Cytochemical Effects of Substituted Pyrimidines on Psittacosis Virus.* (26146)

MORRIS POLLARD, THEODORE J. STARR,† YOH TANAMI,‡ AND ARTHUR Y. ELLIOTT
Dept. of Preventive Medicine and Public Health, University of Texas, Galveston

The metachromatic staining properties of acridine orange fluorochrome enables visual study of cytochemical changes which accompany certain viral infections(1). When stained with acridine orange and viewed by fluorescence microscopy, the fluorescence induced in the nucleoplasm of normal cells is yellow-green (DNA-staining) and in both cytoplasm and nucleoli is red-orange (RNA-staining)(2,3). Cytochemical changes associated with maturation of psittacosis virus§ in human cell lines have been described as a color sequence from shades of red to orange to yellow to green(4,5). Fluorescein-tagged antibody localized selectively in the mature virus inclusion. This host-parasite, differential-staining system offers a method for visual study of nucleic acid metabolism. It has been applied by us to studies on the mechanism of antibiotic action(6), and, as described herein, on the mode of action of fluorinated pyrimidines which are nucleic acid antagonists(7). The procedure is based on interference with the normal color sequence associated with virus maturation.

Methods. The procedures used for propagation and examination of tissue cultures infected with psittacosis virus have been described(5). Therefore, only a brief outline is given. A human synovial cell line (McCoy)(8) was propagated on coverslips in Leighton tubes. Tissue cultures were inoculated with a dose of virus (designated TTS) calculated to infect 99% of the cell population(9). Nutrient medium, without antibiotics, consisted of Mixture 199 with 2% heat-inactivated calf serum, and 0.5% lactalbumen hydrolysate. The effect of 5-fluorouracil (FU)‖ and of 5-fluoroorotic acid (FO)‖ on the maturation of psittacosis virus was tested. Graded concentrations (6-25 γ/ml) of FU and FO were each added at intervals from 0-24 hours to both infected and uninfected 3-day-old tissue cultures. After incubation at 37°C, coverslip preparations were fixed in acid-alcohol, stained with acridine orange, and examined by fluorescence microscopy(5). Fate of virus in treated and untreated cultures was followed by inoculation of whole culture extracts intracerebrally into mice and by the microscopic counting method(9).

Results. Within 14 hours after infection, the host-cell contributed an RNA-staining (red-orange) matrix around the initial DNA-staining (yellow-green) virus particle. The resulting cytoplasmic inclusion was designated the 14 hour "red-ball" stage (Fig. 1). RNAase treatment removed the matrix material and unmasked immature virus which was Feulgen positive, not antigenic to fluorescein-tagged antibody, and not infective for mice. During this period, the inclusion increased in size, number, and color intensity. The 14 hour "red-ball" inclusions were 1-4 μ in diameter. Within 38-72 hours, further development of the inclusion was noted. The RNA-staining matrix underwent a sequence of color changes from shades of red to orange to yellow to green (Fig. 2). Subsequently, pin-point mature virus appeared which was antigenically positive, DNAase-resistant, and infective for mice. Addition of FU prior to the 14 hour "red-ball" stage inhibited further color changes and hence virus maturation. In contrast, if FU was added after the 14 hour "red-ball" stage (during the period of matrix color change from red to orange),

* Supported in part by U.S.P.H.S. grant and by James W. McLaughlin Fellowship Fund.

† Present address: Kaiser Foundation Research Inst., Lab. of Comparative Biol., Richmond, Calif.

‡ Fulbright Fellow on leave of absence from Dept. of Bact., Faculty of Medicine, Shinshu Univ., Matsumoto, Japan.

§ It is recognized that "virus" may be a misnomer.

‖ Courtesy of Dr. Robert Duschinsky, Hoffmann-La Roche, Inc.

