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Received September 19, 1963. P.S.E.B.M., 1964, v115.

A Virus Inhibitor in Pharyngeal Washings from Patients with Influenza.* (28867)

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Several investigators have recently suggested that interferon(1) plays a role in recovery from viral infection(2,3,4,5,6,7). Isaacs and Hitchcock demonstrated interferon in the lungs of mice experimentally infected with influenza virus(2) and correlated its presence with virus content and eventual recovery. Baron and Isaacs failed to find interferon in human lung tissue obtained from fatal cases of viral influenza(8) and speculated that its absence may have been "related to the fatal outcome." To the best of our knowledge, the presence of interferon or similar virus inhibitors has not been demonstrated in *patients* with viral infection.

Widespread A_2 influenza occurring in the winter of 1962-3 afforded the opportunity to look again for a virus inhibitor in human disease. The nasopharynx was chosen as a source of such an inhibitor for two reasons: 1) influenza virus multiplies in the human upper respiratory tract(9), and 2) a virus inhibitor had been previously demonstrated in pharyngeal washings obtained from a young boy with upper respiratory illness due to parainfluenza II virus infection(10).

This communication reports the presence of an inhibitor of Sindbis virus, exhibiting certain of the properties of an interferon, in pharyngeal washings from patients with A_2 influenza infection.

Methods and materials. Epidemic. Localized outbreaks of febrile respiratory illness occurred in New England early in 1963. Influenza A_2 virus was isolated from hospitalized university students in early February, and similar strains were recovered repeatedly during the next 4-6 weeks.

Thirteen patients with influenza were selected for inclusion in the present study on the basis of comparability of clinical specimens and severity of illness. This group was composed of 12 males and 1 female ranging from 17-34 years of age. For comparison, 5 patients with rubella (female, 17-23 years), 2 patients with infectious mononucleosis (female, 19 and 21 years), 5 patients with leu-

* This study was supported by grant from Nat. Inst. Health, U.S.P.H.S.

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kemia (5-18 years), 3 patients with mild upper respiratory disease (male, 30-34 years) and 12 normal adults (male and female, 22-37 years) were also studied.

Virus isolation. Pharyngeal washings (for virus isolation and inhibitor) were obtained by having each patient gargle 6 ml of Hanks' balanced salt solution for 10-15 seconds. Penicillin, 100 units/ml, streptomycin, 100 μg/ml and amphotericin B, 2 μg/ml, were added to the specimens shortly thereafter. For influenza virus isolation 0.2 ml of undiluted throat washings were inoculated into the allantoic cavity of 8-10 day old embryonated eggs. After 96 hours' incubation at 36-37°C, the allantoic fluid was harvested and tested for hemagglutination with 0.5% suspension of guinea pig or chicken erythrocytes. All strains of influenza virus were identified as A_2 influenza by hemagglutination inhibition with A_2/Japan/305/1957 antiserum,§ and the absence or low titer of inhibition by A/Pr8/antiserum.

Serological tests. Acute and 3-6 week convalescent plasmas or sera were obtained from all patients with influenza. For assay of hemagglutination inhibiting (HI) antibody all specimens were heat inactivated (56°C for 30 minutes) and treated with sodium periodate(11) for 18 hours at 4°C to reduce nonspecific inhibition. Four to 8 hemagglutinating units of a representative strain of influenza virus (current epidemic) were utilized in all tests. A 4-fold or greater rise in HI antibody titer from acute to convalescent specimens was considered diagnostic of influenza infection.

Assay of inhibitor. An aliquot of throat washings was centrifuged for one hour at 110,000 *g* in a Spinco model L ultracentrifuge to sediment influenza virus. The supernatant fluids were later tested and shown to have lost infectivity for embryonated eggs. Two-fold dilutions (in tissue culture nutrient medium) of all supernatant samples were incubated with primary cultures of human amnion cells prepared and maintained as previously described(12). As in the assay of human interferon(13), these and control cultures were "challenged" 24 hours later with approximately 100 $TCID_{50}$ of Sindbis virus. Cultures were considered to have demonstrated viral inhibition when there was less than 25% CPE, and 75% or more in control cultures. To determine the extent of inhibition of Sindbis virus multiplication aliquots of nutrient medium from representative cultures were titrated in primary cultures of chick embryo cells.

All acute phase pharyngeal washings inhibiting Sindbis virus were subsequently retested with the patients' convalescent phase specimens collected 3-6 weeks later.

Results. Twenty pharyngeal washings were obtained from 13 patients with clinical influenza 0-5 days after onset of illness. Specimens were collected on successive days from several of the patients. The clinical diagnosis was confirmed in all patients, either by isolation of influenza A_2 virus (9/13 patients) and/or demonstration of a 4-fold or greater rise in HI antibody (12/13 patients).

