A Histoautoradiographic Study of Deoxyribonucleic Acid Synthesis in Tissue Cultures of Chicken Embryo Liver Cells Infected with RPL-12 Lymphomatosis Virus.*

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The growth of RPL-12 lymphomatosis virus in tissue cultures of chick embryo liver (1, 2) induces the appearance of large intranuclear inclusions which exhibit a strongly positive Feulgen reaction (Fig. 1). After treatment with DNase the Feulgen reaction in the inclusion is negative. Other cytochemical characteristics of these inclusions have been reported elsewhere (2). To study the localization and synthesis of DNA in these preparations histoautoradiographic experiments with thymidine-H^3 were carried out. Thymidine was the compound of choice for these studies because of its specific incorporation into DNA (3, 4) and because of the high resolution which can be obtained (5). It is the purpose of this report to demonstrate: (*a*) that tritiated thymidine is incorporated in the intranuclear inclusions produced by the RPL-12 virus and (*b*) that the chromosoma DNA does not play any major role in the formation of the inclusions, confirming the fact that a large portion of the DNA of the inclusions is synthesized *de novo* under the influence of the virus.

Methods

Virus.—The RPL-12 virus used in these experiments was at its 19th passage through cultured liver cells of chicken embryos. (1). It had a titer ($TCID_{50}$) of $10^{7.3}$.

Tissue Culture.—A suspension of 14 to 15 day old chicken embryo liver cells was prepared by trypsinization, and 100,000 to 150,000 cells in 1 ml. were seeded in a Leighton tube containing a coverslip. The tubes were then incubated at 37°C. During the first 48 hours the growth medium was composed of 30 per cent horse serum and 70 per cent medium 199 (6) to allow formation of a continuous cellular sheet. This medium was then replaced with medium 199 and the cultures were infected with the virus preparation. 70 to 72 hours after infection the coverslips were collected, fixed in Carnoy's solution for 30 minutes, and stained by the Feulgen technique, the hydrolysis being carried out with N HCl at 60°C. for 8 minutes. The coverslips were then mounted on normal microslides, covered with AR-10 Kodak stripping film, and exposed in sealed dark boxes at 4°C., for 4 days at which time the autoradiographs were developed. Incorporation of labeled thymidine could be estimated from the amount of reduced silver grains. The background in these experiments varied between 0.5 to 4.0 grains per nucleus. Tritiated thymidine (New England Nuclear Corporation, Boston) with a specific activity of 1 mc./3.68 mg. was used. The H^3-thymidine was diluted in medium 199 and the equivalent of 1 microcurie (3.68 μg./ml.) was added to each culture at the time indicated in the various experiments.

RESULTS

Once the trypsinized cell suspension was seeded on the flat surface of the coverslip, the hepatic cells reaggregated, so that the monolayers appeared as distinct and discrete islets of hepatic cells surrounded by bands of fibroblasts. The growth of the hepatic cells was favored during the first 48 hours by medium containing 30 per cent serum, whereas growth of fibroblasts proceeded very rapidly 48 hours after preparation of the culture, as it was favored by the absence of the serum. Thus the proportion of hepatic and fibroblast cells in a culture depended essentially on the time at which the cultures were examined.

Control Cultures

(*Series 1*) *H^3-Thymidine Present in the Medium during the First 48 Hours, Absent during the Following 70 Hours.*—Discrete grains were present on the nuclei of most of the hepatic cells; the number of grains per hepatic nucleus was fairly constant. Only some of the nuclei of fibroblasts were labeled with grains; the number of grains in such nuclei was variable. There was a rather large variability from one experiment to another in the percentage of labeled fibroblasts, and in the number of grains per nucleus as compared to that of hepatic nuclei.

(*Series 2*) *H^3-Thymidine Absent in the Medium during the First 48 Hours, Added at the Time of the Change to Medium 199.*—Nuclei of hepatic cells were essentially devoid of grains; only occasionally did some cells show a number of grains significantly higher than that of the background; these represented hepatic cells that had undergone DNA synthesis after the change of medium. Fibroblasts, on the other hand, were heavily labeled throughout the culture (Fig. 2), confirming

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the fact that proliferation of this cellular type occurs mainly 48 hours after cultivation.

