reported separately indicates that rats sensitized and treated for 17 days with cyclophosphamide do not develop evidence of EAE at least for 6 weeks after discontinuing drug treatment and probably longer. Furthermore, such rats if reinjected with spinal cord-adjuvant still do not develop typical EAE. This extended immunosuppressive action of cyclophosphamide in rats is in direct contrast to the short-lived action of nitrogen mustard and 6-mercaptopurine reported in work with guinea pigs and rabbits(1-4). In the cyclophosphamide-EAE study in guinea pigs reported by Calne and Liebowitz(7,8), animals were followed only 8 days after an immunosuppressive 16-day course of treatment, an observation period too short to determine how lasting the immunosuppressive effect may be in this species.

Summary. Experimental allergic encephalomyelitis can be inhibited in Wistar rats treated with doses of cyclophosphamide which carry little or no toxicity for this species of laboratory animal. Inhibition of the disease lasts for at least 3 weeks after discontinuing cyclophosphamide treatment. The suppressive effect of this alkylating agent can be achieved starting treatment as late as 9 days after nervous tissue-adjuvant sensitization. Cyclophosphamide inhibits production of complement-fixing antibrain antibody in parallel with its EAE-inhibitory effect.

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Human Cytomegalovirus. Studies on the Mechanism of Viral Cytopathology and Inclusion Body Formation.* (31889)

ROBERT M. MCALLISTER, JEAN E. FILBERT, AND CLYDE R. GOODHEART†
(Introduced by D. Hammond)
Department of Pediatrics, University of Southern California School of Medicine, and Children Hospital of Los Angeles, Los Angeles

Several investigators have attempted to define the biochemical events leading to viral cytopathic effects (CPE) by the use of metabolic inhibitors(1-3). Cells infected with human cytomegalovirus (CMV) are especially suitable for certain aspects of such study because they slowly develop, during a 72-hour period, CPE and intranuclear and intracytoplasmic inclusion bodies. By means of cytochemical(4), immunofluorescent(4,5), and autoradiographic techniques(6), previous studies have correlated the morphological changes with the synthesis of RNA, DNA and protein as well as the synthesis and release of infectious virus during one cycle of virus infection.

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† Present address: Inst. for Biomedical Research Education and Research Foundation, Am. Med. Assn., Chicago, Ill.

Twenty-four hours after infection, viral CPE can be seen by microscopic observation of living unstained cells. Stains for nucleic acids revealed that cells with early CPE (24 hours after infection) contained a ring-like aggregate of RNA adjacent to the nucleus. Seventy-two hours after infection, the cytoplasmic inclusion body evolved from its early stage of the RNA-containing ring-like structure into a body containing DNA in its center. Autoradiographic studies suggested that this latter DNA apparently migrated to the intracytoplasmic inclusion from the intranuclear inclusion(6). At about this time, infectious virus was first found intracellularly and shortly thereafter, release into the medium began.

Fluorodeoxyuridine (FUdR) partially disrupts these processes(7). In the presence of FUdR, infected cells develop viral CPE and the ring-like RNA halo in the cytoplasm. The cells do not, however, develop DNA-containing intranuclear inclusions, nor does DNA appear in the intracytoplasmic inclusion body. Mature virus is not synthesized in these cells.

In view of the observed effects of FUdR upon CMV-infected cells, it was of interest to determine the effects of other inhibitors of DNA synthesis, as well as the effects of an inhibitor of RNA synthesis and an inhibitor of protein synthesis, upon the development of viral CPE and inclusion bodies. during one cycle of virus infection. Biochemical studies of the macromolecular metabolism of CMV-infected cells would be useful in understanding the mechanisms by which a virus can cause such profound changes in a cell. However, such biochemical studies would require techniques not presently available. For instance, virus titers rarely exceed 5×10^6 cell infectious units/ml; purification procedures attempted to date have not resulted in sufficient recovery to be useful for increasing the titer. Thus, the input multiplicity in these experiments has been limited with the result that only some of the cells in a culture can be infected, yet it has been pointed out(1) that all the cells in a culture must be infected for this kind of biochemical study. Despite these and other technical difficulties, it was felt that information obtained by cytochemical studies, even though qualitative, subject to extension and confirmation by definitive biochemical procedures when they become possible, would be of sufficient interest to warrant this report, describing the results of these studies in which cytochemical techniques were used to observe the virus-infected cells exposed to these inhibitors.

