THE NATURE OF NON-SPECIFIC INHIBITION OF VIRUS HEMAGGLUTINATION*

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The agglutination of red blood cells by certain viruses is the function of a specific receptor substance associated with the cells, which combines with the virus (1). This reaction has provided *in vitro* methods for detecting these viruses and also their antibodies, since the latter specifically inhibit the agglutination reaction. It has been observed, however, that non-specific inhibition of virus hemagglutination may be caused by a variety of materials including normal sera (2), extracts of animal tissues (3), normal allantoic fluid (4, 5), and human tears (6). Of interest in this respect is the finding that hemagglutination with PVM virus in mouse lung extracts is not manifest unless the extracts are heated to 70°C. (7), a procedure which apparently releases the virus from a non-specific inhibitory factor (8).

In the course of studies on influenza virus antibodies in human tissues, a factor other than specific antibody was encountered which prevented hemagglutination with influenza or mumps viruses. Further investigation of this reaction has provided evidence that the virus receptor substance which has been released from cells may be responsible for the inhibition reaction. The experiments are reported in the present paper.

Methods

Preparation of Virus Suspensions.—The PR8 strain of influenza A virus and the Lee strain of influenza B virus were used in these experiments. Suspensions of the viruses were prepared by inoculating egg-passaged virus into the allantoic sac of 11 day old chick embryos. After 48 hours at 37°C., the eggs were chilled overnight at 4°C. and the blood-free allantoic fluid removed. The virus suspensions were then cleared by centrifugation at 3,000 R.P.M. for 15 minutes.

The strain of mumps virus used was obtained through the courtesy of Dr. J. F. Enders as amniotic fluid of the 36th passage in embryonated eggs. The virus was inoculated into the amniotic sac of embryonated eggs on the 7th or 8th day of incubation. After further incubation at 37°C. for 4 days, the amniotic fluid was removed and cleared by low speed centrifugation.

All virus suspensions were stored at −76°C.

Hemagglutination Tests.—Titration of the hemagglutinin in influenza and mumps virus suspensions was done according to the method of Salk (9). In the agglutination inhibition

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test 0.25 cc. of dilutions of serum or tissue extract was mixed with an equal volume of virus suspension containing a known concentration of hemagglutinin. After 1 hour at room temperature 0.5 cc. of a 0.25 per cent suspension of washed chicken red blood cells was added with a pipetting machine. The final concentration of agglutinin in the system was 2 units, unless otherwise indicated. The results were read after 1½ hours at room temperature. The titers were recorded as the reciprocal of the highest dilution producing complete inhibition.

EXPERIMENTAL

Agglutination Inhibition Tests with Human Serum and Tissue Extracts.— Human autopsies were first used as a source of material for studying agglutination inhibition titers of serum and tissue extracts.

Serum and tissues were obtained from a total of ten autopsies, which were performed from 1 to 7 hours after death. Only tissues which showed no gross or microscopic evidence of disease were included in this study. Ten per cent saline extracts of the tissues were prepared by grinding weighed portions with alundum and suspending in saline. The extracts were cleared by centrifugation first at 3,000 R.P.M. for 15 minutes and again at 12,000 R.P.M. for 15 minutes. Blood was obtained from the heart, and the serum was separated after clot retraction. Serial dilutions of the extracts and serum were tested for capacity to inhibit 2 units of the PR8 and Lee strains of influenza virus and the mumps virus, as described under Methods.

Table I summarizes the results obtained with tissue extracts and serum from three autopsies, which are representative of the findings as a whole. Patient 1 was a normal full term infant who died during delivery. The autopsy was performed 2 hours after death. The second patient was a 4 year old boy who died shortly after arrival at the hospital. The cause of death was not determined. The only findings at autopsy, which was performed 1 hour after death, were cardiac hypertrophy and dilatation, some pulmonary edema, subendocardial hemorrhages, and submucosal hemorrhages of the stomach. Cultures of the lung and blood showed no growth and a nasopharyngeal culture was negative for *C. diphtheriae.* Patient 3 was a 56 year old man who died suddenly following myocardial infarction. Autopsy was done 4 hours after death.

