# Effects of 5-Fluorouracil on Infectious Canine Hepatitis Virus Infection of Dog Kidney Cells.* (30724)

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Various antimetabolites such as 5-fluorouracil (FU) have been used to study biosynthetic events in virus-infected cells. FU has at least 2 methods of action: 1) It is converted in the cell to the deoxyribonucleotide form and this compound combines with and inactivates the enzyme or group of enzymes called thymidylate synthetase. This inhibits the synthesis of thymidylate and subsequently the synthesis of DNA(1). 2) It can also be incorporated into RNA molecules as an analog of uracil. This may result in the synthesis of abnormal proteins(2,3).

The synthesis of virus-specific polymers in the absence of mature virus formation has been demonstrated in the presence of FU. Empty pseudorabies virus capsids were formed by infected cells in the presence of FU(4). In SV40-infected cells, abundant virus-specific antigens, demonstrable by immunofluorescence, were formed in the presence of FU, but only rare virus particles or empty viral capsids were seen by electron microscopy(5). Work with type 4 and type 5 adenoviruses has shown that with these viruses, normally functional virus-specific antigen synthesis is dependent upon DNA synthesis(6).

Infectious canine hepatitis virus (ICHV), an adenovirus of canine origin, has been used in this study of the anti-viral activity of FU. This paper presents evidence that FU markedly inhibits DNA synthesis and completely inhibits mature virus particle formation in ICHV-infected cells, but has little effect on protein synthesis in these cells.

*Materials and methods. Virus.* The virus pool used in this study had been passed repeatedly in dog kidney (DK) cells in this laboratory, and had a titer of $1 \times 10^{6.5}$ $TCID_{50}$ per ml.

*Tissue culture.* A single line strain of DK epithelial cells‡ was used for all procedures except virus titrations which were carried out on a different DK epithelial cell line strain.§

*Tissue culture medium.* Medium used was Earle's salt solution containing 0.5% lactalbumin hydrolyzate (LE medium). This was supplemented with lamb serum at levels of 10% for cell growth, and 1.0% for cell maintenance. Penicillin and streptomycin were added to the LE medium at levels of 200 units per ml and 200 $\mu$g per ml respectively.

*Inhibitor.* The FU‖ stock solution was prepared in antibiotic-free LE medium at a $10^{-2}$ M concentration, and was sterilized by filtration through a Millipore filter with an average pore diameter of 0.45 $\mu$. It was stored in sealed glass ampules and frozen until needed, at which time it was diluted to $10^{-3}$ M concentration in LE medium containing antibiotics.

*Cell infection procedures.* The stock virus pool was diluted $10^{-2.5}$ in LE medium, and 0.2 ml was placed on DK cell monolayers grown on coverslips in Leighton tubes, after first washing the monolayers with phosphate buffered saline (PBS) at pH 7.2. After a 2-hour virus absorption period at 37°C with frequent shaking of the tubes, unadsorbed virus was removed by aspiration and the monolayers were again washed with PBS. Cells were then fed with LE containing 1.0% lamb serum. The inhibitor was introduced into designated tubes at this time.

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‡ Obtained from Dr. S. Madin, Naval Biological Laboratory, Berkeley, Calif.

§ Obtained from Dr. A. H. Fieldsteel, Stanford Research Inst., Menlo Park, Calif.

‖ Kindly supplied by Drs. R. Duschinsky and W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, N. J.

*Virus titration.* Serial 10-fold dilutions were made in LE medium of free virus and cell-associated virus. Titrations were performed on monolayers of DK cells in roller tubes. The $TCID_{50}$ was calculated by the method of Reed and Muench(7).

*Acridine orange staining.* Coverslips were fixed for 5 minutes in cold Carnoy's fixative. The staining technique of Mayor(8) was then followed.

*Feulgen staining.* Coverslips were fixed for 30 minutes in cold Carnoy's fixative. The Feulgen reaction was carried out by the method of Leuchtenberger(9).

*Sakaguchi staining.* Coverslips were fixed for 30 minutes in cold Carnoy's fixative, then stained by the method of Deitch(10). All arginine microspectrophotometric measurements were made within 24 hours of staining.

*Fluorescent antibody staining.* The preparation of materials for the indirect fluorescent antibody (FA) technique has been described(11). Coverslips were fixed for 30 minutes in acetone, then stained by the method of Weller and Coons(12). Staining was done within 24 hours of fixation.

