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Effects of Multiplicity of Poliovirus Infection of HeLa Cells Upon Regulation of DNA Synthesis.* (31901)

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Infection of HeLa cells with poliovirus can either stimulate or inhibit the rate of DNA synthesis(1,2). The effects of virus are determined in part by environmental factors (2), in part by an intrinsic programming of viral genetic information(3), and in part by the multiplicity of infection. These are interrelated variables.

It has been suggested that poliovirus inhibition of DNA synthesis is a two-part process: First, a development, after the third hour of infection, of a potential for inhibition (possibly a messenger RNA) and second, the expression of this potential in the form of protein synthesis. The latter is sensitive to the concentration of certain amino acids in the medium whereas the former is not(3). Thus the amino acid composition of the culture medium which affects the rate of DNA synthesis in the uninfected cell(2), influences as well the viral effects upon this synthesis in infected cells. The concentrations of arginine or histidine in the medium required for viral multiplication, for viral inhibition of DNA synthesis and for normal rates of DNA synthesis in uninfected cells, increase in the order listed(2).

This paper is concerned with how the multiplicity of infection interrelates with the stage of infection and amino acid composition of the culture medium in the regulation of DNA synthesis in poliovirus infected HeLa cells.

Materials and methods. Cells. HeLa cells were grown in monolayers at 37°C with Eagle's basal medium(4), twice concentrated, and 10% calf serum and passaged every 7 days. The cells were grown in Roux bottles for experiments which involved isolation of DNA for chemical analysis and in 2 ounce prescription bottles for studies of viral replication. During the experimental period, the medium was replaced with one modified with regard to serum and amino acid content as described in specific experiments. Periodically, cells and fluid were inoculated into special media to ensure they were free of mycoplasma and bacteria(5).

Virus. The Mahoney strain of type 1 poliovirus was passaged routinely in HeLa cells, washed and concentrated by centrifugation and used as a suspension in phosphate buffered saline. The virus was assayed, using a plaque assay, on monolayers of HeLa cells in 2 ounce prescription bottles and concentration expressed in plaque forming units.

Deoxyribonucleic acid. DNA was extracted by the method of Colter and associates(6), isolated as described by Martinez-Segovia *et al*(7), and quantitatively determined by the method of Burton(8).

Radioactivity. For measurement of radioactivity, 0.1 to 0.5 ml of sample was added

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to an appropriate volume of scintillation fluid to give a volume of 10 ml and counted 10 minutes in an automatic Ansitron Model 1300 liquid scintillation counter.

Sources. Guanidine hydrochloride, Eastman Organic Chemicals, Distillation Products Industries; H^3-thymidine monophosphate 1.8 c/mmole, Schwarz BioResearch; 2-dimethylaminoethanol, Matheson Coleman and Bell, Division of Matheson Co.

Experimental and results. The conditions employed in these experiments are the same as in previous related studies(2,3). Under the cultural conditions used, inhibition of DNA synthesis occurs in HeLa cells only after three hours of infection with poliovirus. Hence the effects upon DNA synthesis of varying multiplicities of exposure of cells to virus were determined in the intervals of 1 to 2 and 4 to 5 hours after infection. The loss of infectious virus from the medium at the end of the 2-hour interval was found to be, by plaque assay, in each instance approximately 60%, regardless of the amount of virus added initially. The multiplicity of infection thus may be estimated as 60% of the exposure multiplicities which are recorded for the various experiments. This estimation has been used by others for similar considerations(9). However, as discussed previously, the number of cells infected is not related by a simple Poisson distribution to the disappearance of viral infectivity from the culture medium(10).

The following experiments which relate multiplicity of exposure, presence or absence of histidine or guanidine and the rate of DNA synthesis are essentially of a single design. The growth medium was removed from Roux bottles containing monolayers of HeLa cells and replaced with Eagle's medium (2× concentrated) from which histidine was deleted and supplemented with 1 percent calf serum and 4 μg/ml of each of the deoxyribonucleotides of DNA. The rationale for using the deoxyribonucleotides was discussed previously(2). After 16 hours at 37°C, poliovirus and guanidine were added to appropriate cultures, as indicated in the individual experiments. Those cultures containing histidine received it 16 hours prior to virus addition. H^3-thymidylate was added at the various times indicated and the experiments were terminated one hour after addition of isotope. DNA was isolated and the incorporation of thymidylate determined.