In general, it will be seen (Table I) that inhibition of agglutination with the influenza and mumps viruses was obtained in high titer with all of the materials tested. Extracts of human spleen were also found to cause inhibition in high titer (not shown in the table). Of particular interest was the finding that the titers of the organ extracts were often higher than the serum titers. Previous work on comparative antibody titers of serum and tissue extracts against the typhoid bacillus (10) and the papilloma virus (11) has revealed a much higher titer of specific antibodies in the serum than in the tissue extracts. It was suspected, therefore, that some factor other than specific antibody was responsible for the inhibition of the agglutination reaction.

Effect of Heat on the Inhibitory Factor in Human Serum and Tissue Extracts.— Tests were next done to determine the effect of heat on the capacity of human serum and tissue extracts to inhibit virus hemagglutination.

Aliquots of serum diluted 1:10 and 10 per cent extracts of human tissues were heated at 56°, 65°, and 75°C. for 30 minutes. The precipitates which formed were removed by centrifugation at 3,000 R.P.M. for 15 minutes and again at 12,000 R.P.M. for 15 minutes. Serial dilutions of unheated and heated serum and tissue extracts were then tested with 2 units of PR8, Lee, and mumps viruses.

Table II shows the results of an experiment with material from a 2 months old infant, which had unusually high inhibition titers. It will be seen that

TABLE I

Agglutination Inhibition Titers of Human Serum and Tissue Extracts

Patient No.*	Test virus‡	Agglutination inhibition titer§				
		Serum	Saline extracts			
			Lung	Liver	Kidney	Muscle
1	PR8	640	640	2560	640	40
	Lee	640	1280	640	1280	320
	Mumps	1280	1280	640	1280	320
2	PR8	1280	2560	10,240	2560	
	Lee	320	1280	2560	640	
	Mumps	320	1280	640	640	
3	PR8	1280	640	640	1280	80
	Lee	320	320	640	160	80
	Mumps	1280	1280	1280	2560	320

* See text for description of cases and autopsy findings.

‡ Two hemagglutinating units of virus.

§ Titers are expressed as reciprocal of highest dilution causing complete inhibition of hemagglutination. The dilution factor of the extracts is calculated in terms of wet weight of the tissue.

heating up to 75°C. for 30 minutes had no significant effect on the inhibitory substance in serum. In general, however, the titers of the tissue extracts were reduced by heating to 75°C.

It is obviously difficult from experiments with human serum and tissue extracts to decide whether we are measuring virus antibodies or a non-specific factor, since most human sera contain influenza and possibly mumps virus antibodies. It is well known, however, that the generality of antibodies are destroyed by heating at 75°C. Furthermore, some of the serum and tissue extracts with high inhibition titers had no demonstrable neutralizing antibodies. It seemed reasonable, therefore, to conclude that at least a part of the inhibition titers observed with serum and extracts of human tissues were due to some factor causing non-specific inhibition of the agglutination reaction.

It is recognized that the preceding findings might be criticized since they

TABLE II

Effect of Heat on Inhibitory Substance

Test virus	Material*	Agglutination inhibition titer			
		Not heated	Heated 30 min.‡		
			56°C.	65°C.	75°C.
PR8	Serum	5120	5120	5120	5120
	Lung	10,240	20,480	5120	2560
	Liver	40,000	2560	640	160
	Kidney	20,480	20,480	10,240	10,240
	Muscle	2560	1280	2560	320
Lee	Serum	2560	5120	2560	2560
	Lung	20,480	20,480	5120	5120
	Liver	10,240	320	160	<80
	Kidney	20,480	2560	1280	640
	Muscle	1280	160	80	<80
Mumps	Serum	1280	640	640	640
	Lung	10,240	5120	1280	640
	Liver	2560	160	80	<80
	Kidney	5120	320	80	<80
	Muscle	1280	80	80	80

* Tissues obtained from 2 month old infant with congenital heart disease, 1 hour after death.
‡ Serum and extracts 1:10 in saline.

