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Viral Inhibitors of Biological Origin. II. A Viral Inhibitory Factor Obtained from *E. coli* 0111 and Inhibition of Viral Replication by Nucleic Acid Derivatives.* (29310)

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Suppression of viral multiplication by crude materials derived from a variety of sonically disrupted bacteria has been described(1,2). The inhibitory material from each bacterial species tested suppressed certain viruses without affecting the multiplication of others. Thus, while arboviruses are inhibited by material derived from an unclassified Corynebacterium, ECHO 11 is suppressed by material from *Corynebacterium diphtheriae* (both non-toxigenic and intermedius strains) and vaccinia is suppressed by material derived from various *Escherichia coli* serotypes and *Salmonella typhimurium*. The present report is concerned with further characterization of the material from *E. coli* 0111, using the multiplication of vaccinia virus in primary human amnion cell cultures as an indicator system. Preliminary observations concerning viral inhibition by nucleic acid derivatives are also included in this report.

Preparation. An inoculum of *E. coli* 0111, obtained from Dr. J. G. Michael, was grown in one liter of Bacto nutrient broth for 18 hours. The entire culture was used to prime a Biogen containing 40 liters of medium consisting of 7 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g sodium citrate. 2 H_2O, 1 g $(NH_4)_2SO_4$, 0.12 g $MgSO_4$, and 5 g glucose in each liter of water. After 24 hours' growth at 37°C the culture was centrifuged and dried at room temperature. Sixty g of dried *E. coli* 0111 were obtained in this manner. Acetone soluble substances were removed from the dried culture by 3 extractions with 600 ml of acetone. Following this the bacteria were redried at room temperature. The acetone extracted bacteria were taken up in 100 ml of water and the insoluble residue was discarded. The water soluble material was dialyzed against running tap water for 24 hours. The dialysand was concentrated by lyophilization and then dissolved in 100 ml of distilled water. Microsomal elements were discarded after being sedimented by centrifugation at 35,000 rpm for 1 hour in the #40 head of a Model L ultracentrifuge. The final material was separated by further centrifugation of the supernatant fluid at 35,000 rpm for 3 hours and collection of the viscous green fluid in the lower halves of the centrifuge tubes. Lyophilization of an aliquot of this collected fluid enabled the determination that 5.5 g of material were obtained from the initial 60 g of dried *E. coli*.

Physicochemical properties. The inhibitory activity against vaccinia virus was destroyed by heating at 100°C for 5 minutes but was not affected by heating at 56°C for one hour and at 37°C for 3 hours. The activity was lost following dialysis against a citric acid disodium phosphate buffer at pH 2.3. Treatment of the active fraction with either DNAase or RNAase at a final concentration of 1 μg/ml for 3 hours at 37°C did not alter the viral inhibitory effect. The activity was destroyed by exposure to twice crystallized trypsin at a final concentration of 0.12% for 3 hours at 37°C. These findings are consistent with the characterization of the original crude preparations of the soluble ma-

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TABLE I. I^{131} Uptake in Cells.

	Corrected counts/min/tube
Control cells	3112
Cells + BIF (1:80 dilution in BAF medium)	90
Cells + vaccinia	4154
Cells + vaccinia + BIF (1:80 dilution in BAF medium)	112

terial from sonically disrupted bacteria. The inhibitory activity could be precipitated by ammonium sulfate saturation at 50-70%. Ultraviolet absorption of the final prepared material at 260/280 mμ yielded a ratio of 2, indicating the presence of a large amount of nucleic acid in this material. Using the Ouchterlony technique† a cell wall (O) antigen was found in the concentrated material which gave a single band of precipitation identical with that obtained with whole bacteria.

