ADSORPTION OF INFLUENZA VIRUS ON CELLS OF THE RESPIRATORY TRACT

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(Received for publication, May 14, 1943)

Influenza virus can be adsorbed onto various substances, for example, protamine (1), the amorphous precipitate of freshly thawed allantoic fluid (2, 3), and aluminum hydroxide (4). In none of these cases was the reaction shown to be reversible without a change in the conditions under which the adsorption took place. Some time ago it was demonstrated that influenza virus could be adsorbed onto avian red cells (5), and the striking and peculiar thing about this adsorption was that the virus was released from the red cells after a time, although the conditions under which adsorption took place were not altered. The release of virus was accompanied by inactivation or destruction of the red cell receptor substance, as illustrated by the fact that the cells became incapable of adsorbing further virus. This reaction between red cells and influenza virus was so unusual that it seemed likely that a kindred phenomenon might play some rôle in the pathogenesis of natural infection with this virus. In order to investigate this possibility the experiments herein reported were performed. They concern mainly the reaction between influenza virus and the respiratory epithelial cells of the ferret. These cells are particularly convenient to work with and are undoubtedly susceptible cells in laboratory or contact influenza infection of this animal.

Methods

Titrations of Hemagglutinins and Virus.—The virus strains used were the PR8 strain of influenza A virus (6) and the Lee strain of influenza B virus (7). All of the virus material used was prepared from allantoic fluid by the method which is routine in this laboratory (5). The tests for agglutination titer were done with the aid of a photoelectric cell by the method described by Hirst and Pickels (8). 50 per cent red cell sedimentation was used as the arbitrary end point of titrations, and titers were expressed in terms of the dilution factor necessary to reach this end point (reciprocal of the dilution). The titrations of virus in mice were done by the usual method.

Perfusion of Mammalian Lungs.—In most of the experiments, which were done with anatomically intact lungs, the organs were perfused *in situ* before they were removed from the thorax. The animal was put under deep ether anesthesia, and the chest cavity was opened widely and quickly with scissors, but carefully to avoid large blood vessels. A 19 gauge needle, connected by a rubber tube to a large elevated flask of saline, was then inserted into either the right auricle or right ventricle. Saline was

allowed to flow in under low pressure (20 cm. of water), and at the same time a large incision was made into the left ventricle. Under these conditions the lungs became quite pale in 1 or 2 minutes, but perfusion was continued until the perfusate was quite free of blood. Before the perfusing needle was removed, the systemic vessels leading to and from the heart were clamped and tied off to prevent any backflow of blood into the pulmonary circuit. The lungs were then dissected out together with the trachea as far as and including the larynx. Usually all the lobes were pale, almost white in color with no evidence of remaining blood. Whenever such perfused lungs were ground in saline, the supernatant fluid was not colored with hemoglobin. Though not perfused by this method, the trachea, being nearly avascular, did not introduce appreciable blood into the system.

Tests for the Behavior of Virus in Perfused Lungs.—After excision of the perfused lungs, the larynx and end of the trachea were slipped over a small glass tube inserted in a one-hole rubber stopper. The trachea was fixed to the glass tube by thread tied tightly about the upper portion. With the lungs suspended from this tube, the stopper was placed in an Erlenmeyer flask of 1000 cc. capacity, to the side arm of which was attached a long rubber tube and mouthpiece by which the pressure in the flask could be regulated. Virus suspensions were put into the lungs by means of a 30 cc. syringe and 5 inch 20 gauge needle. The point of the needle was inserted through the trachea to a point just above the bifurcation. The fluid was slowly injected in order to prevent bubbles from being carried down into the lungs. As the fluid was added, the lungs gradually expanded, and usually 25 to 35 cc. were introduced without difficulty. Generally, the lower lobes filled first, and if the fluid was added too rapidly, large bullae formed on the margins of the lower lobes, causing considerable leakage. The leakage of a small amount of fluid (2 to 5 cc.) over a period of hours generally could not be prevented even with extreme care. Occasionally also the upper lobes failed to fill with fluid, but this was not often troublesome.

