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Inhibitory Effect of Mononucleotides on Virus Plaque Formation.* (30083)

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Ribonucleic acids obtained from foreign cells will often induce formation of interferon in cells treated with such preparations(2). It was the original intent of this study to determine the minimal molecular weight of ribonucleic acid preparations required to induce interferon. However, it was found that when yeast ribonucleic acid preparations were completely hydrolyzed, the resultant hydrolysates were more inhibitory to virus plaque formation than the original RNA preparations. This observation led to the present study. Recently, it has been shown that mononucleotides would induce small amounts of interferon in chick cell systems(3) and would also induce resistance to influenza virus in embryonated eggs or mice(4).

Materials and methods. Viruses. Vaccinia and chikungunya viruses were employed in the assays. Vaccinia virus was either the V-1 strain employed by Lindenmann and Gifford (5) or a similar strain(6), and was grown on the chorioallantoic membranes of 10- to 12-day-old chick embryos. Chikungunya virus was grown in the brains of newborn mice. Viruses were stored at —60°C in sealed glass capillaries or ampules.

Tissue cultures. Chick embryo tissue cultures were prepared essentially as described (5). Gey's, instead of Hanks' balanced salt solution was employed in both the growth and maintenance media. Proteose peptone, 0.1%, was routinely added to growth and maintenance media(6). For studies with mononucleotides, the yeast extract was omitted from the maintenance medium since it was thought to contain unknown amounts of these compounds. Omission of the yeast extract had no measurable effect on the plating efficiency of vaccinia virus nor on plaque size.

Virus assays. Vaccinia virus was assayed as previously reported(5) except for the slight change in medium composition and for a reduction in final volume from 2.5 to 2.0 ml. Chikungunya virus was assayed by plaque technique described by Ruiz-Gomez and Isaacs(7).

Compounds. Yeast nucleic acid was purchased from L. Light and Co., Ltd., Colnbrook, England. Mononucleotides were obtained from the same source or from the California Corporation for Biochemical Research, Los Angeles.

Results. Effect of yeast nucleic acid and alkaline hydrolyzed yeast nucleic acid on virus plaque formation. Yeast nucleic acid was hydrolyzed with 0.3 M potassium hydroxide for 18 hours at 37°C(8). It was then neutralized with 0.3 M hydrochloric acid and tested on chick cells to determine the effect on the plating efficiency of vaccinia and chikungunya viruses. Preparations were added with virus in the case of vaccinia virus assays and usually 1 hour before virus when chikungunya virus was employed. Chikungunya virus was allowed to adsorb for 1 hour before the agar overlay was added and the RNA or RNA hydrolysates were permitted to remain in the overlay. Yeast RNA preparations were usually dialyzed against balanced salt solutions before use, especially after the effect of mononucleotides became known. Results of one such experiment are

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TABLE I. Effect of Yeast RNA and RNA Hydrolysates Upon Plating Efficiency of Vaccinia and Chikungunya Viruses.

Treatment	Virus: Chikungunya Avg No. of plaques	Chikungunya % inhibition	Vaccinia Avg No. of plaques	Vaccinia % inhibition
Dialyzed RNA, 10 mg	19	51	160	38
Hydrolyzed RNA*	12	69	103	60
Controls	39	—	258	—

* Hydrolyzed for 18 hr with 0.3 M KOH and then neutralized with 0.3 M HCl. Equivalent amount of dialyzed preparation employed as for original RNA preparation.

shown in Table I. Other studies showed that yeast RNA had no effect on the rate of adsorption of vaccinia virus. The hydrolyzed preparations consistently inhibited plaque formation to a greater extent than the unhydrolyzed preparations. Chikungunya virus was usually inhibited to a greater extent than vaccinia virus. These findings are consistent with the greater sensitivity of chikungunya virus to interferon(9). However, the vaccinia assay was used in all subsequent studies because of the simplicity of the assay and the greater number of plaques that could be counted on equivalent monolayers. The inhibitory effect of the hydrolyzed preparations was completely lost upon dialysis against 20 volumes of balanced salt solution overnight indicating that small molecular weight compounds were responsible for the inhibition. Subsequent studies were thus made with mononucleotides.

