THE QUANTITATIVE DETERMINATION OF INFLUENZA VIRUS AND ANTIBODIES BY MEANS OF RED CELL AGGLUTINATION

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In a recent brief communication we described the agglutination of chicken red blood cells by materials containing the PR8 and Lee strains of influenza virus (1). Furthermore, it was shown that the addition of specific immune serum inhibited the agglutination in the presence of the homologous virus, but not when heterologous strains were used. This paper presents the results of further experiments related to these phenomena. The first experiments deal primarily with the correlation between the agglutinating capacity of virus suspensions and their infectivity for mice. A greater number of experiments have been directed toward correlating the agglutination-inhibiting power of various sera with their virus-neutralizing capacity. Experiments primarily concerned with the mechanism of agglutination will be discussed in a later report.

Methods

Preparation of Virus Suspensions.—The allantoic fluid of infected chick embryos has been used as the principal source of virus throughout the work reported here. Eleven-day old white Leghorn embryos were used for inoculation. The shell was first sterilized with alcohol, and a small puncture was made over the air sac with a blunt dissecting needle. A second hole was made over the region where the chorioallantoic vessels were visible. The point of a 23 gauge needle was inserted into the latter hole, and 0.1 cc. of virus suspension was injected just beneath the egg shell into the allantoic sac. Usually infected allantoic fluid, diluted to 10^{-3} or 10^{-5} with saline, was used for the inoculum. The holes were sealed with paraffin, and the eggs were incubated at 37°C.

After 48 hours the allantoic fluid was removed from the eggs with a minimum of contamination with embryonic red cells. While this was slightly difficult technically, the increased care was justified by the higher virus titer of the fluid obtained. A moderate number of red cells, although quickly removed from the fluid by centrifugation, took out 50 to 90 per cent of the virus originally present. The eggs were opened by removing the shell over the air sac with forceps, and care was taken not to tear the chorioallantoic membrane. Closed, pointed forceps were used to pierce the exposed membrane near its outer margin. The forceps were then allowed to open,

and the membrane was held up like a tent. This opened the allantoic sac so that the fluid could be aspirated with a syringe and large bore needle (13 gauge) or with a rubber bulb on a capillary pipette. Care was taken not to aspirate any blood which ran down the torn membrane. An average of 4 to 5 cc. of cloudy fluid was obtained from each egg, and occasionally as much as 10 cc. could be aspirated.

The allantoic fluid from several hundred eggs was pooled and then redistributed into small lusteroid tubes. These tubes were quickly frozen and stored at −72°C. in a CO_2 ice box. Under these conditions of storage both the agglutinating capacity and the virus titer as determined in mice have remained essentially unchanged for several months.

Strains of Virus Used.—Allantoic fluid was prepared in the manner described above using four different strains of influenza virus: the mouse-adapted PR8 and the W.S. strains of influenza A virus, the Lee strain of influenza B virus, and swine influenza virus. All except the swine strain had had several passages in eggs prior to use. The allantoic fluid containing swine virus was prepared from eggs which had been inoculated with infected mouse lung.

Preparation of Chicken Red Cells.—Except when otherwise noted, the cells used for all the tests have been a 2 per cent suspension of adult chicken red cells. Chickens were bled from a wing vein into a syringe containing 10 cc. of 2 per cent sodium citrate. The citrated blood was filtered through gauze to remove the small clots, and the cells were washed three times in saline. The cells were removed by low-speed centrifugation after each washing. After the third washing the packed cells plus two volumes of saline were put into 15 cc. graduated centrifuge tubes and spun at 900 R.P.M. for 8 minutes. The sedimented red cells were diluted with saline to fifty times their volume. The red cell count on such 2 per cent suspensions was 160,000 to 180,000 per c. mm. The chickens were usually bled on the first day of the week, and the red cells were washed and stored at 4°C. after packing the final time. They were diluted just before use. The red cells, when stored in this way, could be preserved in a satisfactory condition for at least a week.

Titration of the Red Cell Agglutinating Capacity of Influenza Virus Suspensions.—The test tubes used in all the *in vitro* agglutination experiments were 7 cm. long and had an internal diameter of 0.8 cm. For agglutination titrations, series of twofold dilutions of the virus suspensions were made in saline. To 1 cc. of each dilution was added 1 cc. of the 2 per cent red cell suspension. The tubes were immediately shaken until the cells were well mixed. The titrations then stood at room temperature, without being shaken or disturbed, for 1 hour before reading.