Inhibitor was demonstrated in 5 specimens collected from 4 patients (Table I). From 4 of these 5 specimens influenza virus was isolated. The 5 specimens containing inhibitor were stored at 4°C and retested with the convalescent specimen obtained 3-6 weeks later. No fall in titer of the inhibitor was observed. Inhibitor was not present in any of the convalescent throat washings.

Table II presents results of assays of the inhibiting capacity of pharyngeal washings as measured by reduction of cytopathic effect (CPE) and multiplication of Sindbis virus in human amnion cell cultures. Four of the 5 specimens effectively inhibited Sindbis virus even when diluted 8-fold. The end point dilution was not determined. In cultures exhibiting limitation of CPE, viral multiplication was reduced by a factor of 2-3 logs.

Pharyngeal washings were obtained from 12 normal adults and patients with illnesses other than influenza. This latter group was composed of 5 patients with rubella (0-2nd day of illness), 2 patients with infectious mononucleosis and B streptococcal pharyngi-

§ Microbiological Associates, Inc., Washington, D.C.

TABLE I. Incidence of Virus Inhibitor in Pharyngeal Washings from Patients with Influenza.

Day of illness	Total No. of specimens	Virus isolated	Specimens positive for both virus and virus inhibitor	Specimens negative for virus but positive for virus inhibitor
1	9	7	3	0
2	4	2	1	0
3	4	1	0	0
4	2	0	0	1*
5	1	0	0	0
Total	20	10	4	1

* A specimen obtained from this patient on the second day of illness was positive for both influenza virus and virus inhibitor.

tis, 5 patients with acute leukemia (2 in the acute phase and 3 in remission) and 3 patients with mild upper respiratory disease. In none was an inhibitor demonstrable.

Characterization of inhibitor. All pharyngeal washings were ultracentrifuged to sediment influenza virus. No infectious virus could be recovered from the supernatant fluids. Further evidence that influenza virus itself was not responsible for inhibition of Sindbis virus was obtained in the following manner: Primary cultures of amnion cells were inoculated with 1, 10 and 100 EID_{50} of 2nd or 3rd chick embryo-passaged influenza virus of the current A_2 strain. Twenty-four hours later these cultures and suitable control cultures were "challenged" with 100 $TCID_{50}$ of Sindbis virus. No interference was observed.

The inhibitor did not directly inactivate Sindbis virus. Three specimens containing inhibitor (Table II, specimens 1, 4 and 5) were incubated with approximately 500 and 50 $TCID_{50}$ of Sindbis virus for 1 hour at 37°C. Aliquots of the mixtures were inoculated into primary cultures of human amnion

TABLE II. Assay of Virus Inhibitor in Pharyngeal Washings from Patients with Influenza as Demonstrated in Human Amnion Cell Cultures Infected with Sindbis Virus.

		Dilution of throat washing					
		1:2		1:4		1:8	1:16
Patient	Specimen	CPE*	Titer†	CPE	Titer	CPE	CPE
A.L.	1. Acute (day 1)	±	4.5	++		+++	
	Convalescent (day 20)	++++	6.7	++++			
	Virus control	++++					
M.G.	2. Acute (day 1)	0		0	≤2.5	0	+
	Convalescent (day 21)	++++		++++	4.7		
	Virus control	++++					
F.L.	3. Acute (day 1)	0	3.7	0	4.5	±	
	Convalescent (day 35)	+++	6.7	+++	7.0		
	Virus control	+++	7.0	+++	7.0		
G.V.	4. Acute (day 2)	0	2.7	±	4.5	±	++
	Convalescent (day 21)	++++	5.5	++++	6.5		
	Virus control	++++					
	5. Acute (day 4)	0		0		0	
	Virus control	++++					
	Acute (day 4)			0			
	Acute with trypsin			++++			
	Virus control			++++			

* CPE due to Sindbis virus. 0 = No CPE. ± = Foci of CPE. + = 25% cell destruction. ++ = 50% cell destruction. +++ = 75% cell destruction. ++++ = 100% cell destruction.

† Titer of Sindbis virus in fluid from test cultures as assayed in primary cultures of chick embryo cells and expressed as log TCD_{50}/.1 ml.

cells. No inhibition of Sindbis virus CPE was observed.

Specimen 5 of patient G.V. containing inhibitor was retested at a dilution of 1:4 after pre-incubation with crystalline trypsin‖ for 1 hour at 37°C. The inhibitor was destroyed by this proteolytic enzyme (Table II).

Discussion. An inhibitor of Sindbis virus has been demonstrated in pharyngeal washings of 4 patients with A_2 influenza in the acute phase of illness but not in their convalescent phase specimens collected several weeks later. Inhibition did not depend upon direct interference by influenza virus since 1) all specimens were ultracentrifuged prior to assay and the supernate was shown to be noninfective for embryonated eggs; 2) inoculation of amnion cultures with A_2 influenza virus did not inhibit Sindbis virus; 3) under the same experimental conditions as employed here the infectivity of several other strains of influenza A virus had not been diminished by the concentration of trypsin(14), shown in this study to inactivate the inhibitor.

