Cytochemical Observations of Ribonucleic Acid in the Nucleolus and Cytoplasm of Cultured Cells Infected with Canine Hepatitis Virus.* (29629)

J. E. MOULTON, L. M. FRAZIER AND D. LAU (Introduced by D. R. Cordy)
Department of Pathology, School of Veterinary Medicine, University of California, Davis.

Genetic information for protein synthesis comes from the nucleus while actual assembling of amino acids into protein occurs in the cytoplasm. Since proteins are not synthesized in the nucleus, this demands the existence of an intermediate carrier of information, which, according to the hypothesis of Jacob and Monad(1) is an unstable intermediate or "messenger" ribonucleic acid (RNA). For some time we have been interested in protein synthesis in cells infected with deoxyribonucleic acid (DNA)-containing canine hepatitis (CH) virus and the role of RNA in the process. In the present study, the quantity of RNA was determined in the nucleolus and cytoplasm during infection with CH virus.

Materials and methods. (1) *Cell cultures.* Secondary cultures of DK cells were propagated on cover slips in Leighton (L) tubes and infected with CH virus (final concentration 10^5 $TCID_{50}$/ml) as described previously (2). After 12, 24, and 30 hours, infected and uninfected cultures were removed, fixed in Carnoy's fixative, and stained for RNA with azure B(3). Cover slips in each experiment were stained simultaneously to minimize variations.

(2) *Microspectrophotometry.* The microspectrophotometric method(4) for quantitation of RNA using azure B stain was modified from Swift and Rasch(5). Specificity of staining was determined by pretreatment of cultures with ribonuclease(3). Cytoplasmic absorptions were made in areas 3.67 μ in diameter, in 50 cells selected at random in each culture. These measurements were made in different areas of the cytoplasm of each cell using the two wavelength (508 and 540 mμ) method(5) to correct for irregular distribution of RNA. Mean absorptions at each wave length were corrected using the table of Swift and Rasch(5). Nucleolar measurements were made in areas 2.45 μ in diameter at the single wavelength of 540 mμ. Background absorptions were made outside the cell. Since fixed cells in monolayers can be considered as flat plates, the square area rather than volume of nucleoli and cytoplasm may be used for determining total RNA(5). The cytoplasm and nucleoli of the DK cells were irregular and areas could not be measured, so cell images were projected onto paper with a prism and outlines of the nucleoli, nucleus, and cytoplasm were drawn. The drawings of the nucleoli and cytoplasm were then cut out of the paper and weighed. The final amount of RNA (in arbitrary units) for the nucleoli or cytoplasm was calculated as extinction $\times$ weight.

(3) *Virus titration.* Virus titer was determined after 12, 24, and 30 hours of infection by a modification of the infected cell count method(6). This is based on the assumption that one infectious unit is sufficient to infect one cell so the number of infected cells is proportional to concentration of virus inoculated. Details of the procedure are as follows: Roller-tube cultures of DK cells were infected with CH virus and after 12, 24, and 30 hours the cells were removed and frozen and thawed to release virus. The cell suspension was diluted 100-fold and 0.2 ml aliquots were added to cover slip cultures of DK cells in L tubes for 1-hour adsorption periods. This was done in triplicate. Monolayers were washed with phosphate buffered saline, growth medium was replaced, and tubes were incubated at 37°C for 26 hours. Only one complete cycle of infection can occur during this period. Cover slips were removed, washed, fixed in Carnoy's, and stained with azure B(3). Since this stain was used at pH 4, RNA, but not DNA, was stained in the

* This work was supported in part by General Research Support Grant 5457 from N.I.H., U.S.P.H.S.