Titration of Agglutination-Inhibiting Substances in Sera.—The sera to be tested were diluted in twofold steps in saline. To 0.5 cc. of each serum dilution was added 0.5 cc. of virus suspension, using the same concentration of virus in each tube. The virus suspension had previously been diluted to four times the desired final concentration. To the mixture of serum and virus was added 1 cc. of a 2 per cent red cell suspension, and the tubes were shaken until the cells were well dispersed. The agglutination was read at 1 hour. Throughout this paper the concentrations of serum and virus suspension are given in terms of the final concentration, after the red cells have been added. In certain inhibition experiments the dilutions of serum were made in normal horse serum or normal ferret serum instead of in saline, so that the total amount of

serum in each tube (normal plus immune) would be the same. All ferret sera were inactivated at 56°C. for 30 minutes before use. The human sera were not inactivated.

Two controls were included in each test: a positive control containing red cells, virus suspension, and the same diluent used in making the serum dilutions, and a negative control containing red cells and saline but no virus.

Grading the Agglutination Tests.—The tests were all read at 1 hour's time and were viewed against a bright white background. For this purpose a 15 watt "daylight" fluorescent light proved to be the most useful. The racks were placed directly against the light.

The amount of red cell sedimentation, rather than visible agglutination, was taken as the index of the degree of reaction since it was easier to see. In the negative control tube the red cells slowly settled during the hour before the test was read. About 3 to 4 mm. from the top of the fluid column was a sharp sedimentation boundary above which the saline was clear. The cells settling out at the bottom formed a small, round, sharply outlined disk, but the density of the lower three-fourths of the cell suspension remained unchanged.

In the tubes in which agglutination occurred, the masses of aggregated cells usually settled to the bottom in 1 hour. Those cells which remained in the supernatant fluid were usually finely dispersed and not granular, although occasionally clumps adhered to the sides of the tubes. In the tubes in which agglutination was most marked, only a thin veil of non-granular cells was left in suspension. With decreasing degrees of agglutination the density and cell concentration of the supernatant fluid approached that of the negative control tube. It was the density of the cells remaining in suspension which was used for reading the tests.

In order to make the grading of the reaction more objective, the density of the cellular suspensions in the various tubes was compared with the density of standard suspensions of red cells in saline. These standard suspensions were made from the same red cell preparation used in the tests. The tube being read was placed between two tubes of the same standard red cell concentration, and the density of the cells in the lower half of the tube was compared with the known dilutions. For the standard suspension, red cells were prepared in concentrations of 1.0, 0.75, 0.50, and 0.30 per cent. Tubes in which the density fell between that of the 1.0 per cent and the 0.75 per cent standard were called one plus. Those with a density between that of the 0.75 and the 0.50 per cent standards were called two plus, those between 0.50 and 0.30 per cent were graded three plus, and all tubes with a density less than that of the 0.30 per cent suspension were designated four plus.

With the increasing degrees of agglutination the size of the disk in the bottom of the tube also increased. In the negative control tube the disk in the bottom was small and sharply outlined. When agglutination occurred, the margins of the bottom disk often had a characteristic irregular lacy pattern made up of clumps of cells. This was especially true with very slight degrees of agglutination. When a tube had this granular pattern on the bottom with no decrease in density of the supernatant suspension, it was called plus-minus.

The end point in all agglutination titrations and serum inhibition tests has arbitrarily been taken to be the dilution where two plus agglutination occurs. If two plus agglutination does not occur in any tube, the end point is assumed to be half way

between two serial dilutions, one showing more and the other less than two plus agglutination.

Tests in Mice.—The serum neutralization tests in mice were done in the manner described by Horsfall (2) except that twofold instead of fourfold serum dilutions were used. The virus titrations in mice were done in the usual way except that dilutions of virus were made in steps of $10^{-0.5}$ instead of 10^{-1}. The immune ferret sera used were obtained by bleeding ferrets 14 days after inoculation intranasally with living virus.

EXPERIMENTAL

Correlation of Agglutination Titers and 50 Per Cent Mouse Mortality Titers of Suspensions of Influenza Virus.—In the first experiment different preparations of the same strain of influenza virus (PR8) were tested by *in vitro* agglutination and by intranasal inoculation of mice in order to compare the titer obtained.

Two preparations of infected mouse lung, one of allantoic fluid, and two of ground whole chick embryo, were used. Each suspension was centrifuged at low speed until it was clear. Some of the preparations were stored for as long as 6 weeks at −72°C., while others were tested immediately after harvesting. *In vitro* titrations were set up as described under Methods. At the same time mouse titrations were done on these suspensions using dilutions of virus in $10^{-0.5}$ steps.