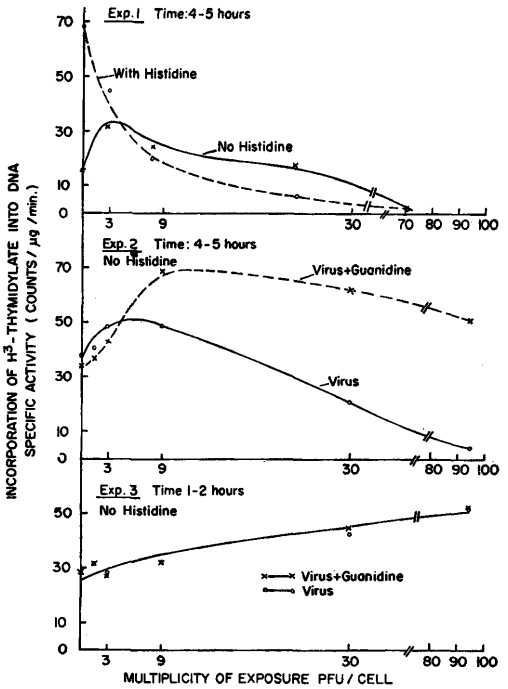

Fig. 1. Effect of the multiplicity of poliovirus infection of HeLa cells upon rate of DNA synthesis in presence and absence of either histidine (50 μg/ml) or guanidine (75 μg/ml) or both.

Effect of histidine on multiplicity dependent DNA synthesis. In Exp. 1, Fig. 1, effects of multiplicity of infection upon DNA synthesis in the 4-5-hour interval are compared in cells maintained on regular medium with those in medium from which histidine was deleted 16 hours prior to infection and during the course of infection.

With the complete medium, exposure multiplicities of 3 produced a partial inhibition of DNA synthesis which increased progressively as the multiplicity reached 71. It is unlikely that this multiplicity dependent inhibition can be accounted for in terms of the fraction of cells infected since it remains observable throughout the range between 24 and 71 PFU/cell. Rather, it would appear that the multiplicity of infection of an individual cell affects the degree of inhibition

at this particular time interval.

With medium deleted of histidine, the dependence of DNA inhibition upon multiplicity of infection was more clearly evident, as it occurred only with multiplicities greater than 24. However, multiplicities under 24 showed a stimulation of DNA synthesis above the rate observed in the absence of infection. The stimulated rate was still less than the maximal rate observed with complete medium but with a multiplicity of 3 it approaches that of the culture with complete medium of the same multiplicity of infection. With intermediate multiplicities, the rate of DNA synthess may result from an imposition of a degree of inhibition upon this stimulation.

Effect of guanidine on multiplicity dependent DNA synthesis. To evaluate independently the role of the multiplicity of infection upon these two simultaneous viral effects, the rate of DNA synthesis was determined as a function of the multiplicity of exposure in the 4-5-hour interval in HeLa cells (all cultured in the absence of histidine) with and without addition of guanidine at the time of infection. Previously it was found that guanidine not only inhibited the multiplication of the virus but also prevented virus-induced inhibition of DNA synthesis(2).

In the presence of guanidine (Fig. 1, Exp. 2), inhibition of DNA synthesis did not occur even with high multiplicities of infection. The slight drop in the rate with 94 PFU/cell is probably associated with an observed inability to block the multiplication of high concentrations of poliovirus with guanidine, possibly due to the presence of resistant mutants. The degree of stimulation was multiplicity-related, at least in the range of 1.5 to 9, and approached the maximal rate (70 counts/μg/DNA) for these cells, *i.e.*, the rate in the presence of histidine (Fig. 1, Exp. 1).