normal cell. However, the mature inclusion body in the infected cell contains a large amount of DNA(2) and will stain with azure B and can be distinguished easily from the smaller, more darkly stained nucleoli. The inclusion can often be identified by its halo. Inclusion bodies were counted in 500 high power fields per cover slip; 3 cover slips were counted for each period of infection. The titer of the virus was calculated as follows:

$$\text{Virus units/ml} = \frac{\text{aliquot infecting virus}}{\text{virus dilution}} \times \frac{\text{mm area of L tube well}}{\text{mm area of micro. field}} \times \frac{\text{No. cells with inclusions}}{\text{No. micro. fields counted}}.$$

Results. The histograms (Fig. 1, 2) illustrate that nucleolar and cytoplasmic RNA remained almost constant in average amount and distribution in control and infected cultures. A few scattered cells had increased

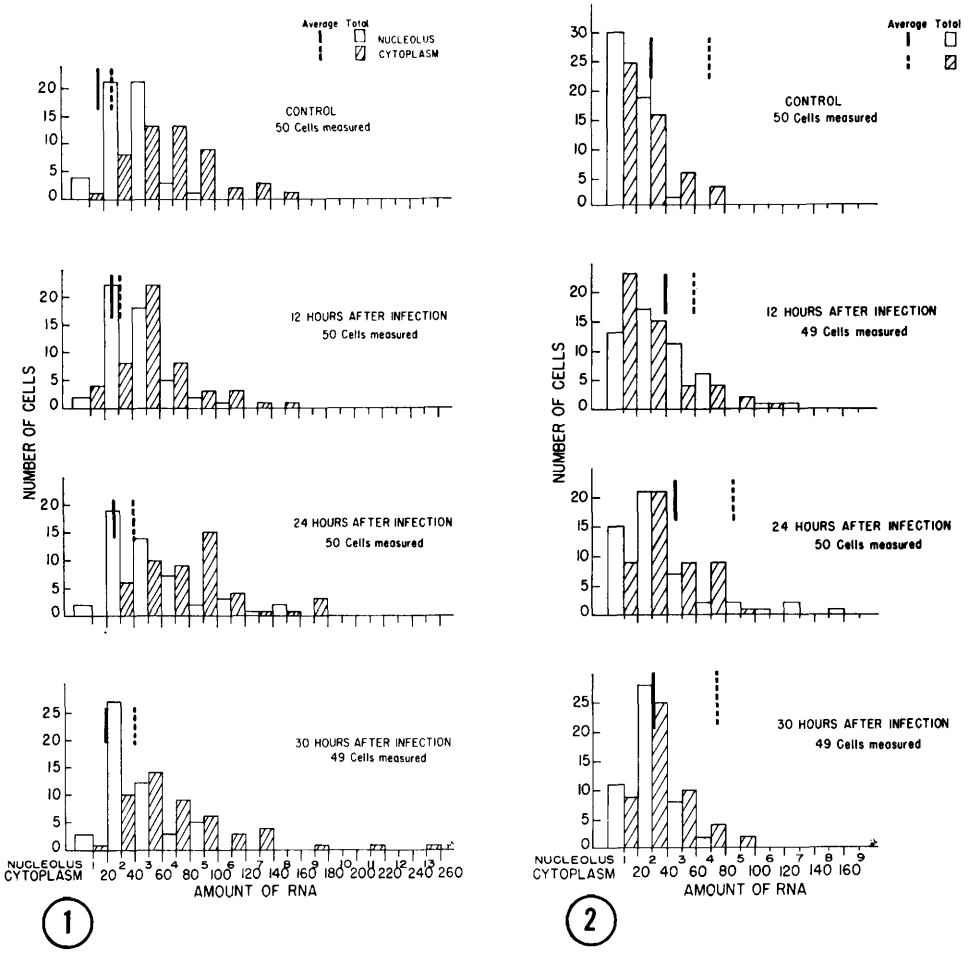

FIG. 1. Histogram showing levels of RNA in nucleoli and cytoplasm of uninfected and infected DK cells at varying periods of infection. Height of each column represents number of cells; columns are placed along abscissa according to amounts of RNA (in arbitrary units). Nucleolar RNA is about the same in control and infected cells in distribution and average amount. Cytoplasmic RNA is increased in distribution and average amount at 24 and 30 hours of infection.

