THE ACTION OF IMMUNE SERUM ON HUMAN INFLUENZA VIRUS IN VITRO

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The mechanism by which immune serum inhibits the disease-producing function of filterable viruses has been subjected to repeated study by different workers and with different viruses. In most instances amounts of serum just, or not quite sufficient to "neutralize" the selected dose of virus have been employed. Bedson (1), Todd (2), Andrewes (3), and Craigie and Tulloch (4) have shown that by dilution of such a mixture it is possible to recover active virus. It has also been shown that a serum-virus mixture may produce no evidence of infection when administered to susceptible animals by one route but may produce typical infection if administered by another route (2, 3, 5–7). On the basis of these results the conclusion has been generally drawn that no union between immune substance and virus occurs *in vitro*. Sabin (8) reached similar conclusions by demonstrating that virus could be recovered from a neutral serum-virus mixture by high speed centrifugation, and Long and Olitsky (9) have recovered vaccine virus from immune animals by cataphoresis.

In most of these instances, however, the treatment of virus with immune serum has resulted in a reduction of the infectious titer of the virus suspension when compared with the effect of normal serum. The reduction of virus titer has been most pronounced in the presence of an excess of immune serum or when a period of several hours' incubation has preceded dilution of a mixture. Certain workers have, therefore, postulated that some type of reaction, agglutination or inactivation, between the immune substances and the virus occurs *in vitro* (1, 10–13).

In studies with human influenza virus cultivated in chick embryo-Tyrode culture medium, it was noted, in agreement with observations

of others, that the addition of immune serum to the medium before the introduction of virus resulted in a failure of the virus to survive or multiply. The fact that the virus was well adapted to growth under artificial conditions which readily lent themselves to a study of the influence of immune serum upon virus in the presence of cells suggested further investigation of the problem. The present report deals, therefore, with certain experiments which have a distinct bearing upon the problem of the reactions between immune serum and virus *in vitro*.

Materials and Methods

The strain of human influenza virus employed was the PR8 strain which has been maintained in chick embryo tissue culture medium through 200 consecutive transfers during a period of 16 to 18 months (14). The strain of swine influenza virus was the S15 strain,[1] transferred to tissue culture medium in which it has been maintained through 150 transfers. The tissue cells consisted of minced 12 day chick embryos in a concentration such that 5 cc. of culture medium contained approximately 200 mg. of minced embryo tissue. The immune sera were obtained from ferrets convalescent from infection with PR8 human influenza virus or from rabbits immunized by means of intraperitoneal inoculation with the virus. Tests for the presence or absence of active virus in a given mixture were made by inoculation of the material to be tested into the nasal passages of anesthetized susceptible mice. The lungs of all the mice, either at the time of death or at the end of the observation period of 9 to 10 days, were examined for gross pathological lesions characteristic of infection by influenza virus.

Centrifugation of cells was performed in the ordinary centrifuge at 2000 to 3000 revolutions per minute. The high speed centrifuge used for sedimentation of serum-virus mixtures was the air-driven one described by Bauer and Pickels (15). In each instance the use of the term "washing" indicates that the sedimented material was resuspended in the wash fluid and this suspension again subjected to centrifugation.

The Effect of Immune Serum upon Tissue Cells

Since only three immunologically active components—virus, cells, and serum—are present under the conditions of study, the effect of immune serum in preventing virus infection must be exerted in one of three ways: the serum must act on the tissue cells, on the virus, or only in the presence of both virus and cells. Sabin (16) has interpreted his results to indicate that the immune substances in serum act primarily upon the tissue cell rather than upon the virus. An experi-

[1] Obtained through the kindness of Dr. R. E. Shope.

ment was conducted, therefore, to determine whether the exposure of the tissue cells to immune serum had any effect, persisting after washing, upon the growth of influenza virus subsequently added.

Two flasks containing 4.0 cc. of Tyrode solution and 0.2 cc. of minced 12 day chick embryo were prepared. To one was added 0.5 cc. of immune ferret serum; to the other, 0.5 cc. of normal ferret serum. After incubation at 37°C. for 24 hours, the cells from each flask were removed by centrifugation at 3000 R.P.M., washed twice with about 8 cc. of Locke's solution, and then reintroduced into flasks containing 4.5 cc. of Tyrode solution. To each flask was added 0.5 cc. of influenza culture virus and the cell-virus suspensions were incubated at 37°C. for 48 hours. The cells were again removed by centrifugation and disrupted by grinding in Locke's solution. Both the suspensions of ground cells and the supernatant fluids of the cultures were tested for their virus content by means of mouse tests.