*Electron microscopy.* Coverslips were fixed for 45 minutes in 1.0% osmium tetroxide buffered at pH 7.4. They were then dehydrated in an ethyl alcohol series before embedding, sectioning, and uranyl acetate staining.¶

*Hemagglutination.* Cells were scraped from roller tubes into PBS (pH 8.0), and were disrupted by repeated freezing and thawing. This suspension was clarified by centrifugation and the supernatant was examined for its capacity to agglutinate chicken erythrocytes, using the method of Fastier(13).

*Microspectrophotometry.* The microspectrophotometer** was used in conjunction with a photometerhead containing a light sensitive phototube. Light transmission was registered on a photovolt meter.††

Feulgen and Sakaguchi microspectrophotometry was carried out at wave lengths of 546 and 517 mμ respectively. Light transmission was measured through the nucleus or the nucleus and the cytoplasm of individual stained cells. Insertable, fixed aperture diaphragms permitted measurement of light transmission through desired, limited areas of a cell. The diaphragm size used in nuclear measurements was large enough to measure transmission through almost the entire nuclear area at once. The diaphragm size used for cytoplasmic measurements was small enough to fit completely inside of the rim of cytoplasm surrounding the cell nucleus. Three measurements were made in the cytoplasm of each cell to determine the average light transmission through the cytoplasm. Relative cell size was determined by projecting each cell's image, using a projecting prism, onto a sheet of heavy gauge paper with a uniform weight per unit area. The paper was fixed at a right angle to the plane of projection and was at a constant distance from the projecting prism. This assured equal magnification of cell images. The projected image was traced on the paper. The traced pictures were cut out and the drawn nucleus and cytoplasm were weighed separately to the nearest mg. Thus, the nucleus and cytoplasm of each cell had comparative, although arbitrary, sizes in mg determined for them. Light transmission through cells was compared with background light transmission.

Basic formulas used to calculate the arbitrary absolute units of DNA or arginine in a cell were:

$$1)\quad E = \log_{10} \frac{I_O}{I_S}$$

where E is the coefficient of extinction, $I_O$ is the intensity of light transmission from a cell-free background area, and $I_S$ is the intensity of light transmission through the cell being measured(8).

$$2)\quad A = EW$$

where A is the number of arbitrary absolute units of arginine in the cytoplasm, or arginine or DNA in the nucleus of a cell, E is the coefficient of extinction calculated in the preceding equation, and W is the weight in mg of the paper cut outs of the nuclear or cytoplasmic images.

¶ Electron microscopic procedures were performed by R. B. Addison and J. Pangborn, Univ. of California, Davis.

** Gartner Mechanical Engineering Co., San Francisco, Calif.

†† Model 501 M, Photovolt Corp., New York.

*Results. Morphological observations.* Acridine orange stained, infected cells first showed a diffuse intranuclear pinpoint stippling of inclusion body type DNA. These individual pinpoint structures grew by enlargement and coalescence until finally a large, single, dense inclusion body formed, nearly filling the nucleus and usually surrounded by a clear halo. FU stopped inclusion body maturation at stages that normally were intermediate ones. No unique pattern of inclusion body formation resulted from FU.

Fluorescent antibody first revealed virus-specific antigen as fine pinpoint stippling in the nucleus and cytoplasm. In the nucleus, these structures enlarged and coalesced to form granules and clumps of fluorescent material. Mature inclusion bodies often had dull or no central fluorescence. Rarely were they brightly fluorescent throughout. Cytoplasmic fluorescence usually remained as diffuse pinpoint granules of varying intensity. FU stopped the changing antigen pattern in the nucleus at stages of granules or clumps. It had no effect on the appearance of cytoplasmic antigen.

Electron micrographs of infected cells showed typical crystal-like arrays of intranuclear virus particles. No virus particles or empty viral capsids were seen in FU-treated cells.

*Microspectrophotometric measurements.* DNA and arginine levels did not change in uninfected control cells throughout the experimental period. The level of FU used in this study had no effect on DNA or arginine levels in these cells.

Fig. 1 and 2 show that nuclear and cytoplasmic arginine respectively increase markedly in infected cells. FU has little or no quantitative effect on these increases. Fig. 3 shows that DNA increases markedly in infected cells and FU greatly inhibits this increase.