The results, recorded in Table I, show that the mouse lethal titers and the agglutination titers of these suspensions parallel each other over a wide range of virus concentration. The figures in the last column of the table represent the concentrations of 50 per cent mouse lethal doses in the *in vitro* end point dilution (++) of the various virus suspensions. The relatively slight variation in these concentrations ($10^{3.5}$ to $10^{3.9}$) when virus from very different sources was tested suggests that under these conditions the *in vitro* titration may be a good index of the amount of lethal influenza virus in a suspension.

However, it must be emphasized that this correlation holds only when freshly prepared or well preserved virus suspensions are tested, that is to say when all of the virus presumably is pathogenic, for it can be demonstrated that the infectivity of the virus can be destroyed without destroying the capacity of agglutinating red blood cells. For example, when infected allantoic fluid was heated at 56°C. for 15 minutes, the infectivity of the preparation was completely lost, while the *in vitro* agglutination titer remained undiminished. Likewise, when infected allantoic fluid was allowed to stand at room temperature for several days, the mouse lethal titer slowly diminished but the *in vitro* titer remained constant. Therefore, to measure the infectivity of a preparation by the *in vitro* test, the suspension must contain no great proportion of inactivated virus.

Another exception to the correlation between the two methods of titration is afforded by infected ferret lung. A suspension of ground infected ferret lung

TABLE I

Comparison of Agglutination and Mouse Mortality Titrations on Different Preparations of PR8 Virus

Source of virus	Dilution of virus										Saline control	*In vitro* titer	50 per cent mouse mortality titer	Mouse lethal titer/agglutination titer*
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560				
Mouse lung 335th passage		++++	++++	++++	++++	++++	++++	+++	++	±	0	1,280	$10^{-6.7}$	$10^{3.6}$
Mouse lung 336th passage		++++	++++	++++	++++	++++	++++	+++	±	0	0	960	$10^{-6.3}$	$10^{3.5}$
Allantoic fluid		++++	++++	++++	++++	++++	++	+	0	0	0	320	$10^{-6.0}$	$10^{3.5}$
Ground whole chick	++++	++++	++	0	0	0	0	0	0	0	0	20	$10^{-5.2}$	$10^{3.9}$
Ground whole chick	++++	+++	±	0	0	0	0	0	0	0	0	15	$10^{-4.9}$	$10^{3.7}$

* Expressed as the number of 50 per cent mouse mortality doses in 0.05 cc. of the dilution of virus suspension which caused two plus agglutination of red cells.

containing a mouse-adapted strain of PR8 virus at a high titer would not agglutinate red cells in any dilution. That this may be due to inhibitory substances in the ferret lung is shown by the fact that when ground normal ferret lung is added to infected allantoic fluid, red cell agglutination is inhibited.

While the experiment summarized in Table I shows a certain consistent relationship between mouse lethal titer and agglutination titer when different preparations of the same strain are tested, an experiment recorded in Table II shows the relationship between these two titers when different strains of influenza virus in allantoic fluid are compared. It will be seen that four different strains, some of them antigenically unrelated, showed a remarkable constancy in their *in vitro* titer, differing by not more than twofold. Simultaneous mouse titrations on the same preparations, however, demonstrated widely differing capacities to kill mice. For example, the W.S. strain, which in this experiment has

TABLE II

Comparison of Agglutination and Mouse Mortality Titrations on Four Different Strains of Influenza Virus

Virus strain	Dilution of virus						Agglutination titer	50 per cent mortality titer
	1:32	1:64	1:128	1:256	1:512	1:1,024		
PR8	++++	++++	+++	++	±	0	256	$10^{-6.0}$
W.S.	++++	++++	++	0	0	0	128	$10^{-6.5}$
Swine	++++	++++	++	0	0	0	128	$10^{-4.2}$
Lee	++++	++++	+++	++	0	0	256	$10^{-4.0}$

the highest mortality titer in mice, gives a lower *in vitro* titer than the Lee strain, which has the lowest mortality end point in mice.

It is clear from this experiment that the agglutinating capacity of virus suspensions and their ability to cause lesions or death are independent variables when different virus strains are compared. In view of their wide variation in this latter respect, it was surprising that the allantoic fluid preparations of all the strains so far studied showed such a constant agglutination titer.