Effect of mononucleotides on plating efficiency of vaccinia virus. The effect of the 4 mononucleotides which would be present in yeast RNA hydrolysates was tested individually for their effect on vaccinia virus plaque formation. Adenylic acid (AMP) was the most inhibitory of the 4 mononucleotides. In the initial experiments the other mononucleotides showed little or no effect on vaccinia virus replication. However, when yeast extract was omitted from the maintenance medium, the addition of these compounds also resulted in inhibition of plaque formation, but to a lesser extent than AMP. Since mononucleotides were probably present in the yeast extract, we considered that mixtures of the mononucleotides might be less inhibitory than the single compounds alone. This was subsequently tested. Table II shows the effect of the 4 mononucleotides on vaccinia virus plaque formation. For comparative purposes, the di- and tri-phosphates of the most inhibitory compound were also tested. Thymidine monophosphate was also included in this study as a representative of the other pyrimidine found in nucleic acids and also because of the finding that large quantities of thymidine inhibit DNA synthesis(10).

Reversal of inhibition of plaque formation by mixtures of mononucleotides. Mixtures of the mononucleotides were made and tested for their ability to inhibit vaccinia virus plaque formation. The results are shown in Table III. Various combinations of the mononucleotides were shown to be less inhibitory than other combinations. Marked reversal of the inhibitory effect was observed when all 4 nucleotides were present in equimolar concentrations.

Discussion. Nucleic acids, if foreign to the host cell, stimulate interferon production(2). One group of workers(3,4,11) has reported that DNA and RNA from a variety of sources as well as certain nucleic acid deriva-

TABLE II. Inhibition of Vaccinia Virus Plaque Formation by Nucleic Acid Derivatives.

Compound	PDD_{50} dose,* micromoles
Adenosine monophosphate (AMP)	1.1
" diphosphate (ADP)	2.3
" triphosphate (ATP)	4.0
Cytidine monophosphate (CMP)	8.1
Uridine monophosphate (UMP)	3.4
Guanosine monophosphate (GMP)	—†
Thymidine monophosphate (TMP)	21.4

* PDD_{50} dose is that amount of compound which depressed the plaque count to 50% of controls, or 50% plaque depressing dose.

† PDD_{50} not calculated since saturated solutions (1.1 micromole/ml) resulted in 45% inhibition.

tives would induce interferon in chick embryo fibroblast cultures. Hydrolyzed RNA as well as mononucleotides protected mice against influenza and EMC infection. Adenine nucleotides were the most effective of the compounds tested by that group. In the present study, yeast RNA and alkaline hydrolysates of yeast RNA were found to inhibit both DNA (vaccinia) and RNA (chikungunya) containing viruses. We have found small amounts of interferon-like substances in cell cultures treated with inhibitory doses of mononucleotides. The amount of inhibitory substance produced was variable and never sufficient to explain the inhibition shown by the compounds(1). In addition, control cultures also show inhibitory substances to vaccinia virus plaque formation when employed at high concentrations which we have interpreted as due to a normal level of interferon. Interferon production does not seem to be responsible for the inhibition reported here. It is possible that interferon induction is the mechanism for the inhibition but that the interferon remains intracellular and therefore not detectable in amounts needed to explain the inhibition.

TABLE III. Effect of Monocleotides and Mixtures of Monocleotides on Vaccinia Virus Plaque Formation.

Compounds and mixtures	mg/ml	Avg No. of plaques* Exp No. 1	2	3	% inhibition Exp No. 1	2	3
AMP	.5	42	7	12	84	95	93
	.25	—‡	16	—	—	89	—
	.125	—	112	—	—	23	—
UMP	1.0	—	51	—	—	65	—
	.5	—	86	107	—	41	40
	.25	—	129	—	—	12	—
CMP	1.0	—	97	—	—	34	—
	.5	—	126	151	—	14	15
	.25	—	138	—	—	5	—
GMP	1.0	—	82	—	—	44	—
	.5	—	80	84	—	45	53
	.25†	—	80	—	—	45	—
AMP+	.5						
GMP+	.5						
CMP+	.5						
UMP	.5	231	77	126	13	47	29
AMP+	.5						
CMP+	.5						
UMP	.5	201	62	155	24	58	13
AMP+	.5						
GMP+	.5						
UMP	.5	—	45	109	—	69	39
AMP+	.5						
GMP	.5	—	38	33	—	74	81
AMP+	.5						
CMP	.5	93	35	60	65	76	66
AMP+	.5						
UMP	.5	77	28	20	71	81	89
AMP+	.5						
CMP+	.5						
GMP	.5	—	12	29	—	92	84
GMP+	.5						
CMP+	.5						
UMP	.5	—	59	—	—	60	—
Controls	—	265	146	178	—	—	—