If the effect of guanidine is merely to prevent the inhibitory phenomenon and thus allow an uncomplicated view of the stimulatory phenomenon, then guanidine should not affect the rate of DNA synthesis observed in the 1-2-hour interval of infection since the inhibition is naturally programmed to occur after the third hour. The correctness of this view is supported by the data of Exp. 3, Fig. 1, where the rate of DNA synthesis is plotted as a function of the multiplicity of exposure of cultures with and without guanidine. In these cultures (from which histidine was deleted) at the 1-2-hour interval, there was a clear multiplicity dependent stimulation of DNA synthesis at all multiplicities tested. The maximum stimulation obtained with 94 PFU/cell was twice the value of the uninfected culture. There was no observed effect of guanidine under these conditions.

Relation of stage of infection to multiplicity dependent DNA synthesis. High multiplicities of infection in the absence of histidine stimulated the rate of DNA synthesis in the early stages of infection (Exp. 3, Fig. 1), but later inhibited it (Exp. 2, Fig. 1). This is clearly shown in Fig. 2 where in a similar type of experiment, DNA synthesis was determined at intervals during the first 5 hours of infection. Cultures with and without guanidine, infected with a single multiplicity (79 PFU/cell) were compared with corresponding control cultures which contained no guanidine. All were depleted of histidine. The inhibition is dependent upon viral development while the stimulation is not, hence high concentrations of virus in the presence of guanidine stimulate DNA synthesis even in the 4-5-hour interval.

Reversal of effect of guanidine with dimethylaminoethanol. The basis of the viral inhibitory activity of guanidine is not established; however, several compounds have been found to antagonize its action. Dimethylaminoethanol has been reported to be one of the most effective(11). As shown previously, and verified in Table I, it will prevent completely the inhibition of viral multiplication (as indicated by plaque formation) by more than minimal inhibitory levels of guanidine. In the present experiments, plaque formation is completely prevented by 50 μg/ml guanidine but not by 25 μg/ml. In all experiments, 75 μg/ml of guanidine were employed. The action of this concentration on plaque formation was completely reversed by 90 μg/ml of dimethylaminoethanol. To learn whether the action of guanidine in preventing virus-induced inhibition of DNA synthesis could be differentiated from the action upon viral replication, a reversal of the former

TABLE I. Comparison of Sensitivity of Viral Replication and Virus-Induced Inhibition of DNA Synthesis to Guanidine and Dimethylaminoethanol (DMAE).

Virus*	Additions: Guanidine†	Additions: DMAE†	Incorporation H^3-thymidylate, counts/μg/DNA‡	Viral replication, No. plaques§: No. 1	No. 2	No. 3
—	—	—	22.0	—	—	—
—	—	+	23.8	—	—	—
+	—	—	1.71	47.5	53.5	55.5
+	—	+	3.41	46.5	51.0	65.0
+	+	—	20.2	.0	.0	.0
+	+	+	5.90	45.0	23.0	54.0

* Virus inoculated into cell culture in Roux bottles was 3.03×10^9 plaque forming units (PFU).

† Concentrations of guanidine and dimethylaminoethanol were 75 μg/ml and 90 μg/ml, respectively.

‡ Incorporation of H^3-thymidylate into DNA was determined in the interval 4-5 hr after infection.

§ Viral replication measured in terms of average number of plaques formed in 3 cultures which received indicated additions and inoculated with approximately 50 PFU. Data from 3 separate experiments.

action with dimethylaminoethanol was attempted. Monolayer cultures of HeLa cells in Roux bottles under the conditions of the previous experiments were infected with a multiplicity of 60 PFU/cell. At the time of infection, the cultures were supplemented with guanidine and dimethylaminoethanol alone and in combinations as indicated in Table I. Isotopically labeled thymidylate was added at the fourth hour and the experiment terminated on the fifth. Dimethylaminoethanol when added alone was not inhibitory. A small stimulation, 9%, was actually observed. Similarly, when present in a virus-inhibited culture, there was a small stimulation which, however, was a doubling of the low residual rate of DNA synthesis. In contrast, addition of dimethylaminoethanol to an infected culture containing guanidine reduced the rate of DNA synthesis from 20.2 count/μg/DNA to 5.9. It thus appears that the dimethylaminoethanol supplies a deficiency essential to both viral replication and the DNA inhibitory mechanism.