Infected Cultures

(*Series 3*) *H^3-Thymidine Present in the Medium during the First 48 Hours; Culture Washed and New Growth Medium Added for 24 Hours; Cultures then Infected with the RPL-12 Virus and Collected 72 Hours Later.*—Nuclei of uninfected hepatic cells showed diffuse labeling as in the control cultures. The inclusions, however, were devoid of grains in the central portion; some grains were present at the periphery of the inclusions and in their immediately surrounding areas (Fig. 4). In these areas fragments of Feulgen-positive material were frequently seen, and could be topographically identified with the grains. Nuclei of fibroblasts were irregularly labeled as in control cultures.

(*Series 4*). *H^3-Thymidine Added at the Time of Infection or at Various Times Thereafter.*—The inclusions were intensely labeled when the H^3-thymidine was added at the time of infection. Within the same slide the number of grains per inclusion was from 5 to 8 times higher than that observed in fibroblast nuclei. The grains were distributed throughout the inclusions, and within any one slide there was no much variability in the number of grains per inclusion (Figs. 3 and 5).

In order to investigate the dynamics of inclusion formation, H^3-thymidine was added to the medium at various times after virus infection. H^3-thymidine was introduced in the medium for a standard duration of 2 hours beginning at various times after infection. At the end of the exposure period, the tracer was removed by changing the cells to a medium containing non-tritiated thymidine (17.5 μg./ml.). The percentage of labeled inclusions was then calculated. The number of labeled inclusions progressively increased from the 3rd hour up to 12 to 16 hours after exposure to infection, at which time 100 per cent of the inclusions were labeled. The percentage of labeled inclusions did not decrease after this time, remaining constant up to 56 hours after infection or 16 hours before harvesting. In these experiments coverslips were collected 72 hours after infection. The number of grains per inclusion during this late stage decreased, and became more variable.

DISCUSSION AND CONCLUSIONS

The results indicate that labeled thymidine is incorporated in notable amounts into the intranuclear inclusions induced by the RPL-12 lymphomatosis virus in chicken embryo hepatic cells grown in tissue culture. They also indicate that this incorporation is independent of the cell division, as accumulation of labeled thymidine is observed in cells which apparently have ceased to undergo mitosis under the conditions of the experiment. Furthermore, it appears that incorporation of thymidine continues at least up to 56 hours after the infection. The chromosomal DNA in the nucleus prior to infection does not play any major role in the formation of the inclusions, as cells labeled prior to infection have grains only at the periphery of the inclusions. This can be interpreted to mean that the preexisting DNA is localized at the periphery of the inclusions, as one of the first morphological manifestations of virus infection is margination of the chromatin at the nuclear membrane. The actual amount of DNA per inclusion is not known, although work is in progress to investigate it by cytophotometry and chemical estimation. Davies *et al.* (7) have demonstrated that virus particles arranged in a lattice formation are found within the DNA-containing inclusions. Initial experiments (8) with 5-fluorodeoxyuridine, an inhibitor of DNA synthesis (9), indicate that the number of inclusions and the amount of virus produced in cultures treated with this compound is noticeably decreased. It is thus apparent that active DNA synthesis is an essential part of the process of viral reproduction. Studies on the actual chemical composition of the RPL-12 lymphomatosis virus are in progress.