Measurement of Agglutination-Inhibiting Substances in Serum.—In a previous paper (1) it was stated that when sera (human and ferret) which contained a high titer of influenza-neutralizing antibodies were added to virus suspensions, these virus suspensions would no longer agglutinate red cells, even though the immune serum was present in very low concentration. It was also shown that the amount of this inhibiting substance in a serum could be titrated by determining at what dilution of serum the inhibitory effect was no longer demonstrable. Before going further into the study of this phenomenon it was considered of importance to investigate the inhibitory properties of normal sera.

Serum inhibition tests were done on a number of normal ferret sera, using

both the PR8 and the Lee strain of virus. The sera were heated at 56°C. for ½ hour, and serial twofold dilutions in saline were made. To these dilutions a constant amount of infected allantoic fluid was added, and the amount of agglutination was read 1 hour after adding red cells. The results with three such sera, shown in Table III, demonstrate that there was an inhibitory substance present in normal ferret serum, and that with low dilutions of serum the inhibition was active against both viruses tested. Similar inhibition was demonstrated with mouse, rabbit, and guinea pig sera, but practically no inhibitory effect was demonstrable with horse serum. This inhibitory factor was partly destroyed by heating the serum to 56°C. for ½ hour. The possible presence of a normal inhibitory substance in human serum will be discussed later.

Although the presence of an agglutination-inhibiting substance in normal serum complicated the titration of inhibition due to influenza virus antibodies, it was relatively easy to obviate this difficulty. When a difference in inhibition titer between two sera from the same individual or from the same animal was measured, the change in titer was assumed to be due to antibodies, since the amount of normal inhibitory substance was probably the same in both sera. Also the inhibition titer of most immune ferret sera was very high, considerably beyond the range where the normal inhibition was active. When titers from different animals of the same species were compared, the immune serum dilutions were made in normal serum from the same species so that the total amount of serum in each tube (normal plus immune) was the same. This made it possible to dilute out the inhibitory effect due to antibodies while the normal inhibitory effect was kept constant.

The Serological Specificity of the Serum Inhibition Test Using Immune Ferret Sera.—The antigenic relationships of various strains of influenza virus have been extensively studied by several authors. These studies have shown that influenza A virus strains are antigenically distinct from the Lee and T.M. strains of influenza B virus (3, 4). Also differences have been demonstrated between various strains of influenza A virus (5–7), and swine influenza virus has been shown to be distantly related to influenza A strains (8, 9). The following experiment was performed to see whether or not the inhibition test is sufficiently sensitive to detect the antigenic differences and similarities demonstrable by other methods.

The PR8 and W.S. strains of influenza A virus, the Lee strain of influenza B virus and swine influenza virus were used. Allantoic fluid preparations of the virus strains and ferret antisera were prepared as described under Methods. Since high concentrations of normal ferret serum inhibit agglutination of the red cells, all immune serum dilutions were started at 1:32. All dilutions of the immune sera were made in normal ferret serum so that the final concentration of serum in each tube (normal plus immune) was 1:32. Agglutination titrations of the four virus preparations were also

TABLE III

Agglutination Inhibition Titrations on Normal Ferret Sera Using PR8 and Lee Viruses

Serum No.	PR8 (A)								Lee (B)							
	Dilution of serum						Virus control	Titer	Dilution of serum						Virus control	Titer
	1:4	1:8	1:16	1:32	1:64	1:128			1:4	1:8	1:16	1:32	1:64	1:128		
A-17-67	0	++	++++	++++	++++	++++	++++	8	±	+	+++	++++	++++	++++	++++	12
A-17-52	0	+	++	++++	++++	++++	++++	16	0	±	++	++++	++++	++++	++++	16
A-17-72	0	0	0	++	+++	++++	++++	32	0	±	+	+++	++++	++++	++++	24

TABLE IV

Serum Inhibition Tests Using Four Virus Strains and Their Respective Ferret Antisera