*Virus production.* Fig. 4 shows that high titers of infectious virus, both free and cell-associated, are produced by infected cells. FU blocked completely the formation of infectious virus.

*Hemagglutinin production.* Uninfected cells produce no hemagglutinin. Infected cells produce hemagglutinin by 48 hours after infection. The highest titer of hemagglutinin in infected cells was 1:256. Infected cells treated with FU also produced hemagglutinin but the highest titer reached in these was 1:8.

*Discussion.* FU stopped ICHV inclusion body maturation at intermediate developmental stages. Acridine orange staining distinguished between cellular and inclusion body types of DNA by their differing tinctorial properties. The inhibition of inclusion body maturation appeared morphologically to be due to a simple deficiency of inclusion body type DNA. This was in accord with the microspectrophotometric results which showed that FU inhibited DNA synthesis in infected cells. FUDR has been shown to affect inclusion body formation in this system by diminishing DNA synthesis(14). Stages of pseudorabies virus inclusion body formation that are dependent upon DNA synthesis are blocked by FU(4).

Arginine is present in all proteins and arginine levels may therefore be used as indicators of protein levels(15). Arginine increased markedly in the nucleus and cytoplasm of infected cells. FU had little quantitative effect on this increase.

It appears that only a fraction of the usual amount of DNA synthesis seen in ICHV-infected cells is actually needed, if indeed any is necessary, for the synthesis of normal quantities of protein to occur. Whether this protein in FU-treated cells is normal, functional virus-specific protein was not determined. However, the virus-specific antigenicity of these proteins was shown by imuunofluorescence, and they were capable of producing hemagglutination. This indicates that at least in these respects, some of these proteins had a degree of normalcy. In studies with types 4 and 5 adenoviruses, the synthesis of normally functional virus-specific antigens was prevented by inhibition of DNA synthesis(6).

The limited amount of DNA synthesized in FU-treated, infected cells, showed typical inclusion body type DNA staining with acridine orange. No further criteria were applied to determine if this DNA was normal in other respects.

The failure to form infectious virus or morphologically identifiable virus particles in FU-treated cells appeared complete. This failure would be expected to have been only partial if it was due solely to reduced numbers of normal viral DNA genomes. Since some increased DNA synthesis occurred in FU-treated, infected cells, a simple numerical shortage of normal viral genomes does not appear to explain this failure. A likelier possibility is that the virus specific DNA and/or some or all of the virus specific protein synthesized was functionally abnormal. Functional abnormalities in virus specific polymers could prevent virus particle formation in FU-treated cells.

*Summary.* It has been shown that FU, at a $10^{-3}$ concentration, markedly inhibits DNA synthesis in DK cells infected with ICHV. In the absence of FU, DNA levels in