Materials and methols. Cells and media. Human fetal skin-muscle cells were used for the experiments reported here. The cells were cultivated as monolayers either on coverslips in Leighton tubes or in plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). Eagle's medium with 2% calf serum (Microbiological Associates, Bethesda, Md.) was used for maintenance of fully grown cell sheets.

Virus. The Rib strain(4) of CMV in 50-90th passage was used throughout. Supernatant fluid from infected cultures with advanced CPE was used without sonication or centrifugation. The virus was assayed by counting infected cells(8).

Inhibitors. The sources of the inhibitors used are as follows: 5-iodo-2′-deoxyuridine (IUdR): Pfizer and Co., Maywood, N. J.; Mitomycin C and puromycin: Nutritional Biochemicals, Cleveland, Ohio; Actinomycin D: Merck Sharp & Dohme, West Point, Pa.; Cytosine arabinoside (1-β-Darabinofuranosylcytosine HCl): Upjohn Co., Kalamazoo, Mich.

Experimental procedure. The methods for infecting cells and for fixing and staining the virus-infected cells have been described (4). Cell sheets were rinsed with buffer solution and exposed to virus at a multiplicity of about 0.1 to 1.0 cell infectious units/cell. After 1 hour at 37° the cells were rinsed 3 times before adding maintenance medium containing one of the inhibitors. After 24, 48, and 72 hours, the cells of representative cultures were fixed and stained with May-Grünwald-Giemsa, acridine orange or "fluorescent" Feulgen (Fig. 1A and B).

The titer of infective virus in the infected cells was determined by scraping the cells off the petri dish cultures, suspending them in buffer, grinding with Alundum, and assaying the centrifuged supernatant(8).

TABLE I. Effect of Inhibitors upon Occurrence of Viral Cytopathology, Intracellular Inclusion Bodies and Infective Virus in CMV-Infected Cells.*

Experimental group	Viral CPE unstained	Stained cells Inclusion bodies† IC	IN	Intracellular virus (CIU/culture) Untreated	Treated
No additions	Present	Orange halo Green center	Present	—	—
Added IUdR (100 μg/ml)	"	Orange halo	Absent	2.0×10^5	1.4×10^4
Added IUdR and thymidine (100 μg/ml each)	"	Orange halo Green center	Present	"	5.3×10^5
Mitomycin C (15 μg/ml)	"	Orange halo	Absent	1.1×10^5	<10
Cytosine arabinoside (100 μg/ml)	"	" "	"	"	"
Actinomycin D (0.5 μg/ml)	Absent	Absent	"	1.7×10^5	"
Puromycin (0.5 μg/ml)	Present	Orange halo	"	2.0×10^5	20

* Experimental procedure: One 60-mm petri dish of human fetal skin-muscle cells was used for each group. Each dish was rinsed with buffer solution, inoculated with 0.2 ml of suspension containing about 10^5 CIU of CMV and incubated at 37°C for 1 hr. After rinsing 3 times, medium containing 2% calf serum with or without one of the inhibitors was then added. After 4 days the culture fluids were discarded and the cell sheets were rinsed once and scraped off the dish; the cells of each group were ground with Alundum in 1 ml of buffer, cell debris was removed by low-speed centrifugation and the supernates were assayed.

† IC = intracytoplasmic inclusion body; IN = intranuclear inclusion body.

Effects of inhibitors on human fetal fibroblasts. The cytotoxic effects of the inhibitors on uninfected human fetal fibroblasts were tested in preliminary experiments. Concentrations of inhibitors used in further experiments, when the cells were exposed to them for 72 hours, were below cytotoxic levels and were: IUdR, 100 μg/ml (3×10^{-4} M); mitomycin C, 5-15 μg/ml; cytosine arabinoside, 100 μg/ml (4×10^{-4} M); actinomycin D, 0.1 to 0.5 μg/ml (4.5×10^{-7} to 9.0×10^{-8} M); and puromycin, 0.5 μg/ml (1.0×10^{-6} M).

Effects of inhibitors on extracellular virus. Preliminary experiments were made to test the effects of the inhibitors upon infectivity of extracellular CMV particles. Viral suspensions were diluted in buffer with or without the inhibitors and incubated 4 hours at 4°C. The surviving virus was assayed by the cell count method; inhibitor was removed by rinsing the cell sheet 3 times with buffer following the adsorption period so that the inhibitor would not interfere with the assay.

The inhibitors in the concentrations indicated above caused no increased loss of infectivity of CMV during 4 hours at 4°C when compared to untreated CMV.