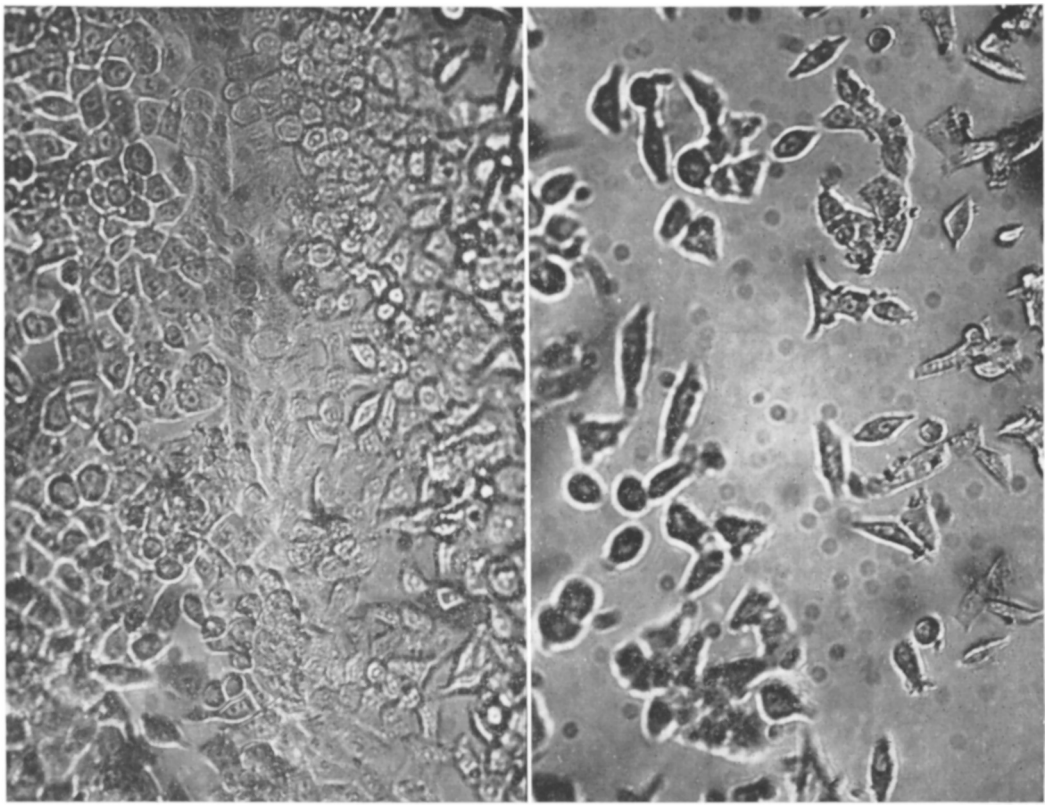
*Cellular I*DU absorption.* It was previously shown that the bacterial interference factor (BIF) affected the intracellular replication of vaccinia, herpes simplex and adenovirus type 2, so that little or no infective virus was produced(2). In an attempt to ascertain whether viral DNA synthesis was suppressed by the BIF, 0.5 μc of I^{131} labelled 5-iodo-2-deoxyuridine (I*DU), kindly supplied by Dr. W. L. Hughes, Brookhaven National Laboratories, were added to the bovine amniotic fluid (BAF) medium(3) in each of duplicate tubes containing uninfected primary human amnion cells, cells infected with 100 $TCID_{50}$ of vaccinia virus 24 hours previously, cells treated with BIF 24 hours previously, and cells both infected with 100 $TCID_{50}$ of vaccinia and treated with BIF 24 hours previously. After 24 hours' incubation each cell culture was rapidly washed 10 times with BAF medium so that the final wash contained no detectable I^{131}. The I^{131} in the cell cultures was then assayed in a well-type scintillation counter. The I^{131} uptake by the cells is shown in Table I. The fraction containing the viral inhibitory activity suppressed the uptake of I*DU into the cells whether or not the virus was present. The suppression of I*DU uptake by BIF treated cells was confirmed by repeating the experiment. BIF did not suppress the cellular uptake of C^{14} labelled glycine, leucine, valine or adenine. The viability of the BIF treated cells was shown by their ability to support vaccinia virus replication when the BIF was washed out with fresh BAF medium.

Cell multiplication. Since the radioactive I*DU experiments suggested that BIF interfered with the uptake of a DNA precursor in treated cells, experiments were undertaken to determine whether BIF would affect the multiplication of cells in tissue culture. When suspensions of primary human amnion cells (300,000 cells/ml) and HeLa cells (30,000 cells/ml) were placed in culture tubes, addition of BIF (diluted 1:80 in BAF medium) suppressed the multiplication of cells as shown by the failure of the cells to grow into a confluent sheet. Fig. 1 shows BIF treated HeLa cells and untreated controls. Table II gives cell counts for individual tubes which had been inoculated with 30,000 HeLa cells. The cells in the BIF treated tubes did not multiply as well and the number of viable cells in the BIF treated tubes actually decreased while untreated control cells continued to multiply. It is of interest that BIF treated cells which were washed with Eagle's medium on the third day thereafter proceeded to grow to a confluent sheet of cells in contrast to the cells which remained in contact with BIF. There was no histologic evidence of toxicity when BIF was added to fully confluent sheets of HeLa cells in the same con-

TABLE II. Cell Counts for HeLa Cells. Initial inoculum 30,000 cells.