FIG. 2. Histogram of RNA in nucleoli and cytoplasm of DK cells. See legend of Fig. 1 for explanation of figure. In this experiment there was little change in distribution or average amount of nucleolar and cytoplasmic RNA.

TABLE I. Comparison of Cytoplasmic and Nucleolar RNA Concentrations and Sizes.

	Exp I				Exp II			
	Cytoplasmic RNA	Cytoplasmic size	Nucleolar RNA	Nucleolar size	Cytoplasmic RNA	Cytoplasmic size	Nucleolar RNA	Nucleolar size
C	26	171	1.0	4.1	69	344	2.1	7.6
V12	30	219	1.9	6.8	57	320	2.3	7.8
V24	41	234	1.8	5.3	85	313	2.7	5.6
V30	36	227	1.6	4.0	75	297	2.1	5.8

Avg cytoplasmic and nucleolar RNA concentrations and sizes in control (C) and virus (V) infected cultures after 12, 24 and 30 hr. See text for interpretation.

amounts of cytoplasmic RNA in 24- and 30-hour infected cultures in one experiment (Fig. 1). The method of calculating total RNA corrected for contraction of nucleoli and cytoplasm, which occurs during the later stages of infection(2). However, it was of interest to express the data in a different manner to determine if extinction (concentration per unit area) of RNA varied with weight (representing size) of nucleoli and cytoplasm. Table I shows that cytoplasmic and nucleolar RNA concentrations and sizes increased slightly over the control values in Exp. I; cytoplasmic and nucleolar RNA increased slightly or remained the same as the control values while size decreased in Exp. II. Virus titers are shown in Fig. 3.

Discussion. Association of RNA with formation of DNA viruses has been reported by others. Increased cytoplasmic RNA was found in vaccinia virus infection of HeLa cell cultures(8) and chick chorioallantoic membrane (CAM) cells(7). Increased incorporation of P^{32} into RNA was observed in adenovirus infection of KB cells(9). In addition, it was shown that RNA inhibitors could block synthesis of vaccinia virus, adenovirus and herpes simplex virus(10).

The present study showed that there was little difference in average amounts of RNA in the nucleoli and cytoplasm during infection, but there was wider distribution of RNA in the infected cultures. The amount of messenger RNA necessary for viral protein production may be so small that the methods employed here were not able to detect it. It is also possible that a change in composition and more rapid turnover of nucleolar and cytoplasmic RNA was more important than increased total amount in association with formation of viral protein. RNA remained the same in spite of severe pathologic changes in the nucleoli and cytoplasm in the later stages of infection. At the time the inclusion body is formed, the nucleoli are usually contracted and marginated in the halo or trapped within the inclusion and the cytoplasm is severely vacuolated or contracted(2).

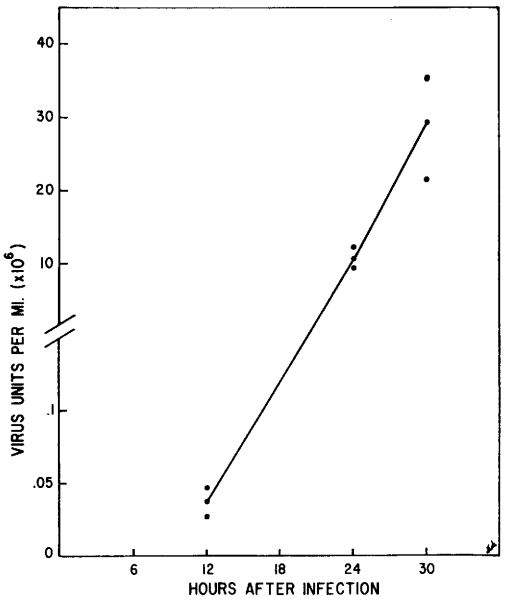

FIG. 3. Virus titers at varying periods of infection.