Results. Effects of inhibitors on development of viral cytopathology and viral inclusions, and on synthesis of infective virus.

1. *Inhibitors of DNA synthesis: IUdR, mitomycin C and cytosine arabinoside.* The results obtained with these inhibitors were similar to those previously obtained with FUdR(7). Infected cells exposed to IUdR (100 μg/ml), mitomycin C (5-15 μg/ml) and cytosine arabinoside (100 μg/ml) developed viral CPE when observed unstained and also developed the RNA-halo of the intracytoplasmic inclusion when stained 24-72 hours after infection (Fig. 1C). These cells did not develop the DNA-containing intranuclear inclusions, nor did DNA appear in the intracytoplasmic inclusion 72 hours after infection, as it did in untreated cells. The titer of infective virus was reduced in cells exposed to this concentration of IUdR and no infective virus was detected in cells exposed to the other inhibitors (Table I).

These results indicate that the development of viral CPE and the early intracytoplasmic inclusion can occur in the presence of inhibitors of DNA synthesis and therefore presumably is not dependent upon the synthesis of DNA. Infective virus is not produced and DNA does not appear in the intracytoplasmic inclusions. These results are consistent with the current concepts of the mechanism of action of these inhibitors(9-11). IUdR is incorporated into DNA and also blocks any

of several enzymes in the pathway of thymidine to DNA; the specific enzyme blocked depends on the cell type tested(9). In the human fetal fibroblast used in the present studies, it may be inferred that, of the enzymes that might be inhibited, IUdR blocks a step leading to thymidylate, such as thymidylate synthetase, as the effect of the compound is reversed by exogenous thymidine (Table I).

2. *Inhibitor of DNA-dependent RNA synthesis: Actinomycin D.* Infected cells exposed to actinomycin D did not develop viral CPE, any elements of the intracytoplasmic or intranuclear inclusions, or infective virus (Fig. 1D, Table I). This is not due to general cell toxicity; control experiments indicated that cells so treated with actinomycin D are still capable of forming infectious poliovirus. This result suggests that DNA-dependent synthesis of RNA is necessary for further changes to occur in the CMV-infected cell.