TABLE III

Inhibition Titers of Normal Rabbit Serum and Tissue Extracts

Normal rabbit	Agglutination inhibition titer			
	Not heated		56°C. 30 min.	
	Test virus		Test virus	
	PR8	Lee	PR8	Lee
Serum	160	160	160	160
Red cell extract	40	40	<40	<40
Lung extract	20,480	20,480	10,240	20,480
Liver "	2560	10,240	40	1280
Kidney "	20,480	10,240	2560	2560
Muscle "	320	320	<40	<40

were obtained with autopsy material. Comparable experiments were undertaken, therefore, with normal rabbit and guinea pig tissues. Table III summarizes the results obtained in a typical experiment with serum and tissue extracts from a normal rabbit. The findings are similar to those observed with

human tissues in that extracts of the tissues had higher inhibition titers than the serum and heating at 56°C. had no detectable effect on the serum or lung extract titers but reduced the titers of the liver, kidney, and muscle extracts. Similar findings were observed with guinea pig serum and tissue extracts. Hirst has already reported that extracts of ferret lung cause marked inhibition of influenza virus agglutination (3). Obviously, specific antibodies cannot be responsible for the inhibition of virus hemagglutination produced by serum and tissue extracts of normal animals.

Effect of Hemoglobin on Inhibition Titers of Organ Extracts.—The extracts tested in the preceding experiments contained considerable amounts of hemoglobin due to extraction of the red blood cells present in the tissues. Attempts were therefore made to obtain extracts with as little hemoglobin as possible. For this purpose, lungs were removed from normal rabbits and guinea pigs. Immediately after removal, one-half of the lung was thoroughly perfused with physiological saline through the pulmonary artery, while the other half was not perfused. Saline extracts of the perfused and non-perfused lungs were then prepared and the capacity of each to inhibit influenza virus hemagglutination was determined, as previously described. Although extracts of the perfused lungs contained no visible hemoglobin, they still caused inhibition. The virus inhibition titers, however, when compared with extracts of non-perfused lung taken from the same animal, were usually reduced two- to fourfold.

The decrease in inhibition titers of organ extracts observed in these experiments indicated that both tissue cells and red blood cells yielded the inhibitory substance. The latter possibility was tested further in the next experiments.

Inhibitory Substance in Red Cell Extracts.—

Human, chicken, sheep, and rabbit red blood cells were obtained in citrate and washed three times in physiological saline. The hemagglutinin titer of PR8, Lee, and mumps virus preparations was determined with each of the red cell suspensions (0.25 per cent) in the usual way. The red cells were then extracted in saline in a Waring blendor (1:20 by volume of packed cells) and centrifuged two times at 3,000 R.P.M. for 15 minutes. Serial dilutions of the supernatant fluids were tested for capacity to inhibit agglutination of chicken red cells as already described, using 2 units of PR8, Lee, and mumps viruses.

Table IV illustrates the results obtained in numerous tests with human and animal red blood cells. The findings clearly indicate a correlation between the capacity of the red cells to agglutinate with these viruses and the inhibition titers produced by extracts of the red cells. Human and chicken red cells agglutinated readily with these viruses and extracts of the cells caused inhibition of agglutination in high titer. Rabbit red cells, on the other hand, agglutinated weakly with the viruses and their extracts caused little or no inhibition (see also Table III). Sheep red cells varied in their capacity to agglutinate with mumps and influenza virus and also in their yield of the inhibitory substance.

In the experiment with sheep cells recorded in Table IV, agglutination was observed only with the mumps virus, and extracts of the cells inhibited hemagglutination only with mumps virus and not with the PR8 or Lee influenza virus.

TABLE IV

Comparison of Virus Receptor Substance and Inhibitory Substance of Red Blood Cells

Red blood cells	Agglutination titer with			Inhibition titer* red cell extracts		
	Influenza virus		Mumps virus	Test virus		
	PR8	Lee		PR8	Lee	Mumps
Human	256	1024	1024	2560	1280	2560
Chicken	512	2048	1024	5120	1280	5120
Sheep	<32	<32	256	<80	<80	2560
Rabbit	<32	<32	<32	<80	<80	<80

* Expressed as highest dilution of extracts causing complete inhibition of chicken red cell agglutination with 2 units of test virus (see text).