Summary. The quantity of RNA remained about the same or slightly increased in the nucleoli and cytoplasm of DK cells during infection with DNA-containing canine hepatitis virus.

1. Jacob, F., Monad, V., *J. Mol. Biol.*, 1961, v3, 318.

2. Moulton, J. E., Frazier, L. M., *Virology*, 1961, v15, 91.

3. Rasch, E., Swift, H., Riesen, A. H., Chow,

K. L., *Exp. Cell Res.*, 1961, v25, 348.

4. Soo, S. F., Moulton, J. E., *Am. J. Vet. Res.*, 1963, v24, 150.

5. Swift, H., Rasch, E., in *Physical Techniques in Biological Research*, Oster, G., Pollister, A. W., Ed., Academic Press, Inc., New York, 1956, vIII, 376.

6. Weiss, E., Huang, J. S., *J. Infect. Dis.*, 1954, v94, 107.

7. Caspersson, T., Thorsson, K. G., *Klin. Woch.*, 1953, v31, 205.

8. Loh, P. C., Payne, F. E., Nakamura, M., Ackermann, W. W., *Fed. Proc.*, 1959, v18, 581.

9. Green, M., *Virology*, 1959, v9, 343.

10. Tamm, I., Bablanian, R., *J. Exp. Med.*, 1960, v111, 351.

Received June 16, 1964. P.S.E.B.M., 1964, v117.

Importance of Horizontal Plane Cell Mass Integrity in Wound Contraction.* (29630)

JASPER L. PHILLIPS AND ERLE E. PEACOCK, JR.

Department of Surgery, University of North Carolina School of Medicine, and North Carolina Memorial Hospital, Chapel Hill

Specialized cells in the deepest layer of the dermis at the edge of a full-thickness skin wound ("picture frame area") recently have been shown to perform an important role in the phenomenon of wound contraction. In their studies on the mechanism of wound contraction, Grillo, Gross, and Watts demonstrated that repeated separation of the "picture frame" area of dermis from underlying subdermal tissue interferes with the contracting process and causes an actively contracting wound to reopen to its original dimensions(1,2). Such data strongly suggest that wound contraction forces are developed in a vertical plane between the deep margin of dermis and the subdermal tissue, and that disruption of the dermis-subdermis interface prevents normal movement of the skin edges.

We have hypothesized that, even if contraction forces are oriented primarily in a vertical direction, horizontal plane changes within the "picture frame" area of dermis also must be occurring as the wound perimeter decreases. The possibility that a reduction in number and/or a more compact arrangement of cells in this area might be contributing actively instead of adjusting passively to the contracting process (horizontally oriented purse string effect) has been investigated by performing the following experiments:

* Supported by Grant #A3664, from Nat. Inst. of Arthritis and Metab. Dis., USPHS.

Materials and methods. Female, Sprague-Dawley rats weighing approximately 150 g each were used in all experiments. Full-thickness skin wounds were produced by excising a 2 × 2 cm area of skin from the mid-dorsal region. India ink marks were tattooed on the wound edges at 1 cm intervals around the circumference of the wound. As healing progressed, changes in wound area were measured by placing semi-transparent paper over the wound and outlining the area encompassed by the perimeter markers.

A group of 42 rats was subdivided into 4 groups and wounded as described above. Group I (15 rats) were allowed to heal without any other manipulation. Group II (15 rats) had 16, equally spaced, radially oriented, 5 mm, full thickness, skin incisions placed in the skin edge of the wounds every day after the fifth postoperative day (beginning of period of maximum rate of contraction). Group III (12 rats) were rewounded 7 days after initial skin excision (time of maximum rate of contraction) by excising a .5 × .5 cm area of skin from the cranial and caudal ends of both lateral wound edges. Removal of these portions of the wound created 2 centrally located opposing peninsulas of actively advancing wound margin, the leading edge of which contained an India ink mark. Rate of movement of these partially isolated wound edges was determined by measuring the distance between the tattoo