TABLE I

Growth of Virus in Cultures Containing Cells Treated with Normal or Immune Serum

Cells treated with serum; incubated 24 hours; washed twice; returned to culture medium; and virus then added.

A. Cells treated with immune serum	Cells	d++++	d++++	d++++	d++++
	Supernatant	d++++	d++++	d++++	d++++
B. Cells treated with normal serum	Cells	d++++	d++++	d++++	d++++
	Supernatant	d++++	d++++	d++++	d++++

d++++ = mouse died before 10th day with complete pulmonary consolidation.

It is seen from Table I that multiplication of the virus was supported as well by the cells previously exposed to immune serum as by those treated with normal serum. No evidence was obtained to indicate that immune substances were retained by the cells in sufficient concentration to interfere with the survival or multiplication of the virus.

The Effects of the Order of Addition of Serum and Virus to Cells upon the Subsequent Survival and Multiplication of the Virus

Rivers, Haagen, and Muckenfuss (17), Andrewes (18), and Sabin (16) found that vaccine virus added to a suspension of minced rabbit testis before the addition of immune serum, not only survives but multiplies and produces inclusion bodies in the cells, whereas

when the immune serum is added before the virus the latter neither multiplies nor produces inclusion bodies.   In the present study, while using immune serum of low titer, it was also observed that the order in which serum and virus are added to cells distinctly affects the survival of virus in the cells.   When virus was added to the tissue culture medium before immune serum, virus was found to be present within the cells after a period of incubation of 24 hours at 37°C.   The following protocol illustrates an experiment of this type.

Two flasks (A and B) containing 3.5 cc. of human influenza virus and 0.2 cc. of minced 12 day chick embryo were prepared. They were allowed to stand at room temperature for 1 hour. To flask A was added 0.5 cc. of PR8 immune rabbit serum and to flask B, 0.5 cc. of normal rabbit serum. The cultures were then incubated at 37°C. for 24 hours.

Two additional flasks (C and D) were prepared as follows:

Flask C.   3.0 cc. human influenza virus
0.2 cc. minced embryo
2.0 cc. PR8 immune rabbit serum

Flask D.   3.0 cc. human influenza virus
0.2 cc. minced embryo
2.0 cc. normal rabbit serum

The constituents of flasks C and D were added in the order given. The virus and cells were allowed to stand together at room temperature for several minutes before serum was introduced. After the addition of serum, the two flasks were incubated at 37°C. for 1½ hours.

At the end of the incubation period the contents of each flask were separately centrifuged at 2000 R.P.M. for 30 minutes, the supernatant fluid decanted, and the cells washed twice in physiological salt solution. The washed cells from each flask were ground in 2.5 cc. physiological salt solution and the virus content determined by mouse tests. The supernatant fluids were centrifuged in the air-driven centrifuge at 14,000 R.P.M. for 3 hours. Each sediment, drained free of supernatant fluid, was resuspended in 5.0 cc. of physiological salt solution and the virus content of the suspension was titrated in mice.

The results shown in Table II demonstrate that after 24 hours' incubation at 37°C. in the presence of specific immune serum, no active virus was present in the supernatant fluid although still present in considerable amounts in the cells.   Nevertheless, these cells contained less virus than those incubated in the presence of normal serum.   That the absence of virus in sediments obtained by centrifugation of the supernatant fluid was not due to a residuum of free immune serum in

TABLE II

Effect of Weak Immune Serum Added after the Virus

Titration in mice of virus in cells and in ultracentrifuge sediment of culture supernatant.