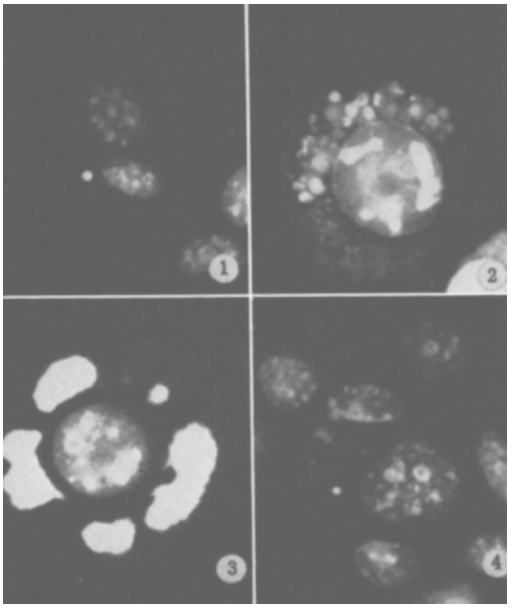

FIG. 1-4. McCoy cells infected with psittacosis virus. With acridine orange fluorochrome and ultraviolet microscopy, normal cytoplasm and nuclei fluoresce red-orange (RNA-staining) and yellow-green (DNA-staining), respectively. ×750.

FIG. 1. 14 hr "red-ball" stage. The cytoplasmic inclusion body which here appears white fluoresces a brilliant red-orange against a lighter cytoplasm.

FIG. 2. 48-72 hr after infection. Maturation of cytoplasmic inclusion bodies is indicated by a sequence of color changes from shades of red to orange to yellow to green which appear here as shades of white to grey. Subsequently, pin-point, DNA-staining mature virus is noted.

FIG. 3. 48 hr after addition of 25 γ 5-fluorouracil to the 14 hr "red-ball" stage (Fig. 1). Instead of the expected color changes indicated in Fig. 2, "fraudulent" RNA-staining inclusions are seen which here appear as white masses.

FIG. 4. 3 days after the stage shown in Fig. 3. The mass of "fraudulent" RNA-staining material disappears and unmasks an inclusion similar to the 14 hr "red-ball" stage.

some inclusion bodies showed evidence of maturation. Within 48 hours after addition of FU to the 14 hour "red-ball" stage, instead of the expected appearance of the yellow-green, DNA-staining mature virus, a mass of RNA-staining material was noted (Fig. 3). This abnormal, RNAase-sensitive, formation has been designated "fraudulent" RNA and may be analogous to that described by Bosch *et al.*(10). It was amorphous in appearance and distinct from both the lighter-stained uninfected cytoplasm and from the more brilliant 14 hour "red-ball" inclusion. Masses of "fraudulent" RNA disappeared gradually within 3-5 days in the course of which time the original 14 hour "red-ball" inclusion remained (Fig. 4). Uninfected control cultures exposed to similar concentrations of FU stained normally with acridine orange. Development of "fraudulent" RNA was more marked with FU than with FO. RNA-staining nucleoli of infected cells treated with FU were enlarged as were some cells. Psittacosis virus multiplied in cultures of McCoy cells; however, virus was absent from infected cultures in which "fraudulent RNA" appeared following treatment with at least 6 γ FU per ml nutrient fluid. This inhibition was partially reversed when 20 times the amount of uracil was added to the FU (Table I).

Discussion. Viruses have been defined as transmissible genetic material(11). As such, they induce changes in infected cells which result ultimately in replication of the original agent. The normal course of psittacosis virus infection of human cell lines showed that the initial DNA-staining virus triggered a reaction in which a matrix of RNA-staining material was formed. That this matrix material gradually faded and disappeared coincident with the appearance of mature virus suggested that it functioned as a metabolic pool or source of nutrients and energy for replication of virus. The matrix material appeared to be involved in production of viral protein-coat and in production of viral DNA itself. These observations are in accord with the roles designated for RNA(12,13). There are indications, also, that FO and FU interfere with DNA formation but not with RNA or protein synthesis(14). In our studies, FO

TABLE I. Effect of 5-Fluorouracil on Replication of Psittacosis Virus in McCoy Cells.