Discussion. The rate of DNA synthesis in HeLa cells which have been depleted of a single amino acid, as histidine, can be stimulated by returning the amino acid to the medium or by infecting the cell with poliovirus(2,3).

Consistent with the experimental observations is the proposal that a primary effect of poliovirus upon some cellular structure makes amino acids available and, further, the degree of the effect is dependent upon the multiplicity of infection. In cells depleted of histidine, the increased availability of amino acids would be reflected as a stimulation in the rate of DNA synthesis in the early stages of infection or even later if guanidine were present to prevent virus-induced inhibition of DNA synthesis (Fig. 2). The considerable viral replication which occurs in media deficient in amino acids essential for that replication(2,12) would also be accounted for by increased amino acid availability. Further, since the concentration of amino acid needed for virus-induced inhibition of DNA synthesis is greater than that for stimulation of DNA synthesis(2), inhibition would occur

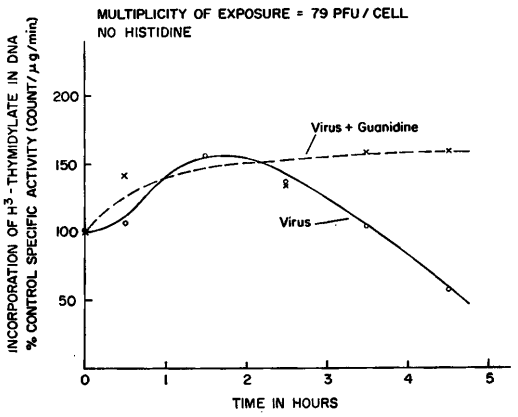

Fig. 2. Effect of a high multiplicity of infection of HeLa cells by poliovirus upon rate of DNA synthesis in presenc and absence of guanidine (75 μg/ml) determined at intervals during the first 5 hours of infection.

late in the infection of amino acid depleted cells only with relatively high multiplicities of infection which would produce a maximal primary effect (Exp. 2, Fig. 1).

Since the stimulatory effect can be observed in the first hour of infection, is multiplicity dependent, and occurs in the presence of guanidine (an inhibitor purported(13) to interfere with synthesis of virus-induced early proteins), the primary viral effect would appear to be an interaction between the parental virion and some cellular structure.

It would seem unlikely that the teleological purpose of such an effect would be to induce a maximal rate of cellular DNA synthesis. Rather the synthesis may be incidental to some other activity essential to viral replication. By the same token, it cannot be assumed that virus-induced inhibition of DNA synthesis is a process directed primarily and exclusively to the prevention of DNA replication, particularly *in situ* in motor neurons.

Summary. The rate of DNA synthesis in HeLa cells cultivated in medium without histidine was stimulated in the early stages of infection with poliovirus and could be inhibited in the late stages. The degree of each effect increased with increasing multiplicities of infection. The inhibition, but not the stimulation, was prevented with guanidine. The effect of guanidine was blocked by dimethylaminoethanol. It is proposed that both viral effects upon DNA synthesis which are dependent upon the multiplicity of infection are indicators of one primary interaction of the parental virion with some cell structure.

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Toxic Effects of Various Penicillins and Cephalosporin Derivatives On Human Amnion Cell Cultures.* (31902)

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The penicillins and available cephalosporin derivatives are, with rare exception, virtually non-toxic for man and animals, even when administered in large doses. However, high concentrations of benzylpenicillin and low ones of cephalothin damage cultured mouse embryo and human amnion cells(1). The purposes of the present study were (a) to study a number of penicillin and cephalosporin C congeners that have not been examined before, (b) to determine the effect of degradation products of these compounds, and (c) to ascertain the activity of combinations of chlortetracycline with the other drugs on cultures of human amnion cells.

Methods. The penicillin compounds studied were 6-aminopenicillanic acid, potassium phenethicillin, sodium methicillin, sodium

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