TABLE V

Effect of Removal of Receptor Substance from Red Blood Cells

Chicken red cells treated with*	Agglutination of red cells‡	Agglutination inhibition titers of red cell extracts		
		Test virus		
		PR8	Lee	Mumps
Saline	+	1280	1280	640
Normal allantoic fluid	+	1280	1280	640
PR8 allantoic fluid	0	<160	<160	<160
Lee allantoic fluid	0	<160	<160	<160

* 37°C. for 17 hours (see text).

‡ With PR8, Lee, and mumps viruses.

Further experiments revealed that extracts of human and chicken red cells did not neutralize influenza virus in mice. Furthermore the extracts failed to fix complement in mixtures with influenza and mumps viruses.

Group O human red cells were ordinarily used in these experiments. However, tests with group A and with group B red cells showed no significant difference in the yield of the virus inhibitory substance. Furthermore, purified group A and B substances[1] caused no inhibition of red cell agglutination with PR8, Lee, or mumps viruses. Extracts of Rh-positive and Rh-negative cells

[1] We are indebted to Sharp and Dohme, Incorporated, Glenolden, Pennsylvania, for the supply of blood group specific substances A and B.

both caused inhibition of virus agglutination. It would appear, therefore, that the blood group substances A and B and the Rh factor are distinct from the substance obtained from human red cells which is responsible for the inhibition of virus agglutination.

Experiments on the effect of heat on the virus inhibitory substance in extracts of human and chicken red cells revealed that a temperature of 56°C. for 30 minutes usually reduced the titer of inhibition. No detectable inhibition was present after heating at 65°C. for 30 minutes. The inhibitory substance in red blood cell extracts appears therefore to be more susceptible to heat than the substance in serum. It has a greater resemblance in this respect to the substance in liver or muscle extract (Table II). Whether this indicates a fundamental difference between the inhibitor in red cell extracts and that in serum or in certain tissue extracts cannot be determined from the available data. It is well known, however, that the effect of heat on biologically active substances is greatly influenced by a variety of factors, for example the concentration of extraneous proteins, including the kind and quantity of extraneous proteins and other constituents of the menstruum.

Effect of Removal of Receptor Substance from Red Cells.—Hirst (1) showed that influenza virus is rapidly adsorbed onto chicken red blood cells and after 4 to 6 hours at 37°C. almost all of the virus is released from the cells. Red cells which had been treated in this manner lost their capacity to agglutinate due to removal of the virus receptor substance from the cells. In the next experiments chicken red blood cells were treated with influenza virus at 37°C. until they no longer agglutinated and then extracts of the cells were tested for capacity to inhibit hemagglutination.

Sterile chicken red blood cells were obtained in citrate and washed three times in sterile saline. 0.5 cc. aliquots of the packed cells were mixed with 19.5 cc. of saline, normal allantoic fluid, PR8, and Lee allantoic fluids, respectively. Although the viruses had been obtained aseptically, penicillin (50 units per cc. final concentration) was added to each mixture as a further precaution. The mixtures were incubated at 37°C. for 16 hours with frequent resuspension of the cells. The red cells were then sedimented by low speed centrifugation and washed twice in sterile saline in the centrifuge. The mixtures were further incubated at 37°C. for another hour. The red cells were then washed twice in saline and suspended in 19.5 cc. of saline. The cells were tested for capacity to agglutinate with influenza and mumps viruses and finally extracted in the Waring blendor. The supernatant fluids, after clarification by centrifugation, were tested for residual virus and for the inhibitory substance as described in the preceding experiments.

Chicken red cells treated with saline and normal allantoic fluid retained their capacity to agglutinate with the viruses tested and extacts of these cells caused inhibition of agglutination. Extracts of virus-treated cells, which contained no residual virus and which no longer agglutinated with influenza or mumps viruses, failed to cause detectable inhibition as illustrated in the experiment shown in Table V. The results clearly indicated that removal of the receptor

substance from red cells also removed the inhibitory factor from extracts of the cells.

