Observations on the Relationship Between Hemagglutinin and Neuraminidase of Influenza Viruses.* (26956)

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Evidence has been presented (1) suggesting that hemagglutinin and neuraminidase may represent 2 distinct and independently functioning attributes of the myxovirus particle, a possibility also suggested by other workers (2). In substantiation of this hypothesis, it was shown that certain strains of influenza virus could be freed of enzyme without significant loss of hemagglutinin (i.e. converted to indicator virus) while others could be stripped of hemagglutinin without significant loss of neuraminidase activity. Moreover the presence or absence of detectable neuraminidase activity in formalinized virus appeared to bear little relation to the enhanced susceptibility of the hemagglutinin in these preparations to inhibition by mucoids over that of the corresponding infective viruses. The relation between the susceptibility of certain mucoids to viral enzyme and the capacity of these substrates to inhibit viral hemagglutination has also been investigated. A lack of correlation has been found which further substantiates the dissociation of hemagglutinin and enzyme. The results of these studies form the subject of this report.

Materials and methods. Viruses were propagated and titrated, and hemagglutination inhibition tests performed as previously described (3,4). Strains of Asian influenza virus (RI/5+ and RI/5—) were generously supplied by Dr. P. W. Choppin and a strain of Newcastle disease virus (NDV) by Dr. P. I. Marcus. Sputum inhibitor was prepared by the acid precipitation method of Marmion, Curtain and Pye (5) as previously reported (6). Urinary mucoprotein was prepared according to the procedure of Tamm and Horsfall (7) from urine of the same donor, a secretor of blood group B. Brain mucolipid was kindly supplied by Drs. A. Rosenberg and E. Chargaff (8); bovine submaxillary mucoid by Drs. R. Heimer and K. Meyer (9); and serum orosomucoid by Dr. S. A. Ellison. While preparing normal human amnion cell cultures for purposes unrelated to the present problem, it had been noted that in the process of trypsinization large amounts of mucoid material were liberated into the suspending medium as the amnionic tissue became disaggregated. After removal of the cells by centrifugation, the supernatants were dialyzed, clarified by centrifugation and lyophilized. The resulting completely soluble colorless material, doubtless largely protein in nature, was found to contain appreciable sialic acid. Amnionic mucoid from placentas of blood group A_1 (cord blood) was devoid of blood group A activity. One preparation from a placenta of group O inhibited agglutination of group O erythrocytes by *Ulex Europaeus* extract but not that by bovine anti-H serum absorbed with A_1B erythrocytes. Receptor substance from human, guinea pig, and sheep erythrocytes was obtained as previously described (3). In addition, the procedure described by Kathan, et al. (10) was employed. This is based primarily on the method originally introduced by Westphal et al. (11) for dissociation of lipoprotein-carbohydrate complexes with hot phenol. As starting material, a lipid-rich (LR) fraction (12) of human erythrocyte stroma was employed. The final product in each

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instance was comparable in all respects to a sample of receptor substance generously supplied by Dr. R. H. Kathan (prep. 1, Table II). Sialic acid was measured by the Bial orcinol and direct Ehrlich reactions (13) as well as by the thiobarbituric acid method (14), using crystalline N-acetylneuraminic acid† (NANA) from sheep serum as primary standard. Chromatographic identification of NANA was carried out as previously described (1).

Results and discussion. It will be seen from Table I that sputum mucoid was relatively active against 4 strains (PR301, FM1, Lee and GL) while the activity of brain mucolipid, both of human and bovine origin, was limited to PR8 and PR301, and that of bovine submaxillary mucoid to PR8, Lee and NWS. The latter finding is in agreement with the observations of other investigators (15,16) who have found bovine submaxillary mucoid to be restricted in inhibitory range among numerous strains of influenza virus. Amnionic mucoid was devoid of detectable inhibitory activity; but there is no way of being certain whether or not receptors may have been present initially in the intact membranes and subsequently destroyed by the trypsinization procedure with which this material was isolated. None of the amnionic mucoid preparations contained hemagglutinin in the concentrations used, although there were traces of residual tryptic activity. The inhibitor of normal horse serum was active against 2 strains of Asian influenza virus (strain RI/5+ of Choppin and Tamm, and our strain of A2/Japan/305/57) and against FM1. The sharp differences noted in the range of inhibitory activity against representative strains of myxovirus is doubtless a reflection of basic structural dissimilarities of the active components in the various mucoids as suggested by the work of Gottschalk and de St Groth (16). Collocalia mucoid inhibited all strains of myxovirus hemagglutinin including RI/5−, a finding which suggests that this material contains inhibitory components resembling those of horse serum.