* Average of 4 bottles.
† Represents saturated solution at 37°C.
‡ — = not done.

The nucleotides with purine bases seem to be more inhibitory than those with pyrimidine bases. CMP seemed to reverse the inhibition of vaccinia virus plaque formation by AMP more than the other 2 mononucleotides although the differences were not great. Reversal of the inhibition by AMP by simultaneous addition of UMP and CMP was almost as great as when GMP was also present. In one case, the reversal was greater in the absence of GMP. This observation is complicated, however, by the low solubility of GMP.

Deoxyadenosine has been reported to apparently inhibit the synthesis of DNA in Ehrlich ascites tumor cells(12,13), in chick embryo minces(14,15) and in the L-M strain of mouse fibroblasts(16). Xeros(10) has shown that thymidine, deoxyadenosine, and deoxyguanosine blocked mitosis in Chang appendix cells and that the block could be reversed by washing. The thymidine inhibition could be reversed by addition of deoxycytidine. This may be explained by the findings of Reichard *et al*(15) that deoxycytidylate synthesis was strongly inhibited by thymidine and the triphosphates of deoxyadenosine and deoxyguanosine. Inhibition apparently occurred during the transformation of the ribosyl to the deoxyribosyl compound. These authors suggest that these phenomena may be components of a homeostatic mechanism governing the control of DNA synthesis. Langer and Klenow(13) showed that deoxyadenosine caused pronounced inhibition

of the incorporation of formate into DNA of Ehrlich ascites cells and that the inhibition could be reversed by simultaneous addition of deoxyguanosine. The same authors showed that deoxyadenosine had no effect on DNA synthesis in the Yoshida ascites tumor cells indicating that the inhibition is not an universal phenomenon. Kit and Dubbs(16) showed that deoxyadenosine drastically inhibited the incorporation of thymidine-H^3 into the DNA of L-M suspension cultures and stated that the inhibition could be largely reversed by addition of deoxyguanosine. Vaccinia virus growth was delayed in deoxyadenosine treated cells. Vaccinia failed to enhance thymidine-H^3 uptake by cells treated with deoxyadenosine. It is possible that the results in the present study may be related to these findings since the deoxyribose and ribose containing compounds are interconvertible in the cell. Since interferon has been implicated in nucleotide inhibition of virus synthesis its mode of action may be involved in this type of control mechanism.

Summary. Alkaline hydrolysates of yeast ribonucleic acid were found to inhibit markedly plaque formation by vaccinia and chikungunya viruses in chick embryo fibroblast monolayers. Mononucleotides were subsequently implicated and shown to inhibit vaccinia virus plaque formation. Adenosine monophosphate was the most effective of the nucleotides tested in inhibiting vaccinia virus and the inhibition could be reversed with mixtures of the mononucleotides.

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Pulmonary Lymph: Demonstration of Its High Oxygen Tension Relative to Systemic Lymph.* (30084)

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Lymph is tissue fluid that has entered the lymphatic capillaries(1). Its respiratory gas composition, therefore, probably resembles that of the immediate tissue environment, barring significant changes along the course of the lymphatics. Bergofsky *et al* found low oxygen tension in systemic lymph sampled from the thoracic duct and its tributaries(2). Data on pulmonary lymph Po_2, not available so far, should reflect the influence of extensive and intimate contact with alveolar gas, and may relate to recently described morphologic(3) and biochemical(4) features of lung tissue. We report here the results of a study of pulmonary lymph oxygen tension and its

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