Dissociation of Virus from Inhibitory Substance.—Influenza virus is eluted from chicken red cells after incubation for varying lengths of time at room temperature or, more rapidly, at 37°C. (1). The hemagglutinin of mouse pneumonia virus can be dissociated from the inhibitory component of mouse lung

TABLE VI

Dissociation of Influenza Virus from Inhibitory Substance

Temperature	Units of Lee virus in mixtures	Agglutination of chicken red cells			
		Virus and inhibitor* incubated			
		1 min.	1 hr.	3 hrs.	6 hrs.
°C.					
	1	0	0	0	0
	2	0	0	0	0
22	4	±	0	0	+
	8	±	0	±	+
	16	+	±	+	+
	1	0	0	0	0
	2	0	0	0	±
37	4	0	0	±	+
	8	±	±	+	+
	16	+	+	+	+

* Mixtures of chicken red cell extract and virus incubated for various periods as indicated, before addition of red cells (see text for details of experiment).

extracts by heating at 70°C. (7, 8). Experiments were undertaken, therefore to see whether influenza virus could be released from the inhibitory substance in red cell extracts by these methods.

Four cc. of serial dilutions of Lee allantoic fluid in duplicate was mixed with an equal volume of a chicken red cell extract (1:40). One set of the mixtures was placed in a 37°C. water bath, while the other was left at room temperature (23°C.). Samples (0.5 cc.) of each mixture were tested immediately and at 1, 2, 3, and 6 hours, by adding 0.5 cc. of a 0.25 per cent suspension of chicken red cells. Agglutination was recorded as usual after 1½ hours at room temperature.

The results of this experiment are shown in Table VI. Inhibition of agglutination was apparent immediately after mixing the virus with the red cell extract. After incubation for 6 hours, however, hemagglutination was again manifest, particularly in the mixtures containing 4 units of virus. There was no striking difference in the reaction at 23°C. and at 37°C. Repeated tests have confirmed these findings and similar results were obtained with human

red cells. In general, it appeared that the dissociation of virus and the inhibitory substance in the red cell extracts was less striking than the elution of virus from intact red cells (1), and that critical amounts of virus and inhibitor were required for its demonstration.

DISCUSSION

The substance (or substances) responsible for inhibition of virus hemagglutination observed in these experiments is apparently widespread in the human and animal body. It can be detected in serum, muscle, various organs, and in the red blood cells of certain species. The experiments with red blood cells (Table IV, V, VI) leave little doubt that the inhibitory substance obtained from these cells is identical with the receptor substance of intact red cells which combines with virus to produce agglutination. It seems likely that the inhibitory effect of tissue extracts is based on the same mechanism, since the virus receptor substance is found in tissue cells as well as in red blood cells. The available data, however, do not exclude the possibility of other substances in tissue extracts producing a similar effect.

The simplest explanation of the non-specific inhibition reaction is that the receptor substance which has been released from cells combines with virus and blocks the union of the virus with the red blood cells. The presence of an inhibitory substance in human tears (6) and also in serum suggests that the receptor substance is released from cells under natural conditions in the body. The findings with the virus inhibitory substance are similar in certain respects to those with the blood group substances A and B. Both are found in many types of mammalian cells in addition to red cells, and also in various body fluids in solution. Furthermore, the blood group substances in solution may combine with isoantibodies to inhibit hemagglutination (12). Evidence that the virus inhibitory substance is not an antibody and that it is distinct from the blood group substances (A, B, and Rh) has been provided in the text.

Further study of the inhibitory substance will be required to assess its significance in the body and in serological tests involving the agglutination inhibition reaction for detecting specific virus antibodies. The failure of the substance to neutralize influenza virus *in vivo* suggests that it is not an important factor in preventing infection. It might be postulated, however, that it plays a rôle in influenza virus infection of individuals with circulating antibodies by preventing union of the virus with antibody. Curnen and Horsfall (8) observed that PVM virus in combination with the tissue factor causing inhibition of hemagglutination failed to fix complement in mixtures with immune serum.