Serum	Virus strain	Dilution of serum										Virus control	*In vitro* titer	Neutralization titer in mice
		1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048	1:4,096	1:8,192	1:16,000			
PR8	PR8	0	0	0	0	0	0	0	+	+++	++++	++++	6,000	16,000
	W.S.	0	0	0	0	0	++	+++	+++	+++	+++	+++	1,024	1,024
	Swine	±	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	96	32
	Lee	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
W.S.	PR8	0	0	0	0	+	++++	++++	++++	++++	++++	++++	768	256
	W.S.	0	0	0	0	0	0	+	+++	+++	+++	+++	3,000	2,048
	Swine	0	0	±	++	+++	+++	+++	+++	+++	+++	+++	256	12
	Lee	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
Swine	PR8	+	++	++++	++++	++++	++++	++++	++++	++++	++++	++++	64	4
	W.S.	0	0	0	0	+	+++	+++	+++	+++	+++	+++	768	16
	Swine	0	0	0	0	0	0	0	++	+++	+++	+++	4,000	1,024
	Lee	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
Lee	PR8	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	<32	<4
	W.S.	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
	Swine	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
	Lee	0	0	0	0	0	0	0	++	+++	+++	+++	4,000	8,000

made in 1:32 normal ferret serum. The dilution of allantoic fluid used in the inhibition tests was twice the concentration which caused a two plus reaction in the agglutination titrations. With the PR8, Lee, and swine strains a final concentration of allantoic fluid of 1:32 was used, and for the W.S. strain a concentration of 1:12. The inhibition titrations were set up as described under Methods, adding each virus to dilutions of each of the four sera. The results are recorded in Table IV.

In examining the antigenic relationships shown by this inhibition test it should be noted that each serum inhibited the agglutination by its homologous virus to approximately the same dilution (3000 to 6000). The agglutination of red cells by the Lee virus suspensions was not inhibited by any of the heterologous sera, nor did the Lee antiserum significantly inhibit the reaction due to any of the heterologous viruses. The PR8, the W.S., and the swine influenza viruses all showed some interrelationship by this method, and of these the PR8 and the W.S. showed the greatest similarity. Both the W.S. and the PR8 sera gave the higher titer with the homologous virus and a four to six times lower titer with the heterologous virus. Swine virus appeared more closely related to the W.S. strain than to the PR8 strain, both when swine antiserum was tested against PR8 and W.S. virus, and when PR8 and W.S. antisera were tested against swine virus.

Neutralization tests in mice were done with these same sera and virus preparations in order to compare the *in vitro* results with one of the generally accepted procedures for the demonstration of antigenic relationships. Approximately 30 fifty per cent mortality doses of each virus were used for the test. The neutralization titers obtained are shown in the last column of Table IV. An interpretation of the *in vivo* results yields essentially the same conclusions about the antigenic relationships of these strains as were obtained from the inhibition test. The only qualitative exception was that the PR8 serum had a higher titer than the W.S. serum when tested with swine virus in mice, while the reverse was true with these sera against swine virus in the *in vitro* test.

Agglutination Inhibition Titrations with Human Sera.—Although we have previously reported the demonstration of a rise in agglutination inhibition titer in serum taken from a patient during convalescence from influenza A, it was considered necessary to ascertain how constant this finding would be in a large series of cases.

Forty-four pairs of acute and convalescent sera from cases of influenza A were used. Half the sera were from an institutional epidemic occurring during the winter of 1938–39 (10), and the remainder were from institutional epidemics in Alabama in the winter of 1940–41 (11). The convalescent serum from each patient had a mouse neutralization titer at least four times as high as that of the acute phase serum, when tested with PR8 virus. For the *in vitro* test twofold dilutions of serum were made in saline, beginning with a final serum dilution of 1:8. A final dilution of 1:64 allantoic

fluid, containing PR8 virus, was used. This concentration of allantoic fluid was four times the amount which causes two plus agglutination in an agglutination titration. In every case the titer of the convalescent serum was at least two, and usually four or more, times higher than the titer of the acute serum.

Similar tests were run on sera from individuals who had been vaccinated with a complex influenza vaccine which contained formalinized PR8 virus (12). Sera were taken before and 2 weeks after vaccination. Fifty-five pairs of sera were tested. Each serum was titered by means of the mouse neutralization test, and in every case the postvaccination serum showed at least a twofold rise in neutralizing antibodies against PR8 virus. When these sera were tested *in vitro*, a corresponding rise in inhibition titer following vaccination was demonstrated in every case.