The susceptibility of the mucoids to the action of viral neuraminidase was determined using virus concentrated by ultracentrifugation of freshly harvested infective chorioallantoic fluid. Substrate and virus were mixed in the proportion mg substrate/ μg "viral" nitrogen averaging 0.1 (range 0.02-0.25). It will be seen from Table I that treatment of any of the mucoids with one or more strains of active virus resulted in the release of appreciable amounts of free or dialyzable N-acetylneuraminic acid. In a few instances (e.g. bovine submaxillary mucoid with PR301, GL and NWS) the quantities of NANA released, albeit small, were readily identified. The limited enzymatic action noted in these instances may perhaps be explained by relatively unfavorable experimental conditions. For example, quantitative study of the enzymatic action of myxoviruses with Collocalia mucoid has revealed the optimum pH for most strains to be 5.5-6.0 (4), there being a sharp drop in activity above pH 7.0. The earlier experiments reported herein, however, had been carried out at 7.2 in calcium borate buffered saline in accord with the methods previously reported by others (17,18) in which receptor destroying activity rather than quantitative measurement of neuraminidase had been the object of study. It might be noted in this connection that the myxovirus neuraminidase is fully active in the absence of calcium ions. Amnionic mucoid, showing no significant inhibition of hemagglutination, was nonetheless susceptible to viral enzyme, resembling in this respect orosomucoid which, while not capable of inhibiting hemagglutination, has also been found susceptible to myxovirus neuraminidase. There appears then to be little relation between the potency of a given mucoid as an inhibitor of hemagglutination

† Kindly supplied by Dr. A. Rosenberg.

TABLE I. Hemagglutination Inhibition in Relation to Enzymatic Activity of Myxoviruses.

	Inhibitor (Substrate)*													
	Sputum mucoid		Bovine submaxillary mucoid		Brain mucolipid				Human amnion mucoid§ Prep.		Horse serum		Collocalia mucoid	
					Human		Bovine		1	2				
NANA content	4%†		27%†		16%†		20%†		3%‡	3%‡	383 μg/ml‡		16%,† 12%‡	
Virus	IR	% NANA released	IR	% NANA released	IR	% NANA released	IR	% NANA released	% NANA released		IR	% NANA released	IR	% NANA released
PR8	9	60	1	22	4	34	1	25			>1200		2	53
PR301	3	36	>85	4	1	19	1				>1200		2	43
FM1	2	26	>89		>40		> 7		65	61	2		1	52
LEE	2	57	1	21	>81	13	>34				>1200		10	50
GL	1	33	>89	7	>40	+‖	> 7				>1200		10	80
A2/Jap	>17	53	> 8		>34		>23				1	4	4	56
Asian RI/5−								78	80	83	>1200	5	10	55
Asian RI/5+											1	3	1	78
Swine											>1200		2	58
NWS	> 6	36	1	2	>44	11	>40						2	63

* IR = Inhibition ratio: Ratio of μg inhibiting 5 HA units/μg inhibiting 5 HA units of the most sensitive strain(s) taken as 1. Enzyme activity expressed as % of total hydrolyzable chromogen liberated from substrate. Avg ratio: mg substrate/μg "viral" nitrogen = 0.1 (range 0.02-0.25).

† Bial orcinol reaction.

‡ Thiobarbituric acid reaction.

§ No significant hemagglutination inhibition with either preparation.

‖ Chromatographic identification only.

and its susceptibility to the enzymatic action of a given strain of myxovirus.