It should be emphasized that a sensitive method for detecting the inhibitory substance was used in the present experiments for purposes of studying the substance. Non-specific inhibition titers of animal sera have been reported to be less than 1:32 when titrated according to the method of Hirst and Pickels

(13), although extracts of ferret lung caused inhibition at a titer of 1:10,000 (3). Salk's method (9) of making serial dilutions of serum in the red cell suspension and then adding virus was found to decrease the non-specific inhibition titer. Apparently, in the latter method, the receptor substance of the red cell and the inhibitory factor in the serum compete for the virus, with less virus bound by the inhibitor than when virus and inhibitor are mixed before the addition of red cells. Francis (14) reported recently that inhibition titers of normal human sera were greatly increased when Type B influenza virus, heated to 56°C., was used instead of unheated virus. It is possible that the factor in serum causing this reaction is the same as the inhibitory substance encountered in the experiments reported here.

At the present time there is no simple method available for differentiating non-specific inhibition from that produced by specific antibody in human sera. The method used to dissociate the hemagglutinin of mouse pneumonia virus from the inhibitory substance in lung extracts, namely heating to 70°C. (7, 8) is not applicable to influenza virus, because the latter is more susceptible to heat than the substance causing inhibition. The dissociation of influenza virus and inhibitor by incubation at 22°C. or 37°C. (Table VI) would not appear to be a practical method for recognizing the non-specific factor, since the reaction proceeds slowly and its demonstration is dependent on critical amounts of virus and inhibitor in the mixtures. A rise in the serum inhibition titer, however, is undoubtedly due to specific antibody, for the fact has been abundantly demonstrated by neutralization and complement fixation tests with influenza virus.

SUMMARY

A study of the component in serum and tissue extracts responsible for non-specific inhibition of hemagglutination with mumps virus and the PR8 and Lee strains of influenza virus has yielded the following results:

1. The inhibitory factor was found in high titer in human serum and in saline extracts of various organs procured at autopsy (lung, liver, kidney, spleen). The inhibition titers of extracts of these organs were usually higher than the serum titers, whereas the titers of muscle extracts were invariably lower.

2. Similar results were obtained with serum and tissue extracts from normal rabbits and guinea pigs.

3. The serum inhibition titers were not affected by heating to 75°C. for 30 minutes, whereas the titers of the tissue extracts were usually reduced by heating at 65°C. or 75°C. for 30 minutes.

4. Saline extracts of human and chicken red blood cells also contained an inhibitory substance in high titer, and these cells showed marked agglutination with influenza and mumps viruses. Rabbit red cells, on the other hand,

underwent little or no agglutination with these viruses and extracts of these cells failed to cause inhibition. Sheep red cells varied in their capacity to agglutinate and also in their yield of the inhibitory substance.

5. When the virus receptor substance was removed from chicken red cells by adsorption and elution with influenza virus, extracts of the cells no longer yielded the inhibitory factor.

6. The inhibitory substance did not neutralize influenza virus in mice and it failed to fix complement when mixed with influenza or mumps viruses.

7. Evidence was obtained that some virus was released from the inhibitory substance after incubation for 6 hours at 22°C. or 37°C.

The implications of these findings are discussed.

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BIBLIOGRAPHY

1. Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.
2. Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.
3. Hirst, G. K., *J. Exp. Med.*, 1943, **78**, 99.
4. Beveridge, W. I. B., and Lind, P. E., *Australian J. Exp. Biol. and Med.*, 1946, **24**, 127.
5. Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, O. G., and Beard, J. W., *J. Immunol.*, 1947, **55**, 69.
6. Burnet, F. M., Beveridge, W. I. B., McEwin, J., and Boake, W. C., *Australian J. Exp. Biol. and Med.*, 1945, **23**, 186.
7. Mills, K. C., and Dochez, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 140; 1945, **60**, 141.
8. Curnen, E. C., and Horsfall, F. L., Jr., *J. exp. Med.*, 1946, **83**, 105.
9. Salk, J. E., *J. Immunol.*, 1944, **49**, 87.
10. Freund, J., *J. Immunol.*, 1927, **14**, 101.
11. Friedewald, W. F., *J. Exp. Med.*, 1940, **72**, 175.
12. Wiener, A. S., Blood Groups and Transfusions, Springfield, Illinois, Charles C. Thomas, 3rd edition, 1946, 275.
13. Hirst,G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.
14. Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.