Pairs of serum from fourteen normal individuals obtained at a 1-year interval were included in this experiment. None of the subjects gave any history suggestive of

TABLE V

Serum Inhibition Titrations on an Immune Ferret Serum Using Different Quantities of Virus

Dilution of allantoic fluid	Dilution of serum*									Virus control
	1:256	1:512	1:1,024	1:2,048	1:4,096	1:8,192	1:16,000	1:32,000	1:64,000	
1:4	0	±	++	++++	++++	++++	++++	++++	++++	++++
1:8	0	0	±	+++	++++	++++	++++	++++	++++	++++
1:16	0	0	0	±	+++	++++	++++	++++	++++	++++
1:32	0	0	0	0	±	++	++++	++++	++++	++++
1:64	0	0	0	0	0	±	++	+++	++++	++++
1:128	0	0	0	0	0	0	±	++	+++	+++
1:256	0	0	0	0	0	0	0	±	±	++
1:512	0	0	0	0	0	0	0	0	0	0
1:1,024	0	0	0	0	0	0	0	0	0	0

* All serum dilutions were made in normal horse serum plus saline, so that the final concentration of serum (normal plus immune) was 1:8 in every tube.

influenza during the intervening period, and none of the pairs of sera showed any difference in the virus neutralization titer. There was likewise no demonstrable difference between the inhibition titers of each of the pairs when tested by the *in vitro* technique.

The foregoing experiments, both with ferret and with human sera, demonstrate a consistent qualitative parallelism between neutralization titer in mice and agglutination inhibition titer *in vitro*. Before attempting to demonstrate a more quantitative correlation between the results of the two tests it was considered necessary to investigate the variables entering into the *in vitro* titration in order to find out what precautions are essential to obtain reproducible results.

Factors Affecting the Results of the Inhibition Test.—In the following series of experiments inhibition tests were performed with a PR8 ferret serum and its homologous virus. In each experiment one variable was introduced into the titrations, in order to see what the effect on the inhibition end point of the serum would be.

In the experiment shown in Table V the ferret immune serum was titrated with a number of different concentrations of the homologous virus. With only minor variations the serum inhibition end points obtained with different quantities of virus were in simple inverse proportion to the amount of virus used in the test. This simple relationship between the amount of virus used and the end point obtained makes it very convenient and easy to compare inhibition tests where different amounts of virus were used. The theoretical implications of this experiment will be more fully considered later.

In another experiment a number of titrations were done on the same serum using different concentrations of chicken red cells for the tests, the results of which are shown in Table VI. While it was difficult to make comparable readings in tests in which there was such a wide variation in red cell concentration, nevertheless it was obvious that the end point decreased as the concentration

TABLE VI

Serum Inhibition Titrations on an Immune Ferret Serum Using Different Quantities of Cells

Final cell concentration	Dilution of serum							Virus control	Red cell control
	1:256	1:512	1:1,024	1:2,048	1:4,096	1:8,192	1:16,000		
per cent									
4.0*	0	0	0	0	±	+++	++++	++++	0
2.0	0	0	0	0	+	+++	++++	++++	0
1.0	0	0	0	±	+	++++	++++	++++	0
0.5	0	0	0	+	+	++++	++++	++++	0
0.25	0	0	+	++	++	++++	++++	++++	0
0.12	0	±	±	++	++	++	++	++	0

* There were 360,000 red cells per c. mm. in the 4 per cent concentration.

of cells used decreased. In this test it was found that by far the most satisfactory concentration of cells for ease in reading the tests was the final 1 per cent concentration used throughout the other experiments in this paper.

Duplicate serum inhibition tests were run simultaneously at 37°C. and at 27°C. with no obvious difference in end point. Although a wider variation in temperatures produced definite changes in the end points, the ordinary changes in room temperature were not sufficient to alter the results significantly.

Duplicate titrations were done with fifteen human sera (high and low titer) and one ferret serum, using six different lots of red cells. Each lot of red cells was prepared separately, and the various lots were stored at 4°C. for from 1 to 6 days before use. There was no systematic tendency of any lot of cells to give high or low results with the various sera. The length of storage apparently had no effect on their agglutinability. In general, duplicate titrations of a given serum gave the same result. In 25 per cent of the tests there was a variation in end point of one-half dilution, and very occasionally there was a variation of a full dilution. From these data it was evident that the use of

different lots of cells was not a serious source of error in the test if the cells had been prepared in a uniform manner.

One factor of considerable importance in obtaining uniform results with the test is the length of time between the addition of red cells and the final shaking for thorough mixing. When the tubes of a serum titration were shaken immediately following the addition of cells, the titer obtained was definitely lower than that of a duplicate titration, when the tubes were shaken 5 minutes after adding the cells. In some of the experiments reported here the cells were added with an automatic pipetting machine, which delivered the suspension with such force that sufficient mixing took place at once.

