DISSOCIATION OF HEMAGGLUTINATING AND ANTIBODY-MEASURING CAPACITIES OF INFLUENZA VIRUS*

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It has been suggested (1) that the virus antigens of new strains of influenza virus, Type B, in allantoic fluid contain some substance which in the presence of normal serum interferes with the agglutination of erythrocytes by the virus. With such materials the apparent virus-neutralizing titer of normal human or animal serum, measured by inhibition of red cell agglutination in a pattern test, reached levels approaching those observed with specific immune serum.

There was no apparent relation detected between a low agglutinating titer of virus, the resultant increased amount of allantoic fluid in a mixture, and the effect observed in the presence of serum. Nor was the effect enhanced by making the titration of serum in dilutions of normal allantoic fluid. Moreover, after an indeterminate number of passages by the allantoic route in eggs the fluid obtained with most of the strains lost, to a large degree, this inhibitor and with such preparations the agglutinin-inhibiting titers of normal or acute stage sera were within the usual low or negative ranges. It was evident then that the inhibitory effect was not due merely to the presence of allantoic fluid.

Mills and Dochez (2) had demonstrated that mouse pneumonia virus in a suspension of mouse lung exhibited the capacity to agglutinate erythrocytes of the mouse only after the suspension was heated. It was of interest in this respect to ascertain whether heating the suspensions of strains of influenza virus had any influence upon the phenomenon observed. With the preparations employed in the present studies, esposure to 56°C. for as long as 30 minutes caused no increase of titer. Ordinarily no significant change in the agglutinating titer of the virus took place. But when the heated preparations were tested as antigen in serum-antibody titrations they were found to have lost much of their capacity to agglutinate red cells in the presence of serum—sug-

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gesting a loss in capacity to combine with antibody although leaving the ability to agglutinate red cells in the absence of serum unimpaired. The results further indicated the probability of a complex antigen partly heat-labile, partly heat-stable. The present paper is a report and an interpretation of these findings.

Materials and Methods

The strains used were of Type B influenza virus, largely those isolated directly in eggs in this laboratory during the epidemic of influenza B of 1945-46 (1). The Lee strain was isolated in 1940, the Chdk strain in the spring of 1945, and the Bon strain was received from F. M. Burnet of Melbourne, Australia.

Virus Preparation.—The preparations of virus employed were obtained as allantoic fluid. Embryonated eggs on the 10th or 11th day of incubation were inoculated in the allantoic sac with egg-passaged virus. After 2 days' further incubation the allantoic fluid was removed by needle and syringe in the absence of obvious bleeding. Upon collection the fluid was tested for its agglutinating titer against adult chicken's washed erythrocytes and stored at 4°C. The interval between collection of a fluid and its use was usually not more than a few days but in some instances a preparation as old as 3 weeks was employed. No deterioration in titer was noted in that time. For routine purposes the virus material was heated at 56°C. for 30 minutes and cooled before use in tests. Infectivity for embryonated eggs was destroyed by the heating process.

Serological Procedures.—Titrations of virus and of serum were made according to Salk's modification of Hirst's procedure (3, 4). In the serum titrations two units of hemagglutinin, four times the final agglutinating dilution of fluid, were employed. The tests were done at room temperature, 20-24°C. All sera were heated at 56°C. for 30 minutes.

Sera.—Sera of both animal and human origin were studied. For the major portions of the study certain representative sera were chosen. The human sera are designated as Nos. 1, 2, 3, normal and immune. Sera 1 are those obtained from subject D. K. on October 2 and October 17, 1945, before and 2 weeks after one subcutaneous injection of influenza virus vaccine. Sera 2 were obtained from subject D. S., on October 17, 1945, in the acute stage of influenza B and late after convalescence on March 1, 1946. Sera 3 were, respectively, a pool of low titer sera from a number of human beings and a pool of high titer sera from human beings after vaccination.

Sera from ferret H30 were normal and convalescent after infection with influenza B virus in January, 1946. Sera from rabbit M48 were the normal and that taken 10 days after one intraperitoneal inoculation of 5.0 cc. of Type B virus in allantoic fluid.