The small volume of material remaining after attempts to isolate virus, centrifugation, and assays for inhibiting activity did not permit full characterization of the inhibitor. Although the method employed to demonstrate its presence has proved unusually sensitive in the detection and measurement of interferon (13,15), it is impossible to state whether the inhibitor in throat washings is, in fact, interferon, particularly in view of the number of tissue extracts(16), bacterial and fungal components(17,18) which inhibit virus growth by acting on cell rather than directly on the virus. The properties of the inhibitor in pharyngeal washings, insofar as determined, are, however, consistent with its identification as an interferon.

It is of interest that this inhibitor was found in specimens from which influenza virus was usually recovered (4/5 instances, Table I). Although the limited number of specimens does not permit comment on its temporal appearance, inhibitor was most easily demonstrated in the first 2 days of illness.

‖ Obtained from Nutritional Biochemicals Corp., and resuspended in phosphate buffered saline (PBS) at a concentration of 500 γ/ml.

Summary. Pharyngeal washings were obtained from 13 patients in the acute phase of influenza and 3-6 weeks later. Five specimens (from 4 patients) collected at the onset of illness contained an inhibitor of Sindbis virus as assayed in primary cultures of human amnion cells. This inhibitor was found to act on the cultured cells rather than on Sindbis virus itself. It was distinct from influenza virus and was not sedimented by ultracentrifugation. It was inactivated by crystalline trypsin. This inhibitor was not demonstrable in specimens obtained from the same patients during convalescence, from normal adults nor from a limited number of patients with other diseases.

The authors are indebted to Dr. John F. Enders for helpful criticisms during these investigations and in preparation of this article. We gratefully acknowledge the excellent technical assistance of Miss Sally Edmunds; and we wish to thank Dr. Curtis Prout and the Medical Staff, Harvard University Health Service for cooperation in obtaining specimens.

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Received September 23, 1963. P.S.E.B.M., 1964, v115.

Effect of Thiazide Drugs on Renovascular Hypertension in Contrast to Their Effect on Essential Hypertension.* (28868)

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Drugs of the benzothiadiazine family have proven extremely useful over the past 5 years for lowering arterial pressure of patients with benign essential hypertension. At least 50% of patients with mild or moderate essential hypertension will get a substantial reduction in blood pressure with the use of the thiazide drugs alone. Even severe essential hypertension is commonly improved by thiazide drugs. Furthermore, thiazide drugs also have the property of potentiating the action of various other types of anti-hypertensive drugs. In a previous study we could find no change in the electrolyte composition of arteriolar walls under the influence of thiazide drugs (1). However, the secretory activity of the granular juxtaglomerular cells appeared to be enhanced by the drug(1), and Winer has shown that a renin-like substance appears in increased concentration in the plasma of human beings taking thiazides(2). Such findings are reminiscent of the effects of a low intake of sodium, and it seems quite likely that the thiazides lower the blood pressure of patients with essential hypertension through the same mechanisms that act in conjunction with a drastic reduction of dietary sodium (3).

We had noted previously that an extreme reduction in sodium intake was quite ineffective in preventing or alleviating the hypertension produced in rats by narrowing one renal artery ("renovascular" hypertension) (4). We reasoned that if the thiazide drugs reacted on various forms of hypertensive disease in the same manner as did a drastic restriction of dietary sodium, then these drugs should be similarly ineffective in "curing" renal hypertension in the rat. The validity of this supposition was tested in the following experiment.

Methods. 20 male rats of the Wistar strain had become hypertensive 4 months after one renal artery had been narrowed, the contralateral kidney remaining intact. The arterial pressure of each rat was at least 160 mm Hg at this time, as measured with the microphonic method(5). The normal arterial pressure of rats of this age and strain averages 132 mm Hg (SD $\pm$ 9 mm Hg). Thus the blood pressure of our experimental rats was distinctly in the hypertensive range. During a 3-week preliminary period, the blood pressure of each rat was determined twice weekly for a total of 6 determinations. At this point, we began dissolving the thiazide drug, methyclothiazide, in the rats' drinking water, adding 86.4 mg of the drug per liter of tap water. In preliminary experiments it was determined that this dosage level produced a near maximum thiazide effect. The rats continued to ingest the thiazide drug in their drinking water for a period of 5 weeks. Then they were taken off the drug and switched back to ordinary tap water for a 4-week follow-up period. During the entire experiment, blood pressure and body weight of each rat were determined twice weekly.

* This work was supported by grants from Am. Heart Assn., Cargill Fund, and Nat. Heart Inst., U.S.P.H.S.