After the lungs were filled with fluid and before each sample was taken, alternate pressure and suction were applied to the flask by means of the mouthpiece. This brought the fluid alternately up into the trachea and down below the bifurcation, and caused a certain amount of mixing so that the samples were more nearly representative of the fluid in the entire lung. For removing samples from the lung the 5 inch needle was again inserted in the trachea and the point placed just above the bifurcation. By applying pressure to the flask the fluid level was maintained above the tip of the needle while a sample was aspirated. After the sample was removed the fluid level was lowered below the tracheal bifurcation by suction, following which the pressure in the flask was returned to that of the atmosphere. All experiments with perfused lungs were carried out in an incubator room at 37°C. In some experiments a buffer solution was used to wash out the virus from the lungs. For this purpose a dilute phosphate buffer (M/100, pH 7.2) in physiological saline was used throughout.

EXPERIMENTAL

The Reaction between Influenza Virus and the Cells of Perfused Ferret Lungs.—When ferret red cells were thoroughly washed, they agglutinated almost

equally well with the PR8 and Lee strains of influenza A and B virus[1] and their reactivity in this respect was comparable to that of human or guinea pig red cells. Ferret red cells also efficiently adsorbed these two strains. In studying the reaction between influenza virus and ferret pulmonary cells it was clearly necessary to eliminate red blood cells completely from the system, and for this reason all ferret lungs were perfused before use.

In the first experiment, the data for which are expressed graphically in Fig. 1, perfused lungs from normal ferrets were used. 35 cc. of allantoic fluid contain-

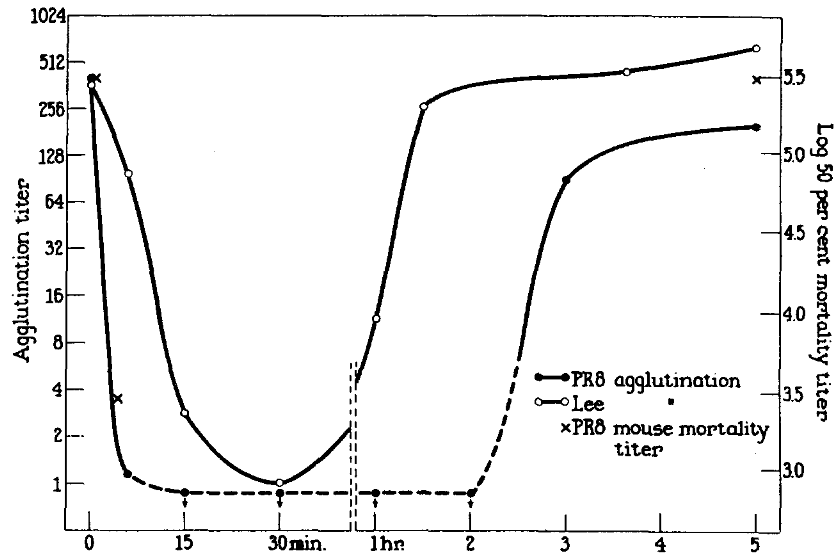

FIG. 1. Agglutination and mouse lethal titrations on suspensions of PR8 and Lee virus inoculated intratracheally in excised perfused ferret lungs. The broken line connects points at which the agglutination titer was too low to be measured.

ing a high titer of PR8 virus was injected as described under Methods. Samples were removed from the lung at intervals, and the agglutination titer of the various specimens was determined at one time. In addition the 50 per cent mouse mortality titer was measured for the original material, the 5 minute, and 5 hour samples. The data show that there was an extremely rapid adsorption of the virus present in the allantoic fluid. Less than 1 per cent remained unadsorbed after 5 minutes, whether measured by mouse mortality or agglutination. On subsequent samples up to 2 hours the agglutination titer was too low to be detected by our method. At 3 hours an appreciable amount of hemag-

[1] Some mammalian red cells, such as those from the *Macacus rhesus* monkey, agglutinate well with the Lee but not with the PR8 strain.

glutinin reappeared in the allantoic fluid, and still more at 5 hours. The lethal titer of the final sample for the mouse was almost back to its original level. Since with the removal of each sample progressively less fluid was left in the lung, the elution of active particles from the respiratory cells took place in a volume considerably less than the original. When this factor was taken into account, it was calculated that about one-fourth of the originally adsorbed virus was released in 5 hours' time.

