl. Cancer Inst. **9**:229–246.
13. SCHECHTER, E. M. 1966. Synthesis of nucleic acid and protein in L cells infected with the agent of meningopneumonitis. J. Bacteriol. **91**:2069–2080.
14. SCHECHTER, E. M., I. I. E. TRIBBY, AND J. W. MOULDER. 1964. Nucleic acid metabolism in L cells infected with a member of the psittacosis group. Science **145**:819–821.
15. STARR, T. J. 1963. Morphological and biochemical integrity of colchicine-induced, psittacosis-infected, micronucleated cells. Texas Rept. Biol. Med. **21**:412–421.
16. STARR, T. J., AND N. SHARON. 1963. Autoradiography with the agents of psittacosis and trachoma. Proc. Soc. Exptl. Biol. Med. **113**:912–914.
17. TAMURA, A. 1962. Studies on location of nucleic acid synthesis in meningo-pneumonitis-infected cells and purification of meningopneumonitis virus. Progr. Virol. (Japan), p. 227–234.
18. TAMURA, A. 1964. Biochemical studies on the mechanism of infection of meningopneumonitis agent. Ann. Rept. Inst. Virus Res. Kyoto Univ. **7**:1–13.
19. TAMURA, A., AND N. HIGASHI. 1963. Purification and chemical composition of meningopneumonitis virus. Virology **20**:596–604.
20. TRIBBY, I. I. E., AND J. W. MOULDER. 1966. Availability of bases and nucleosides as precursors of nucleic acids in L cells and in the agent of meningopneumonitis. J. Bacteriol. **91**:2362–2367.