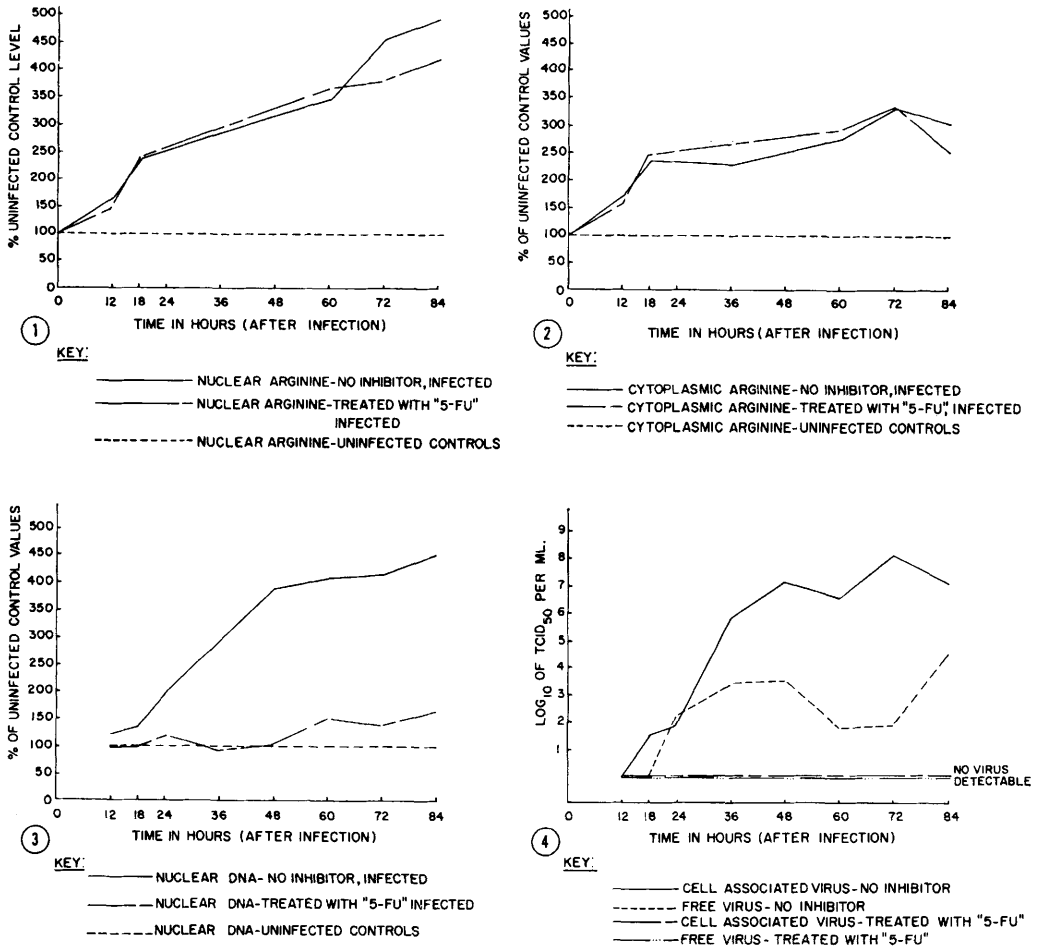


FIG. 1. Comparison of nuclear arginine levels in infected cells, untreated, and treated with FU. Arginine levels in infected cells are given as percentages of levels in uninfected cells. Each point represents an average of measurements from 100 cells made in 2 separate experiments.

FIG. 2. Comparison of cytoplasmic arginine levels in infected cells, untreated, and treated with FU. Arginine levels in infected cells are given as percentages of levels in uninfected cells. Each point represents an average of measurements from 100 cells made in 2 separate experiments.

FIG. 3. Comparison of nuclear DNA levels in infected cells, untreated and treated with FU. DNA levels in infected cells are given as percentages of levels in uninfected cells. Each point represents an average of measurements from 100 cells made in 3 separate experiments.

FIG. 4. Titration of infectious virus produced in untreated cells and cells treated with FU.

infected cells increased to about 450% of uninfected control cell levels by 72 hours post-infection. By contrast, the marked elevation of nuclear and cytoplasmic arginine levels (an index of protein levels) that is seen in infected cells was not affected by the presence of FU. No infectious virus particles were formed in the presence of FU. Electron microscopic examination of infected, FU-treated cells did not reveal the presence of either complete virus particles or empty viral capsids. However, these cells produced hemagglutinins and also virus specific antigen demonstrable by immunofluorescence.

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## Radiation Effects Renal Enlargement in the Mouse.* (30725)

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Earlier work has shown that the kidneys enlarge as animals grow, and that following unilateral nephrectomy, compensatory enlargement occurs in the remaining kidney(1, 2). This enlargement is attributable to both cellular enlargement and cellular proliferation. Thus the hypertrophy response provides a good system for studying radiation effects on cell proliferation and enlargement. The system can also be used to study the influence of radiation on factors which control the initiation of these responses.

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A study by one of us(3) has indicated no noticeable effect of doses up to 1500 r on the enlarging kidney in children following surgery for nephroblastoma. Straube and Patt(4) studied the effect of local irradiation at the time of surgery on renal hypertrophy at 2 weeks after surgery in adult mice. No effect on renal weight gain was found until the doses exceeded 10,000 rads. However, Rosen and Cole(5) found a reduction in mouse kidney weight gain at 3 weeks and in mitoses at 48 hours after nephrectomy when total body exposures below 1000 r were given 3 hours after nephrectomy. Berech(6) and Franklin(7) found decreased compensatory hypertrophy in young mice at 4 to 38 weeks after total body irradiation, and Cole and Rosen(8) reported decreased mitotic activity up to 4 weeks after nephrectomy. It seemed possible that the differences