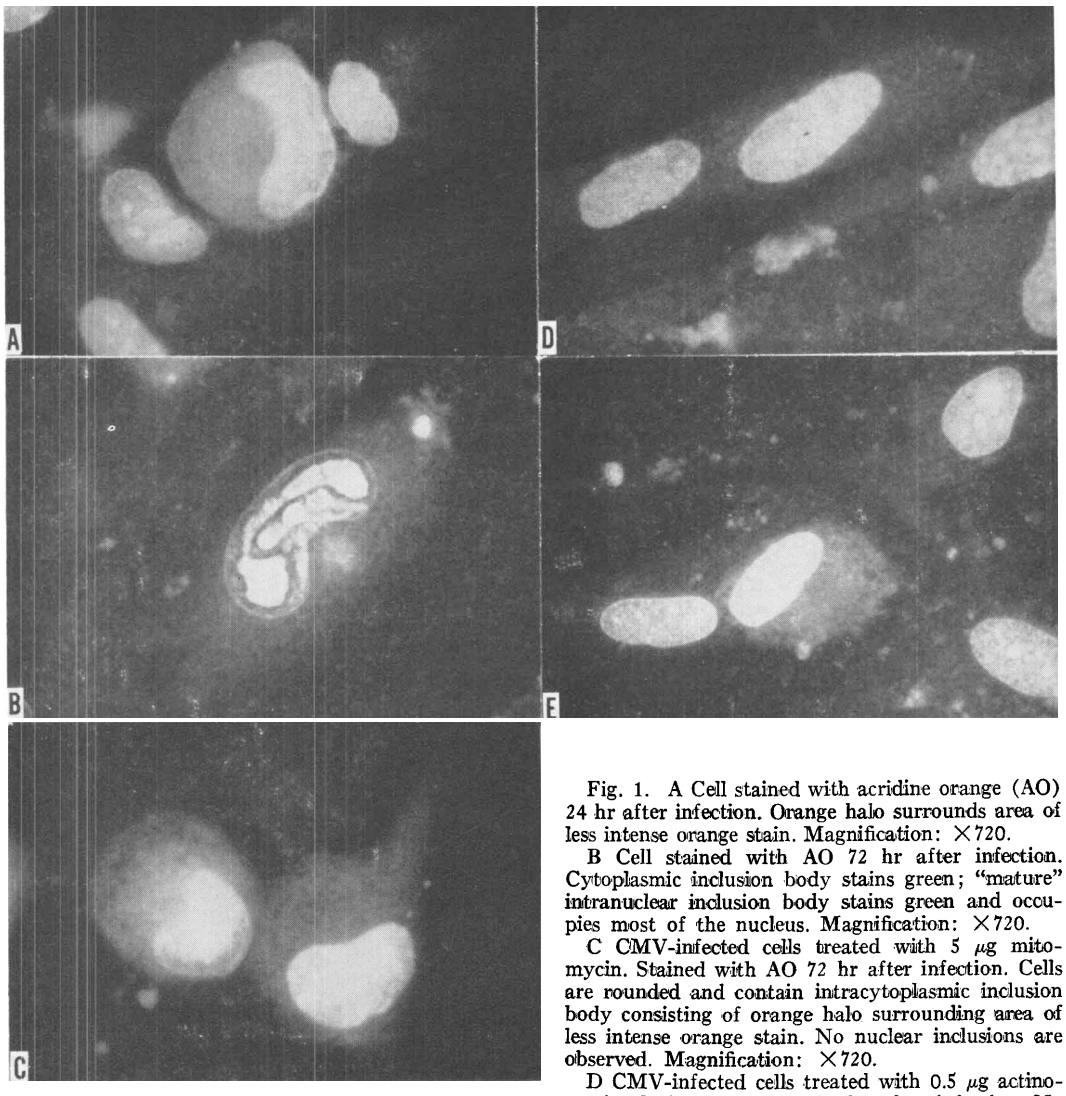

Fig. 1. A Cell stained with acridine orange (AO) 24 hr after infection. Orange halo surrounds area of less intense orange stain. Magnification: ×720.

B Cell stained with AO 72 hr after infection. Cytoplasmic inclusion body stains green; "mature" intranuclear inclusion body stains green and occupies most of the nucleus. Magnification: ×720.

C CMV-infected cells treated with 5 μg mitomycin. Stained with AO 72 hr after infection. Cells are rounded and contain intracytoplasmic inclusion body consisting of orange halo surrounding area of less intense orange stain. No nuclear inclusions are observed. Magnification: ×720.

D CMV-infected cells treated with 0.5 μg actinomycin. Stained with AO 72 hr after infection. No evidence of cell rounding (CPE) or of inclusion bodies. Magnification: ×720.

E CMV-infected cell treated with 0.5 μg puromycin. Stained with AO 72 hr after infection. Cell in center of field is rounded (CPE) and has cytoplasmic inclusion body. No nuclear inclusion body is observed. Magnification: ×720.

3. *Inhibitor of protein synthesis: Puromycin.* Infected cells exposed to puromycin (0.5 μg/ml) developed viral CPE and the RNA-halo and unstained area of the intracytoplasmic inclusion (Fig. 1E). In such cells, the intracytoplasmic inclusion never contained DNA, the intranuclear inclusions did not develop, and infective virus was undetectable (Table I).

The development of viral CPE in the presence of inhibitors of protein synthesis was surprising since other workers have suggested that synthesis of virus proteins may be responsible for poliovirus-induced cytopathology (2,3). Therefore, an experiment was performed to test the effectiveness of the puromycin block. It was found that at a concentration of 0.5 μg/ml and under conditions used for the other experiments, puromycin reduced the incorporation of H^3-leucine into acid-insoluble products in uninfected cells to about half that of the controls. A higher concentration of puromycin could not be used because of its toxicity to the cells when used for the extended period required in these experiments.

Discussion. From these results, it is possible to construct a tentative scheme for the morphological changes induced in human fetal fibroblasts by CMV consistent with the known effects of the inhibitors upon the production of specific macromolecules. It may be postulated that after an infective CMV particle enters a cell and its nucleic acid is set free, it acts as a template for synthesis of messenger RNAs. These RNAs are synthesized using pre-existing cellular DNA-dependent RNA polymerase, since the intracytoplasmic RNA halo is formed in cells treated with puromycin. Alternatively, it is possible that pre-existing RNA is merely rearranged in the cell resulting in the RNA "halo." The CPE results either directly from the accumulation or rearrangement of these RNAs or from the accumulation of new proteins whose synthesis is directed by one or more new RNA species. The synthesis of these proteins is not completely inhibited by puromycin. The nature of the proteins is not specified here, and could be capsid material as suggested for poliovirus-infected cells(2,3). Against this possibility, however, is the time at which CPE occurs. In CMV-infected cells, CPE becomes visible as soon as 18-24 hours after infection and before viral antigen is detectable(4). Further, the CMV system described here appears to be different from the poliovirus system(3). In the latter case, puromycin inhibited virus production and CPE did not occur; cells infected with CMV and treated with puromycin do not synthesize virus but do develop CPE and the cytoplasmic RNA "halo."