Of some importance also was the question of the length of time necessary for the virus-antibody reaction to take place after serum and allantoic fluid had been mixed together. A number of titrations of an immune ferret serum were done. In each case the red cells were added after the virus and serum had stood mixed together for different intervals. Whether the cells were added immediately after adding virus to the dilutions, or whether the virus-serum mixture was allowed to stand for ½ hour before adding the cells, the end point was the same. This rapid interaction between virus and serum obviates any necessity of incubating these two reagents together for any fixed period in performing the inhibition tests.

The preceding experiments emphasize the fact that the serum inhibition test can be performed in a fairly simple manner, without elaborate equipment or precautions, and that under these conditions quite reproducible results may be obtained.

Quantitative Correlation between Virus Neutralization and Agglutination Inhibition Titers of Serum.—After it had been shown that the *in vitro* test gave results which were qualitatively similar to those obtained with the mouse neutralization test and that the results were reproducible, the next step was to see how the serum inhibition titer was related quantitatively to the virus neutralization titer on widely different sera.

The sera used were from sixteen persons acutely ill with influenza A, from twenty-one convalescent from influenza A, from twenty-two normal individuals, and from thirty-one who had been vaccinated 2 weeks previously with a complex chick embryo influenza vaccine. Mouse neutralization tests were done on all these sera, using 200 lethal doses of PR8 mouse passage virus, and the sera were diluted in twofold steps. The *in vitro* tests were done with a 1:64 dilution of allantoic fluid containing PR8 virus. The two plus end point of this same fluid in an agglutination titration was 1:256. The end points obtained in the inhibition tests were plotted against the neutralizing capacity as calculated from the mouse neutralization end points (2).

The results are recorded in Fig. 1, in which it can be seen that the points tend to fall along a straight band. On the whole, the correlation is fairly good between the two methods of measurement over a wide range of antibody levels

and there are no widely discrepant points. The width of the band can be accounted for by the errors in the tests, especially the mouse neutralization test.

This correlation is more significant when one considers that the sera were from four groups of individuals who had had widely differing experience with influenza virus, and yet there is no systematic deviation or scattering of any

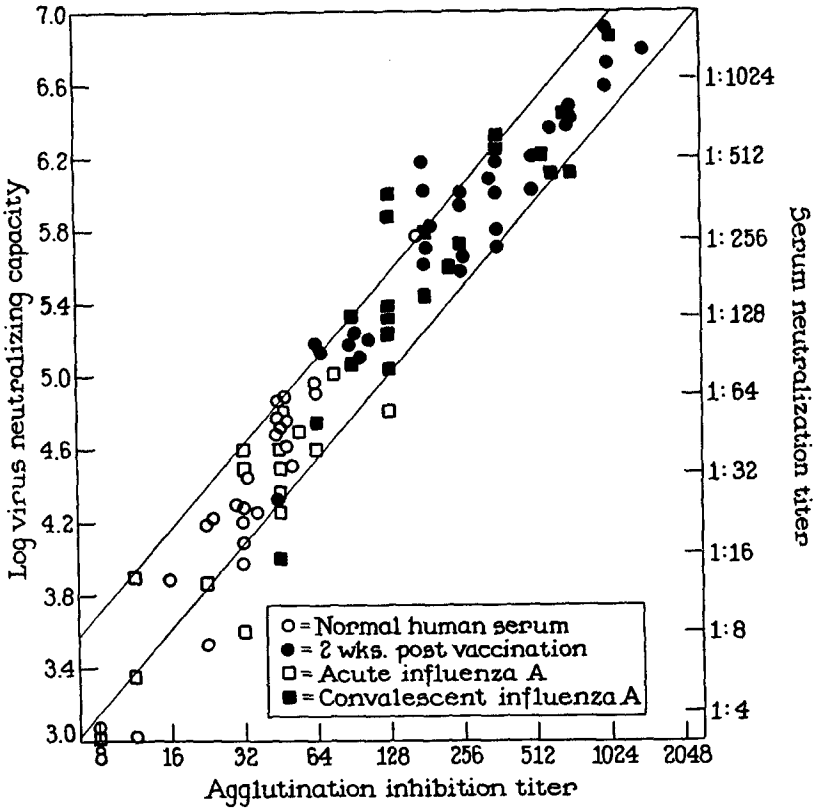

FIG. 1. Correlation of serum inhibition titer with virus-neutralizing capacity of various human sera.

of these groups. The evidence contained in this figure is the best we have obtained so far that the *in vitro* test measures either neutralizing antibodies or something which fairly consistently parallels neutralizing antibodies.