With only one exception, active virus eliminated all detectable hemagglutinin receptors in mucoids normally active against one or more strains of virus. Normal horse serum, however, treated either with concentrated A2/Jap, Asian RI/5+ or RI/5— virus retained most of its inhibitory titer against the first two of these strains. Retention of inhibitory activity following treatment with active virus was also noted by Choppin and Tamm (19). In agreement with their findings also is our observation that all 3 Asian strains showed potent neuraminidase activity against several other mucoids as well, despite the sharp contrast in susceptibility to horse serum inhibition

TABLE II. Interaction of Erythrocyte Stroma Components with Myxovirus Hemagglutinin and Neuraminidase.

Preparation	NANA content‡	μg inhibiting 5 hemagglutinin units*/(NANA released by active virus†) PR8	PR301	FM1	Lee§	GL	A2/Jap	Swine	NWS
Human erythrocyte inhibitor									
Prep. No.‖									
1	10		4/(26)	6	13	10			
2	11		5	10/(56)		5	78	78	
3	14		1/(75)	2	>200/(34)	4	>915/(90)	>100/(64)	
4	10		3	1	12		>100/(64)		
5	16		1(+)	1	143	143	50/(+)	53	
6	10		1(+)	1	10	26	50/(+)	52	
7¶	8		20	1	52	5	52/(82)	52	
8	16		>78	7	>78	30	>78	>78	
9	11	>68	6	1	68	6	>68	>68	
10**	22	>48	48	.2	>48	>48	>48	>48	
11††	20	>50	20	1	>50	>50	>50/(80)	>50	
12††	17	>55	11	.2	>55	>55	>55/(87)	>55	
Guinea pig erythrocyte inhibitor									
1	17	1	1	1	2	1	17	44	2
2‡‡	8		>51	2/(36)	20/(40)	5/(58)	>51/(76)	>51	
Sheep erythrocyte inhibitor									
LR stroma fraction§§			>252	>252	>252	>252	>252	>252	
Ext. 1	21		>221/(0)	>111	>111	>111	>221/(0)	>211/(0)	
2	8		>570	>570	>189	>189	>189	>189	
3‡‡	7		>52	>52	>52	>52	>52	>52	

* All strains except NWS heated to 56°.

† Expressed as % of total hydrolyzable chromogen in substrate. + = identified by chromatography only; 0 = none detected by thiobarbituric acid method. Avg ratio, mg substrate/μg "viral" N = 0.1 (range 0.02-0.16).

‡ Human erythrocyte preparations 1-9, and guinea pig preparations by Bial orcinol method; human preparations 10-12 by thiobarbituric acid method, maximum chromogen being released after hydrolysis in 0.1N H_2SO_4 at 80° for 30 min.

§ Enzyme assays performed at 26°.

‖ No. 1-9 from blood of single donors by method in ref. 3.

¶ 18% of total hydrolyzable NANA released by NDV.

** Sample kindly supplied by Dr. R. H. Kathan.

†† Prepared from stroma from individual donors by method in ref. 11, with modifications.

‡‡ Water soluble fraction from chloroform-methanol insoluble fraction.

§§ μg nitrogen.

between the A2/Jap and RI/5+ strains on the one hand and the RI/5− strain on the other. It might be noted that these 3 viruses possess the most potent and stable neuraminidase activity of any strains thus far studied.