It is further postulated that the DNA present in the intranuclear inclusion is synthesized by using the DNA of the infecting virus as primer and a new DNA polymerase made in response to one of the new messenger RNAs. These syntheses would be blocked by any of the inhibitors used.

Confirmation of the events described in this hypothetical scheme will require precise biochemical evidence that at present is not possible to obtain for reasons described in the introduction.

Summary. When cultured human fetal fibroblasts were infected by human cytomegalovirus, virus-induced morphologic changes consisting of cytopathology, intracytoplasmic and intranuclear inclusion bodies developed during a 72-hour period. Thus, in this system, virus-induced cell changes followed closely upon viral multiplication. Inhibitors of DNA synthesis, IUdR (100 μg/ml), mitomycin C (15 μg/ml) and cytosine arabinoside (100 μg/ml), when added at the time of infection, suppressed the production of infectious virus and the development of mature inclusions but did not interfere with the development of cytopathology and the "early" cytoplasmic lesion. An inhibitor of RNA synthesis, actinomycin D (0.5 μg/ml), when added at the time of infection, prevented all virus-induced morphological changes and the production of infectious virus. An inhibitor of protein synthesis, puromycin (0.5 μg/ml), gave the same results as the inhibitors of DNA synthesis.

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Influence of Intestinal Content on Radiation Lesions of the Small Intestine.* (31890)

NATHAN HIATT AND NANCY E. WARNER (Introduced by A. L. Sellers)
Cedars-Sinai Medical Research Institute and Division of Laboratories, Cedars-Sinai Medical Center, Los Angeles, Calif.

It was observed in this laboratory that the content of the small intestine seems to influence nitrogen mustard enteritis. In dogs with a Thiry-Vella fistula of a segment of jejunum, the intestinal lesions resulting from intravenous injection of an LD_{80} dose of nitrogen mustard (1.5 mg per kilo) are less intense in the isolated segment than in the small intestine in continuity (Fig. 1). A T-V fistula is a loop of small intestine with intact blood vessels and nerves, through which the intestinal content does not pass.

This communication reports the pathological changes observed in an isolated, fistulous loop as compared with the small intestine in continuity, when external radiation instead of a radiomimetic drug is used.

Methods and materials. Five dogs were prepared with T-V fistulas, each consisting of a 15-cm segment of mid-jejunum. One to two weeks post-operatively, when the animals appeared completely recovered, each was lightly anesthetized with sodium pentobarbital. Two dogs were radiated with 1,000r to the abdomen: 3 with 1,500r. These doses are in the lethal range(1). Following radiation, the dogs were maintained on the routine kennel care until the animals succumbed or until gross blood appeared in the stool. These occurred on the third and fourth days in the dogs given 1,500r and the third day in the 2 given 1,000r. The animals with blood in the stools seemed terminal. In these the experiment was ended with intravenous sodium pentobarbital. All the animals were autopsied.

Results. The fistulous segments appeared grossly intact, with glistening, pale pink mucosa covered in part by a little pale yellow mucus. In contrast, the rest of the small intestine was dilated throughout, with a diffusedly injected, edematous mucosa having dark red, elevated, linear, longitudinal streaks; the lumen contained copious, turbid, thick, pink, feculent fluid. The colon also was slightly dilated, with pale pink mucosa having similar red streaks.

In hematoxylin-eosin sections, the mucosa in the fistulous segments was relatively well-preserved. In contrast, the rest of the small intestine had changes that follow exposure to radiation(2). These include mucosal erosions with hemorrhage, reduction in over-all thickness of mucosa, attenuated or absent villi, an inflamed, congested lamina propria, and greater distention of mucosal glands, with stasis of mucus (Fig. 2). In both the fistulous and the non-fistulous small intestine, the usual post-radiation atypias and abnormalities of intestinal epithelial cells were observed, of comparable degree in each location(1).

Discussion. This experiment suggests that the intestinal content exacerbates radiation injury of the small intestine, just as it ag-

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