Dilution	Supernatant fluid		Cells	
	Immune serum	Normal serum	Immune serum	Normal serum
A. Cells and virus in contact 1 hr. before addition of serum. Cells, virus, and serum at 37°C. for 24 hrs.				
Undiluted	0 0 ++	d+++++ d+++++ d+++++	++ +++ ++	d+++++ ++++ ++++
10^{-1}	0 0	++++ ++++ ++++	+++ ++ ++	d+++++ +++ ++
10^{-2}	0 0	+++ +++ ++	0 + +	+++ ++ ++
10^{-3}	0 0	++ ± +		
B. Cells and virus in contact 5 min. before addition of serum. Cells, virus, and serum at 37°C. for 1½ hrs.				
Undiluted	+ + ++	d+++++ +++++ +++	++ +++ +++	d+++++ d+++++ ++++
10^{-1}	0 0	++++ ++++ +++	+ ++ +	d+++++ ++++ ++
10^{-2}	0 0	+++ +++ ++	0	+++ ++ ++
10^{-3}	0 0	++ + ++		

0 to ++++ = degree of pulmonary consolidation.
d = animal died before 10th day.

the fluid is evident, since the serum diluted 1 in 100 failed to neutralize human influenza virus and under the conditions of the experiment a dilution of serum would be reached outside the effective range. It is interesting to note as well that swine influenza virus used as an additional control was apparently unaffected by the PR8 immune serum.

In a second type of experiment the same immune rabbit serum as used in the previous experiment was incubated with human influenza virus for 30 minutes at 37°C. prior to the addition of embryonic cells. Under these conditions there is equal opportunity for free antibody and free virus to act upon the cell.

To a flask containing 4.0 cc. of Tyrode solution was added 0.5 cc. of culture virus and 0.5 cc. of immune rabbit serum; to another, 0.5 cc. of virus and 0.5 cc.

TABLE III

Effect of Weak Serum When Incubated with the Virus for 30 Minutes before Addition of Cells

Serum and virus incubated 30 minutes. Cells added and incubated 2 hours. Cells removed, washed twice, and added to medium containing fresh cells. Incubated 24 hours. Cells ground in supernatant fluid and tested in mice for virus content.

	Degree of pulmonary consolidation			
PR 8 immune rabbit serum	0	0	0	0
Normal rabbit serum	d++++	d++++	d++++	d++++

of normal rabbit serum. Both were incubated at 37°C. for 30 minutes, and then to each was added 0.2 cc. of minced 12 day chick embryo. After 2 hours' incubation at 37°C., the cells from each were removed by centrifugation at 2000 r.p.m., washed twice in Locke's solution to remove excess serum, and then added to flasks containing 4.5 cc. of Tyrode solution and 0.2 cc. of fresh embryonic cells but no serum. The secondary cultures were incubated at 37°C. for 48 hours, the cells removed, ground with sand, and resuspended in the supernatant fluid. The resultant suspensions were tested for virus by inoculating them into mice.

The results given in Table III show that under these conditions virus exposed to the action of immune serum failed to multiply or survive in the cells.

The results of the foregoing experiments, in which serum of low

neutralizing titer was used, agree with the conclusions of other investigators, that when virus is added to cells before immune serum the virus survives in the cells although in a lesser concentration than in the presence of normal serum. On the other hand, when virus was first mixed with immune serum and added to the cells after incubation, no evidence of survival or multiplication of the virus was obtained. In fact, the virus appeared to be completely inactivated.

The Effect of Serum of High Neutralizing Capacity

The preceding observations suggested that even with a serum of comparatively low antiviral titer, a detrimental effect upon multiplication of virus was exerted. In the following experiments convalescent ferret serum was used. The titer of this serum was such that a dilution of 1 in 200 mixed with undiluted tissue culture virus prevented the development of pulmonary lesions in mice inoculated with the mixture intranasally.

Using this serum, the experiments concerned with the effect of immune substances added to the cells before or after the introduction of virus were repeated.

To one of four flasks, each containing 0.2 cc. of minced 12 day chick embryo in 4.0 cc. of Tyrode solution, was added 0.5 cc. of PR8 immune ferret serum, and to another 0.5 cc. of normal ferret serum. The other two flasks each received 0.5 cc. of PR8 strain of culture virus. All four flasks were incubated at 37°C. for 1 hour. Then to each of the first two was added 0.5 cc. of virus; to the third, 0.5 cc. PR8 immune ferret serum; and to the fourth 0.5 cc. of normal ferret serum. All four were again incubated at 37°C. After 24 hours the cells were separated by centrifugation, washed twice in Locke's solution, and ground in 5.0 cc. of Locke's solution. Each suspension was tested for virus by the intranasal inoculation of mice.