EXPERIMENTAL

The Influence of Heating upon the Hemagglutinating Activity of Virus in the Presence and Absence of Serum.—From the data presented in Table I it is seen that in general the ability of a virus preparation to agglutinate chicken's erythrocytes was not obviously impaired by heating at 56°C. for 30 minutes. However, when these heated and unheated aliquots were then employed as antigens for the determination of the capacity of sera to inhibit the hemagglutinin a sharp disparity was observed. The results obtained with unheated material were characteristic of those usually noted with serum obtained before and after antigenic stimulation. With heated antigen there was, with the

exception of strain Bon, a sharp rise in the antiagglutinin effect of the normal phase sera, in many instances to approximate the titers attained by the ac-

TABLE I

Serological Behavior of Heated Influenza Virus

Strain	Passage	State	Agglutinin titer	Dilution used	Inhibition titer of serum					
					1		2		3	
					N	I	N	I	N	I
Gdlo	E 12	Unheated	640	160	64	256	64	256	64	256
		Heated	640	160	2048	4096	2048	2048	2048	2048
Sdki	E 2	Unheated	1280	320	64	512	32	512	32	512
		Heated	1280	320	2048	2048	2048	2048	2048	2048
Mndl	E 10	Unheated	160	40	0	512	0	256	0	512
		Heated	160	40	512	1024	512	1024	512	1024
Peak	E 5	Unheated	640	160	0	128	0	128	0	128
		Heated	640	160	128	256	128	512	64	256
Chdk	E 28	Unheated	1280	320	0	512	0	512	0	512
		Heated	1280	320	128	512	128	512	128	512
Potr	E 9	Unheated	640	160	0	256	0	256	0	512
		Heated	320	80	512	1024	512	1024	256	1024
Skpt	E 6 (*a*)	Unheated	1280	320	0	256	0	256	0	256
		Heated	320	80	1024	1024	512	1024	512	1024
	(*b*)	Unheated	640	160	0	128	0	128	0	128
		Heated	640	160	128	512	256	512	128	512
Lee	M 137–E 101	Unheated	1280	320	64	1024	32	512	32	1024
		Heated	1280	320	512	1024	256	1024	256	1024
Dspl	E 6	Unheated	320	80	0	128	0	128	0	128
		Heated	320	80	64	512	64	512	32	256
Bon	E 68	Unheated	2560	640	0	128	0	64	0	256
		Heated	2560	640	0	128	0	64	0	256

companying immune specimens. The increases in titer of immune sera were not proportionate to those of the normal and the impression is gained that the effect of both the normal and the immune sera tends to approach a limiting upper level.

With strains Potr and Skpt examples are shown of results observed when

a decrease in agglutinating titer of the antigen occurred as a result of heating. The loss was compensated for in the concentration of antigen used for the testing of sera and there was no significant difference between the results obtained in these instances and in others in which the agglutinating titer of the heated antigen was unchanged.

Strains Gdlo and Sdki still exhibited the normal inhibitor at the time of the present observations and there is the indication that the effect of heating of antigen upon the resultant serum titers is greater with these two strains. However, it is clear that sharp effects were noted with old passage strains such as Lee and one of intermediate experience, Chdk. Why the Bon strain should be refractory to the effect of heat is not presently apparent.

The results strongly suggest that the heated preparation of virus in allantoic fluid, while retaining its hemagglutinating capacity to full titer, has given up another property, namely, that of combining readily with a component of serum which ordinarily prevents the agglutination of erythrocytes by the virus. That the enhanced inhibition of agglutination is related to a factor in the serum is indicated by the fact that when a certain dilution of serum is reached agglutination of erythrocytes by the antigen, which is present in a constant amount in all dilutions of serum, again becomes evident.

The capacity of heated antigen to bind the serum factor is not completely lost as seen in the different end-points obtained with the same sera and preparations of different strains. This fact is further demonstrated by the data presented in Table II. In this experiment titrations of the same sera of human and animal origin were made against 1, 2, 4, and 8 units of heated and unheated hemagglutinin of strain Gdlo. The presence of some of the normal inhibitor in the virus preparation is indicated by the titers of the normal sera, especially those of the normal ferret and rabbit. Heating of the antigen resulted in a sharply heightened serum effect. But as the concentration of heated or unheated antigen in the titration series increased the antihemagglutinating titer of normal or immune serum fell progressively. It is apparent then that while the titer of serum is greatly increased in the presence of heated antigen combination between serum and antigen still takes place.

Influence of Time of Heating.—The effect of the length of time heating at 56°C. was continued has not been exhaustively studied. It has been found, however, that exposure to this temperature for periods of 10, 20, or 30 minutes produced essentially the same results with the strains studied. Heating for longer periods has in a limited experience tended more commonly to result in decreases in titer of the antigen.

A few of the same strains of Type B virus maintained by passage in mouse lung have been heated at 56°C. Their hemagglutinin has either been completely or markedly inactivated in 30 minutes. All strains of Type A influenza virus maintained by allantoic passage which have been studied have also lost hemagglutinating activity upon heating under the same conditions.