	Days after cell inoculation			
	1	2	3	5
Exp 1				
BIF 1:80		92,500	130,000	20,000
Control		180,000	250,000	200,000
Exp 2				
BIF 1:20		92,500	40,000	10,000
BIF 1:40		90,000	60,000	30,000
Control		132,000	152,000	127,000
Exp 3				
BIF 1:80	60,000	40,000		
Control	95,000	110,000		

† The antisera against *E. coli* 0111 (AC and BC) were obtained from Dr. John Winn, Laboratory Branch, U.S.P.H.S. Communicable Disease Center.



Control Cells Grown for 2 Days in BAF Medium

Cells Grown for 2 Days in BIF Diluted 1:80 with BAF Medium

FIG. 1. Effect of Coli 0111 BIF on growth of HeLa cells.

centration that suppressed cell multiplication.

Nucleic acid derivatives. Since the uptake of I*DU by primary human amnion cells in culture was almost completely suppressed by the viral inhibitory substance derived from Coli 0111, it was thought that this material might act by interfering with the availability of thymidine for incorporation into viral DNA. However, it was found that addition of thymidine (1 mg/ml) to cultures treated with the bacterial factor did not reverse the inhibition of the cytopathic effects (CPE) of vaccinia virus. Surprisingly, inhibition of viral CPE was demonstrated in control cultures containing thymidine and no bacterial factor. This unexpected inhibition of viral CPE by thymidine led to preliminary experiments on the capacity of various nucleic acid derivatives to inhibit viral replication.

Primary human amnion cell cultures were incubated for 24 hours at 37°C with BAF tissue culture media to which nucleic acid derivative (NAD) had been added prior to inoculation with 100 $TCID_{50}$ of virus. Control cultures incubated with BAF media alone were inoculated with the same dose of virus. In routine tests each NAD was used at a concentration of 1 mg/ml of BAF medium. The pH of each preparation was adjusted up to 7.2 by addition of sodium bicarbonate. Vaccinia, a DNA virus, and Sindbis, an RNA virus, were selected as test viruses.

Thymidine in repeated experiments inhibited the CPE of vaccinia virus when used in concentration of 1 mg/ml. Less inhibition of CPE was demonstrated when thymidine was used in a concentration of 0.5 mg/ml. Thymidylic acid, thymidine triphosphate, deoxyadenosine and deoxy adenylic acid were all effective in inhibiting the CPE of vaccinia virus. In contrast, no inhibition was found when adenosine, adenylic acid, deoxycytidine

TABLE III. Extent of Cytopathic Changes Seen in Treated and Untreated Cultures.

	Day after inoculation with vaccinia virus												
	2	3	4	5	6	7	8	9	10	11	12	13	14
Virus control	1+	2+	2+	3+	3+	3+	3+	3+	4+				
Thymidine triphosphate	0	0	0	0	0	0	0	0	0	0	0	0	0
Virus control	2+	4+											
Thymidine	0	1+	1+	1+	1+	2+							
Thymidylic	0	1+	1+	1+	2+	2+							
Virus control	2+	3+	3+	3+	3+	3+	3+	3+	4+				
Deoxyadenosine	0	0	±	1+	2+	3+	3+	3+	4+				
Deoxyadenylic	0	0	0	±	1+	1+	2+	2+	2+				

1 = 25% of cells affected. 2 = 50% of cells affected. 3 = 75% of cells affected. 4 = 100% of cells affected.

Nucleic acid derivatives at a concentration of 1 mg/ml of BAF media.

triphosphate, and deoxycytidylic acid were used. The degree of CPE found in virus control cultures and those treated with NAD is shown in Table III. Continued metabolism of cells treated with nucleic acid derivatives was demonstrated by continued acid production in all cases. Histologic evidence of definite cell toxicity was lacking except for the cells treated with thymidine triphosphate which had a slightly pyknotic appearance.

Medium was harvested from vaccinia infected cell cultures both with and without NAD in the medium. The titers of vaccinia virus in these media are shown in Table IV. Back titration of medium from cells treated with thymidine triphosphate yielded less infectious virus than medium from the respective virus controls. The lower titer of vaccinia virus found when the medium of thymidine treated cells is compared to that of the virus control is of questionable significance. A single experiment with deoxyadenosine did not show such a difference.