DISCUSSION

The advantages of the agglutination tests over mouse tests for influenza virus and influenza antibody titrations are sufficiently clear so that detailed discussion seems unnecessary. While the agglutination titer of a fresh virus suspension shows a fair correlation with the mouse infective titer in the tests we have

recorded, much more work will be necessary to show what the agglutination titer actually measures. In any case, it is a measurement of a new quality of virus suspensions and as such merits further study. The serum inhibition titrations clearly measure influenza antibodies, and from the quantitative results obtained it seems that the test may very likely measure neutralizing antibodies. The only other *in vitro* test described for measuring influenza antibodies is the complement fixation test. While no direct comparisons have been made between the results of this test and the inhibition test, the outstanding advantage of the latter is its ease of performance and standardization.

If these agglutination tests should come into general use, they would be of value in comparing results from different laboratories, provided some sort of standard procedure were used. Based on our present experience, the procedures outlined under Methods seem to be a satisfactory starting point. For serum titrations it would be necessary to state only the number of agglutination units used for the test. We have generally used four times the amount of virus necessary to cause two plus agglutination. All sera should be heated to 56°C. for 30 minutes before use. This was not done with the human sera in the experiments reported here, but it has been found since these tests were performed that such heating lowers the inhibition end point of low titer human serum but does not affect the end point of high titer serum. Heating probably inactivates a "normal" inhibitory substance present in such sera.

The fact that eggs inoculated with a number of different influenza virus strains yield allantoic fluids of approximately the same agglutination titer is of some theoretical interest. A number of other strains, besides those employed in the present work, have been cultivated in eggs, and all strains so far tested have shown this same constancy in titration end point by agglutination. This similarity in end point suggests two things: (1) that different strains of influenza virus, when grown in eggs, reach approximately the same virus particle concentration in allantoic fluid, and (2) that the individual virus particles of different strains of virus have the same capacity, or nearly so, to agglutinate red cells. If the second assumption is correct, then the agglutination titration method is a simple way of determining the relative number of particles in suspensions of different strains, independently of pathogenicity, something mouse titration fails to tell. In any case, the test provides a method for studying immunologically and in other ways strains which have not been adapted to mice.

The results of the serum inhibition titrations, using different amounts of virus, show that the same amount of serum inhibits agglutination by a constant amount of virus, regardless of the volume in which the reaction takes place, at least over a considerable range of dilution. This result is in keeping with the classical work on antigen-antibody reactions of Dean and Webb (13), which

showed that the optimal proportions point for antigen-antibody reactions was the same, regardless of the volume in which the reaction was carried out. This would tend to show that influenza virus-antibody reactions are the same as other antigen-antibody reactions, at least in this respect.

This result, however, is in apparent conflict with those of Horsfall (2) on the shift in serum neutralization end point in mice, where different amounts of virus are used in the test. Horsfall found that if it took x cc. of serum to neutralize a certain quantity of virus, it took $x/5$ cc. to neutralize one-tenth as much virus. In the mouse test the serum becomes less efficient on dilution. Since the reasons for the discrepancy are not clear, it will suffice to point out that the mouse test is a very complicated test and involves the interplay of many forces over a period of about 10 days, while the *in vitro* test is relatively simple. Because of the complexity of the mouse test, it seems probable that the agglutination inhibition test gives a more accurate picture of the *in vitro* combining ratios of virus and antibody.

SUMMARY

1. The agglutination titer for chicken red cells of freshly prepared or carefully stored suspensions of PR8 influenza virus, that is to say virus of maximum pathogenicity, was found to be proportional to the mouse lethal titer of the same preparations.

2. The agglutination titer of infected allantoic fluid procured in a standard way is relatively constant, regardless of the influenza strain used and its pathogenicity for mice.

3. Virus preparations inactivated by heat or storage may retain their agglutinating power.

4. Certain animal sera contain a partially heat-labile factor which, in low dilution, inhibits the agglutination of chicken red cells by influenza A and influenza B viruses.

5. The agglutination inhibition test, using ferret and human sera, gives qualitative data regarding influenza antibodies which are similar to the information obtained on the same sera by means of the virus neutralization test.

6. There is a definite relationship between the agglutination inhibition titer and the virus neutralization titer of a serum. On a logarithmic scale of both variables, this relationship is essentially linear within the range investigated.

7. The agglutination inhibition titer of immune ferret serum is inversely proportional to the amount of virus used in the test.

Since this paper went to press, McClelland and Hare (14) have published results confirming the work in our earlier publication (1) on the adsorption of influenza virus on red cells and the use of agglutination for measuring influenza antibodies.

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