The results are given in Table IV. Under these conditions no virus was demonstrable in the washed cells after 24 hours' incubation in the presence of immune serum, regardless of whether the serum was added 1 hour before or 1 hour after the cells were exposed to the virus.

That the action of the immune serum is not only on the virus in or adherent to the cells, but is also exerted on that contained in the fluid portion of such "tissue cultures" is evident from the results of an experiment recorded in Table V.

TABLE IV

Effect of Strong Immune Serum Added to Cells before and after the Addition of Virus

	Degree of consolidation of mouse lung			
A. Immune serum 1 hr. before the virus	0	0	0	0
B. Normal serum 1 hr. before the virus	d++++	d++++	++++	++++
C. Virus 1 hr. before immune serum	0	0	0	0
D. Virus 1 hr. before normal serum	d++++	d++++	d++++	++++

TABLE V

Effect of Strong Immune Serum When Incubated with Virus for 15 Minutes before Addition of Cells

Titration of virus in cells and in washed ultracentrifuge sediment of culture supernatant fluid.

	Concentration in terms of original volume				
	10^1	Undiluted	10^{-1}	10^{-2}	10^{-3}
A. Sediment of culture super-natant					
Immune serum	0	±	0	0	
	0	±	0	0	
	0	+	0	0	
	0	0	0	0	
	0	0	0	0	
Normal serum	d++++	d++++	d++++	d++++	
	d++++	d++++	++++	+++	
	d++++	d++++	+++	++	
	d++++	d++++	+++	0	
	+	d++++	+++	+++	
B. Washed cells					
Immune serum		0	0	0	0
		0	0	0	0
		0	0	0	0
		0	0	0	0
		0	0	0	0
Normal serum		d++++	++++	Died	±
		d++++	++++	+++	0
		d++++	++++	+++	+
		++++	+++	++	++
		++++	+++	++	±

PR8 strain of culture virus (0.5 cc.) in 4.0 cc. of Tyrode solution, was incubated with 0.5 cc. of serum for 15 minutes. Then to each flask was added 0.2 cc. of minced 12 day chick embryo and the whole again incubated at 37°C. After 24 hours the cells were separated from the supernatant fluid by centrifugation, washed twice in Locke's solution, ground with sand in meat infusion broth, and the surviving virus titrated in mice. The supernatant fluids were centrifuged in an 8 inch rotor at 14,000 R.P.M. for 3 hours. The sediment was washed once in broth and tests for active virus in the sediment were made by the intranasal inoculation of the material into mice.

After exposure to immune serum, no virus was detected in the cell emulsion or to any significant extent in the sediment obtained from the supernatant fluid.

Experiments such as those already recorded are open to the criticism that the effect may be due to an action of the immune serum which prevents the virus from entering the cells, thus depriving the virus of a protective medium in the cells and subjecting it, therefore, to the destructive effect of a temperature of 37°C. Consequently, it seemed desirable to determine whether the effect of the immune serum can be exerted in the absence of cells and without prolonged incubation. To test this possibility, culture virus was incubated with serum at 37°C. for 30 to 40 minutes, the virus separated and washed free of excess serum, and the washed virus inoculated into mice. The following protocol gives the plan of such an experiment.

Flask A. 2.0 cc. PR8 culture virus
          0.5 cc. PR8 immune ferret serum
Flask B. 2.0 cc. PR8 culture virus
          0.5 cc. normal ferret serum
Flask C. 2.0 cc. Tyrode solution
          0.5 cc. PR8 immune ferret serum
Flask D. 2.0 cc. PR8 culture virus
          0.5 cc. normal ferret serum

All four flasks were incubated for 35 minutes, after which the contents of each were centrifuged in the 8 inch rotor at 14,000 R.P.M. for 3½ hours. The supernatant fluids were discarded and the sediments washed twice in meat infusion broth (pH 7.8), and then suspended in 4.0 cc. of Tyrode solution, the sediments of C and D being combined to serve as a control for the possible sedimentation of the immune bodies of the serum. A portion of each of the three suspensions was diluted and the virus content titrated in mice. The remainder (3.0 cc.) of each was added to a 50 cc. Erlenmeyer flask containing 0.2 cc. of minced 12 day chick

embryo in 1.0 cc. of Tyrode solution. After 48 hours at 37°C., the cells from each of these cultures were ground with sand in their own supernatant fluids and the resultant suspensions tested for virus by mouse inoculation.