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Explanation of Plate

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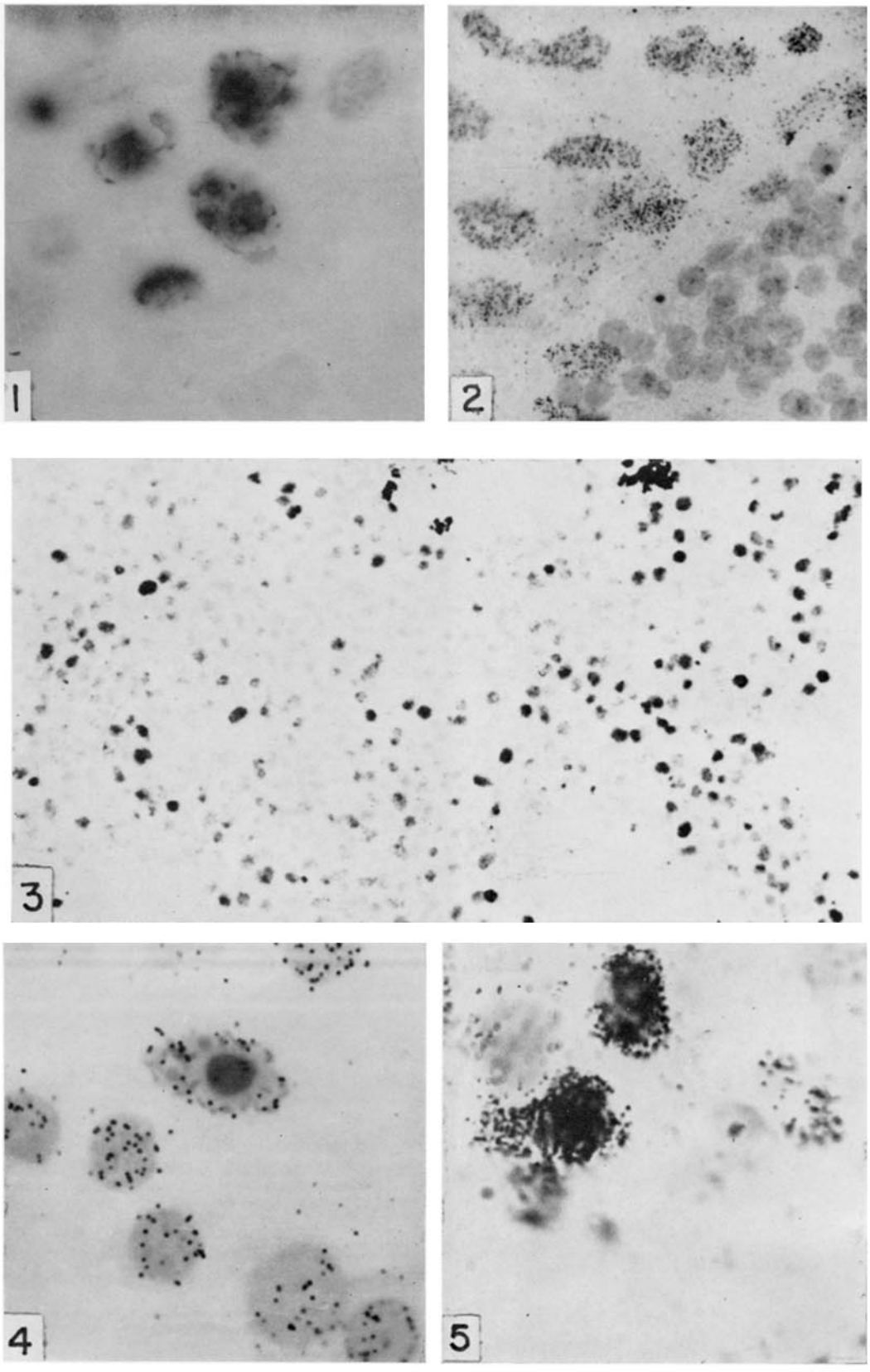
FIG. 1. Intranuclear inclusions in hepatic cells of infected culture. Feulgen. × 1,170.

FIG. 2. Control tissue culture. H^3-thymidine added to the medium 48 hours after preparation of the cultures (series 2). Nuclei of fibroblasts are heavily labeled, while nuclei of hepatic cells are devoid of grains. Feulgen. × 400.

FIG. 3. Infected tissue culture, hepatic cells. H^3-thymidine added at the time of infection (series 4). Dark areas are inclusions covered with large number of grains; nuclei without inclusions do not show overlying grains. 4 days exposure. Feulgen. × 200.

FIG. 4. Infected tissue culture, hepatic cells. H^3-thymidine included in the medium only during the first 48 hours, prior to the infection (series 3). Several grains are seen only at the periphery of the inclusion, on fragments of Feulgen-positive material. Nuclei without inclusions show amount of labeling similar to the one found in series 1 of control cultures. Feulgen. × 1,600.

FIG. 5. As Fig. 3 at higher magnification. The heavy labeling is more clearly seen against the background of the Feulgen-positive inclusions. Feulgen. × 1,200.



(Defendi and Kritchevsky: Tissue cultures of virus-infected liver)