An impression has been gained that preparations of virus which have been stored at refrigerator temperature for 2 to 3 months are somewhat less consistently affected by heat than fresher preparations.

TABLE II

Influence of Increasing Concentrations of Heated and Unheated Hemagglutinin upon Serum Titer. (Strain Gdlo)

Serum			Antigen		Inhibiting titer of serum with increasing units of agglutinin			
Type	No.	Stage	State	Agglutinin titer	1	2	4	8
Human (vaccinated)	1	Prevaccination	Unheated	640	128	64	32	32
		Postvaccination			512	256	128	128
		Prevaccination	Heated	640	4096	2048	1024	512
		Postvaccination			4096	4096	1024	512
Human (illness)	2	Acute	Unheated	640	128	64	<32	<32
		Convalescent			512	256	128	128
		Acute	Heated	640	4096	2048	512	256
		Convalescent			4096	2048	1024	512
Human (pool)	3	Normal	Unheated	640	128	64	32	<32
		Vaccinated			512	256	128	128
		Normal	Heated	640	4096	2048	512	256
		Vaccinated			4096	2048	512	256
Ferret (B)	H30	Normal	Unheated	640	64	32	32	<32
		Convalescent			512	512	256	256
		Normal	Heated	640	4096	4096	1024	256
		Convalescent			8192	4096	2048	1024
Rabbit (Vaccinated)	M48	Normal	Unheated	640	512	512	256	128
		Immune			1024	1024	512	256
		Normal	Heated	640	4096	2048	512	512
		Immune			4096	2048	1024	512

DISCUSSION

The original interpretation (1) offered for the high titers obtained in serum tested for antihemagglutinin against strains of influenza virus, Type B, recently isolated in the egg was that an inhibitor of agglutination was present in the allantoic virus preparations, its influence becoming evident in the presence of serum. With further passage most strains lost the inhibiting effect. In

the present experiments, however, it was shown that irrespective of whether the untreated preparation exhibited this characteristic, heating of the material brought it forth and the effect in titration of serum was the same as that previously observed with freshly isolated strains. The similarity suggests that the same principle is involved in both instances.

Assuming that heat has resulted in the elimination of a property of the virus preparation, it is now suggested that the material from early passages in eggs behaves as it does because a factor is missing; it is acquired during subsequent passage so that later preparations react with immune serum in a specific manner. When those materials are heated, however, that property is again removed. Nevertheless, under any of these conditions the capacity of the virus preparations to agglutinate erythrocytes appears to be unchanged—when measured in the absence of serum.

The demonstration that heating of the virus preparations results in a dissociation of the hemagglutinating activity and the capacity to measure specific antibody leads to the postulate of a complex antigen: a heat-stable component causing agglutination of red blood cells and reacting with specific antibody; a heat-labile component reacting with a component of normal serum which tends to inhibit hemagglutination by virus. In the presence of the intact complex the natural serum factor is countered by the labile component and specific antibody can be measured by its effect in preventing agglutination by the virus. When the labile component is absent or removed by heat the agglutinin-inhibiting effect of the normal serum factor in either normal or immune serum becomes evident in high dilution. The measurement of specific antibody may thus be obscured. Whether the results in the latter instance are due to an antigen-antibody reaction or to the influence of serum *per se* is not clear. The concept bears similarity to the LS antigen of vaccinia extensively studied by Craigie, Rivers, and their associates and reviewed recently by Rivers (5).

It may be that the normal serum factor is the same as that noted early by Hirst to inhibit agglutination by virus (3). The infectious, the complement-fixing, and the toxic properties (6, 7) of influenza virus are destroyed by heating of the same extent as that employed in the present experiments. At present, identification of the postulated reagents with other known activities has not been made. The labile antigenic component may be related to the toxic principle, its influence being to counteract an antitoxic action developed in serum following experience with antigenically similar toxins of various origins.

The problem obviously requires thorough investigation and the interpretation suggested can serve as a working hypothesis for further studies of the antigenic constitution of influenza virus which are being made.

SUMMARY

Preparations of Type B influenza virus, propagated in the embryonated egg and obtained in the form of allantoic fluid, were found after heating at

56°C. for 30 minutes to retain the capacity to agglutinate erythrocytes but no longer measured specific antibody when used as antigen in titrations of serum antibody.

The dissociation of the two activities suggests the presence in such virus preparations of a complex virus antigen comprising, (1) a heat-stable component which agglutinates erythrocytes and reacts primarily with specific antibody; (2) a heat-labile component reacting with a factor of normal serum which ordinarily tends to inhibit the hemagglutinating activity of influenza virus.

The relation of the reagents to other known serological activities of influenza virus is being studied.

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