Drug added	γ/ml	Virus recovered 4 days later: Mouse inoculation	Virus recovered 4 days later: Infective units/ml
FU	6	0/5*	20†
FU + uracil	6 + 120	2/5	1.1×10^8
None	—	5/5	1.1×10^7

* No. infected/No. inoculated.
† Particle count in tissue culture.

and FU interfered with the DNA (virus) to RNA (intermediate) to DNA (virus) sequence. Instead of the expected cycle, these pyrimidines caused accumulations of abnormal RNA-staining material which inhibited virus maturation. Removal of this "fraudulent" RNA-staining matrix with RNAase unmasked DNA-staining immature virus. These observations suggested that FU and FO did not interfere with RNA or DNA synthesis *per se* but did interfere with development of intact virus through inhibition of protein synthesis.

When chlortetracycline HCl was added to the nutrient medium at the "red-ball stage" of psittacosis virus, maturation of the inclusion was arrested and virus was not recoverable from such cultures(6). While chlortetracycline and FU interfered with virus replication, the mechanism of each was different: the former appeared to block the mobilization of an RNA-staining matrix, while the latter interfered with the normal biosynthetic sequence by formation of an abnormal intermediate compound.

Summary. Cytochemical changes associated with psittacosis virus infection of tissue cultures were studied by acridine orange staining and fluorescence microscopy. Normal virus development was interrupted by the addition of 5-fluorouracil and 5-fluoroorotic acid which caused the accumulation of "fraudulent" RNA. It is suggested that the substituted pyrimidines interfered with development of intact virus through inhibition of protein synthesis.

1. Anderson, E. S., Armstrong, J. A., Niven, J. S. F., *Sym. Soc. Gen. Microbiol.*, 1959, v9, 224.

2. Armstrong, J. A., *Exp. Cell Res.*, 1956, v11, 640.

3. von Bertalanffy, L., Bickis, I., *J. Histochem. Cytochem.*, 1956, v4, 481.

4. Pollard, M., Starr, T. J., Moore, R. W., Tanami, Y., *Nature*, 1960, in press.

5. Starr, T. J., Pollard, M., Tanami, Y., Moore, R. W., *Tex. Rep. Biol. and Med.*, 1960, v18, 501.

6. Moore, R. W., Pollard, M., Starr, T. J., Tanami, Y., *ibid.*, 1960, v18, in press.

7. Heidelberger, C., Chaudhuri, N. K., Danneberg, P., Mooren, D., Griesback, L., Duschinsky, R., Schnitzer, R. J., Pleven, E., Scheiner, J., *Nature*, 1957, v179, 663.

8. Fernandes, M. V., *Z. fur Zellforsch.*, 1959, v50, 433.

9. Tanami, Y., Pollard, M., Starr, T. J., Moore, R. W., *Tex. Rep. Biol. and Med.*, 1960, v18, 515.

10. Bosch, L., Harbers, E., Heidelberger, C., *Cancer Res.*, 1958, v18, 335.

11. Lwoff, A., *Bact. Rev.*, 1959, v23, 109.

12. Brachet, J., *Biochemical Cytology*, Academic Press, Inc., N. Y., 1957, p. 516.

13. Caspersson, T. O., *Cell Growth and Cell Function*, W. W. Norton & Co., 1950, p. 185.

14. Ackermann, W. W., Loh, P. C., Payne, F. E., *Bact. Proc.*, 1958, 71.

Received August 3, 1960. P.S.E.B.M., 1960, v105.

Permeability of Ehrlich Ascites Cells to Adenine.* (26147)

JOHN A. JACQUEZ AND FREDDA GINSBERG (Introduced by C. C. Stock)

Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Graduate School of Medical Sciences, New York

Data on the permeability of cell membranes to a considerable number of non-electrolytes are available(1,2). Relatively little is known of the permeabilities of cells to the purines and pyrimidines although nucleic acid metabolism has been a major area of investigation for over a decade.

The present paper reports our work on permeability of Ehrlich ascites cells to adenine.

Methods and materials. The Ehrlich ascites used was a hypotetraploid line carried in Swiss albino mice by weekly intraperitoneal injections of 0.1 ml of ascites. For experiments, 6 day ascites was collected into 50 ml Krebs-Ringer bicarbonate (KRB) containing 0.2 mg heparin. The resulting suspension was diluted with an equal volume of

* This work was supported in part by grants from Am. Cancer Soc. and by CCNSC contract.