As shown in Table II, essentially the same pattern of dissociation was observed with respect to the inhibitors obtained from erythrocytes. Although a number of preparations appeared to be somewhat limited in range against the several indicators, all were susceptible to attack by active virus. The "purer" materials (human erythrocyte preparations 10, 11 and 12) appeared to be the most restricted of all, being active primarily against FM1. On the other hand, guinea pig receptor substance had a much broader spectrum of activity. Preparations of receptor substance from both human and guinea pig erythrocytes were susceptible to the neuraminidase of all strains of active virus tested. In contrast, analogous material from sheep erythrocytes, although containing appreciable sialic acid, was totally inactive and furthermore was unaffected by concentrated active virus. The possibility that inhibitory activity may have been destroyed or altered during the extraction procedure is minimized by the fact that the crude sheep stroma fraction (LR) from which the final material was derived was also inactive. Such findings may also account for the relative inagglutinability of sheep cells by most myxoviruses at room temperature, with the notable exception of some strains of Asian influenza virus. These latter resemble the RI/5+ strain in that they do not elute readily from chicken erythrocytes. They do, however, elute slowly but completely from sheep erythrocytes, with resulting destruction of receptors. The apparent insusceptibility to viral neuraminidase of the sheep substrate may appear to be inconsistent with some of the previous observations. One can only presume that the hemagglutinin acts independently of neuraminidase, the latter still being necessary for elution from the intact erythrocyte. A slow rate of elution, as noted with some Asian strains, may reflect either inaccessibility to viral enzyme or differing configuration of the mucoprotein receptor substances at the sheep erythrocyte surface.

Summary. A number of mucoids of varying NANA content were found to differ widely in their capacity to inhibit the hemagglutinin of several strains of myxovirus when assayed under standardized conditions. This pattern of inhibition bore no quantitative relationship to the content of NANA of individual mucoids, each of which was found to be susceptible to the neuraminidase of 2 or more strains of active virus. Receptor substance from human and guinea pig erythrocytes inhibited hemagglutination and was susceptible to viral neuraminidase. Stroma and sialic acid-containing receptor-like substances from sheep erythrocytes were neither capable of inhibiting hemagglutination nor susceptible to viral neuraminidase. The implications of these findings are discussed with respect to the separate identities of myxovirus hemagglutinin and enzyme.

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Polycythemia Secondary to a Pheochromocytoma with Production of an Erythropoiesis Stimulating Factor by the Tumor. (26957)

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Polycythemia has been noted in association with various neoplasms including renal tumors (1,2), cerebellar hemangioblastomas (3,4), uterine fibroids (5) and pheochromocytomas (6). Hematologically these patients have shown erythrocytosis with no associated splenomegaly or increases in the white cell or platelet count. The elevated hemoglobin and hematocrit returned to normal following resection of the tumors. An erythropoiesis stimulating factor (erythropoietin) has been demonstrated in a saline extract of a renal carcinoma (7) and in the fluid obtained from renal cysts (7,8) and cystic cerebellar hemangioblastomas in patients with associated erythemia (4).

In this report we describe a patient with polycythemia associated with pheochromocytoma tumors and demonstrate production of an erythropoiesis stimulating factor by the tumor.

Case summary. D. R., a 10-year-old white boy, was admitted to the Univ. of Maryland Hospital with a complaint of headache. Six months prior to admission the patient developed progressively severe headaches, drenching night sweats, and a 12 lb. weight loss. Two weeks prior to admission the blood pressure was elevated to 170/140 mm Hg.

On admission the patient was markedly plethoric. Blood pressure was 180/110 mm Hg in the upper extremities and 210/160 mm Hg in the lower extremities. A round non-tender walnut-size mass was found in the left flank just under the costal cage. There was no splenomegaly and the remainder of the physical examination was negative.

Laboratory data. The hematocrit was 65%, hemoglobin 21 g %, red cell count 8.3 million/mm^3, leucocytes 8000/mm^3, and platelet count 745,000/mm^3. The bone marrow showed marked erythroid hyperplasia. Total red cell volume was 40 cc/kg, 14 cc/kg above estimated normal value. Arterial oxygen saturation was 93%. Urinary catecholamine excretion determined on 2 occasions gave results of 792 and 1014 units.

At operation 4 tumor masses were removed, 2 from the left adrenal, one from the bifurcation of the aorta and one from the right adrenal. These tumors were microscopically identified as pheochromocytomata.

The patient gradually became normotensive and has been free of symtoms for the year following surgery. The blood data were as follows: hemoglobin 13.8 g %, hematocrit 39%, red cell count 4.65 million/mm^3, and red cell mass was 26 cc/kg. Catecholamine excretion was 4 units.