The results are given in Table VI. Such experiments seem unequivocal and indicate clearly that after 30 minutes' incubation at 37°C., immune serum affects the virus in such a way as to render it noninfectious for mice and to prevent its multiplication in the presence of

TABLE VI

Inactivation of Virus after Incubation with Strong Immune Serum for 30 Minutes in Absence of Cells

Dilution	Immune serum	Normal serum	Virus and sediment of immune serum
A. Titration of virus in the twice washed ultracentrifuge sediment			
Undiluted	0 0 0	d++++ d++++ d++++	d++++ d++++ d++++
10⁻¹	0 0 0	d++++ d++++ d++++	d++++ d++++ d++++
10⁻²	0 0 0	++++ ++ ++	++ +++ ++++
B. Titration of virus in 48 hr. tissue culture of the washed ultracentrifuge sediment			
Undiluted	0	d++++	d++++
	0	d++++	d++++
	0	d++++	d++++
	0	d++++	d++++
10⁻¹	0	d++++	d++++
	0	d++++	d++++
	0	d++++	d++++
	0	++++	d++++

susceptible cells. Normal serum or the sediment from immune serum was without effect.[2]

DISCUSSION

Most of the studies concerned with the question of union between antibody and virus *in vitro* have been carried out by means of the dilu-

[2] Further experiments have been completed in which it was found that the washed sediment obtained after centrifugation of the immune serum-virus mixture did not inactivate small amounts of added virus.

tion phenomenon in neutral or subneutral mixtures. Nevertheless, through all of them runs the suggestion that some reduction of virus titer occurs if the mixtures are incubated for 1 hour or more. This effect was more pronounced when immune serum was present in excess, frequently resulting in a failure to obtain reactivation. Sabin (8, 16), using high speed centrifugation for separation of vaccinia and pseudo-rabies viruses from serum-virus mixtures, has extended the findings obtained with other technics and has concluded, as have other workers, that no union between virus and protective substances occurs *in vitro* and that prevention of infection of susceptible cells is more closely related to fixation of immune substances by the cell.

The experiments reported were carried out with a strain of human influenza virus fully adapted to maintenance in tissue culture medium and with centrifugation at 14,000 R.P.M. in an air-driven high speed centrifuge for recovery of virus from the liquid portions of the medium.

The results of the present studies with the virus of human influenza have shown clearly that following incubation in the presence of sufficient antibody, virus is inactivated so that it is no longer detectable in sediments of immune serum-virus mixtures even after repeated washings and centrifugation. Under similar conditions in the presence of normal serum, virus so treated is fully infectious for mice. Furthermore, in the presence of cells, the virus is rendered inactive by a potent immune serum, no matter whether the cells are exposed first to virus or immune serum. These results cannot well be interpreted as due to an action of the immune substances on the tissue cells, since cells treated with immune serum and subsequently washed, adequately support the multiplication of the virus. They indicate rather that a pronounced effect of immune serum is exerted directly upon the virus. Procedures such as high speed centrifugation, which readily throws out the virus from normal serum-virus mixtures, fail to disrupt any association which may have occurred between immune substances and virus. Whether cells are required for the final disposal of the inactivated virus cannot be stated upon the basis of the present experiments. If cells are essential, it would appear that their function is to complete the destruction of the virus rendered inactive by the immune substances of the serum.

SUMMARY

Studies have been conducted on the effect of immune serum upon a strain of human influenza virus (PR8) grown in chick embryo tissue culture medium. The results have demonstrated (a) that when cells are exposed to the action of immune serum of high titer and subsequently washed freely, these cells support the growth of virus as well as cells treated with normal serum; (b) that, in agreement with the results of other workers, when virus is added to cell suspensions before the addition of immune serum of low titer, virus survives in the cells; (c) that when mixtures of immune serum of low titer and virus are added to cells, there is little evidence of survival or multiplication of the virus. Furthermore, when immune serum of high titer is used the virus is inactivated regardless of whether the cells are first exposed to virus or immune serum. Finally, virus mixed with a strong immune serum is inactivated in the absence of cells, as shown by the fact that centrifugation at high speeds of such serum-virus mixtures yields no active virus, whereas normal serum-virus mixtures yield fully active virus.

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