CPE of Sindbis virus in 3-4 week old primary human amnion cells was repeatedly suppressed by deoxyadenosine. In one experiment the virus controls showed CPE 2 days after viral inoculation which progressed to involve all of the virus control cells by the fourth day after viral inoculation. Cell cultures from the same human amnion that had been treated with deoxyadenosine prior to viral inoculation did not show any CPE during the observation period of 15 days. Deoxyadenosine did not produce toxic effects in the treated cells judging both by histologic appearance and continued acid production. In other experiments the deoxyadenosine treated cultures showed some degree of CPE although significantly less than that seen in controls. In one experiment, titration of tissue culture media obtained 3 days after viral inoculation revealed a titer of $10^{-6.5}$ for the virus control and no detectable virus in the deoxyadenosine treated cultures. The CPE of Sindbis virus in chick embryo fibroblasts was also inhibited by deoxyadenosine. Thymidine, thymidine triphosphate, and deoxycytidine triphosphate had no appreciable effect in inhibiting the CPE of Sindbis virus in aged amnion cells.

TABLE IV. Titration of Infectious Vaccinia Virus from Virus Controls and Treated Cultures.

Days after viral inoculation	Virus control, titers $\log_{10}$/0.1 ml	Nucleic acid derivative, titers $\log_{10}$/0.1 ml	
3	3.5	Thymidine triphosphate	2.3
10	3.5	*Idem*	1.0
5	3.5	Thymidine	2.8
4	3.5	Deoxyadenosine	3.5

Nucleic acid derivatives at a concentration of 1 mg/ml of BAF media.

It was considered that the suppression of Sindbis and vaccinia CPE might have resulted from interferon production induced in the cells by NAD. Evidence against this hypothesis was obtained by incubating separate amnion cell cultures at 37°C for 2 days with aliquots of medium containing a single nucleic acid derivative (deoxyadenosine, deoxyadenylic acid, and thymidine). The medium with the nucleic acid derivatives was then removed and the cells were washed with medium 199 before incubating them with BAF

medium for an additional 2 days at 37°. The BAF medium was then removed from the treated cells and, when tested, did not inhibit the cytopathic effects of Sindbis virus in amnion cell cultures and thus did not contain interferon.

Discussion and summary. The experimental results show 3 different effects of *E. coli* BIF, namely, suppression of the intracellular stages of vaccinia replication, suppression of the multiplication of human amnion and HeLa cells, and suppression of the cellular uptake of I*DU. Although it is possible that these 3 actions are unrelated, they can be theoretically explained on the basis of interference with cellular DNA metabolism. Further work is necessary to explore this unifying hypothesis. It should be borne in mind, however, that a net gain in cellular DNA synthesized is probably not essential for synthesis of viral DNA.

Inhibition of viral CPE and infectivity in cell cultures can be elicited with certain nucleic acid derivatives. Sindbis virus could be completely suppressed when deoxyadenosine was present in the media at a concentration of 1 mg/ml. Suppression of vaccinia was achieved with the same amount of thymidine triphosphate. Thus a certain degree of specificity of antiviral activity was demonstrated.

Suppression of cellular and bacterial metabolism by nucleic acid derivatives has been reported previously. Lark(4) found that, in synchronously dividing cultures of *Alkaligenes fecalis*, DNA synthesis could be selectively blocked by deoxyadenosine at a concentration of 1 μ mol/ml. The effect could be reversed by adenosine. Klenow(5) showed that deoxyadenosine at a concentration of 1 μ mol/ml inhibits the incorporation of C_{14} and P^{32} orthophosphate into the DNA of Ehrlich Ascites cells *in vitro*. Morris and Fischer(6) demonstrated that the replication of murine neoplastic cells could be inhibited by thymidine, deoxyadenosine, or deoxyguanosine. They were able to reverse this inhibition with deoxycytidine. Reichard, Canellakis, and Canellakis(7) have described inhibition of the enzyme system which converts cytidine monophosphate to deoxycytidine monophosphate by the triphosphates of thymidine, deoxyadenosine, and deoxyguanosine.

Further studies are necessary for learning the specific manner by which the nucleic acid derivatives inhibit viral replication. Inhibition of viral replication by the nucleic acid derivatives studied may result from some interference with cellular metabolic activity as described by the investigators mentioned above, or it may be brought about by still undefined effects on the metabolic activity of host cells. Interference with every stage of viral replication has to be considered.

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