In order to extend the analogy of this reaction to that of avian red cell adsorption, the same experiment was repeated with the Lee strain of influenza B virus, the results of which are also shown in Fig. 1. The agglutination titer of the Lee preparation was of the same order as that of the PR8 suspension just described. The adsorption of this strain was somewhat less rapid than that of PR8 virus, maximum adsorption not being achieved for 30 minutes. While the degree of adsorption at the maximum point was less than with the PR8

TABLE I

Adsorption and Release of PR8 Virus from Perfused Mouse and Rabbit Lungs As Tested by the Agglutination Reaction

	Control	Time after inoculation into lungs		
		5 min.	2 hrs.	8 hrs.
	Agglutination titer			
Mouse	256	<8	20	84
Rabbit	239	32	37	169

strain, nevertheless over 99 per cent of the hemagglutinin was removed from the allantoic fluid. The elution phase was more rapid and complete than with the PR8 strain and the final sample, removed after 5 hours, had a titer of 632 as compared with the original titer of 362. This again was due to the fact that the virus was eluted into a volume far less than the original. When corrections for change of volume were made, it was found that about 75 per cent of the hemagglutinin which was at one time adsorbed onto the respiratory epithelium reappeared in the allantoic fluid.

The same kind of experiment was performed with PR8 virus and perfused mouse and rabbit lungs (Table I). With a perfused mouse lung preparation 1.5 cc. of PR8 allantoic fluid was inoculated intratracheally with a hypodermic needle. This was aspirated at 5 minutes and was replaced with 1.5 cc. of dilute phosphate buffer. The buffer was removed at 2 hours and replaced with more buffer which was finally removed 8 hours after the beginning of the experiment. All the samples were titrated simultaneously, and the data in Table I show that the behavior was strikingly similar to that of ferret lung preparations. Over 95 per cent of the virus inoculated was adsorbed in 5 minutes, and in 8 hours about

one-third of the adsorbed virus was released. A similar experiment was performed with a perfused rabbit lung preparation with essentially the same results although here the degree of adsorption was not nearly so marked. Once again, however, elution of adsorbed virus was demonstrated.

Adsorption of Inactive Virus onto the Cells of Perfused Ferret Lungs—A preparation of PR8 virus was heated at 56°C. for 30 minutes. This treatment resulted in a loss of approximately 60 per cent of the agglutinating activity and rendered the suspension non-infectious for mice. 25 cc. of the heated suspension was inoculated intratracheally into a perfused ferret lung, and samples were removed at intervals. The agglutination activity of these samples is shown in Table II. There was a rapid adsorption of hemagglutinin, but even after 5 hours there was no evidence of release of activity from the cells. A

TABLE II

Effect of Formalin and Heat on Adsorption and Release of Influenza Virus from Cells of Perfused Ferret Lungs

Virus strain	Treatment	Control	Time after inoculation into lung, *min.*					
			6	15	30	60	120	300
		Agglutination titer						
PR8	Untreated	632	<2	<2	<2	<2	417	447
	Heated 56°C.	194	<2	<2	<2	<2	<2	<2
	Formalin 0.1 per cent	97	<2	<2	<2	<2	<2	<2
Lee	Untreated	182	21	11	6	39	84	388
	Heated 56°C.	120	<2	<2	<2	<2	<2	<2
	Formalin 0.1 per cent	74	11	<2	<2	<2	<2	<2

similar result was obtained with PR8 virus inactivated by the addition of 0.1 per cent formalin, 48 hours before use. Heated and formalinized Lee virus also showed no evidence of release after adsorption on ferret lung.

This behavior was somewhat different from that obtained when similarly inactivated influenza virus was allowed to act on avian red cells. As previously observed (5) heated and formalinized PR8 virus is capable of adsorption and release from avian red cells. The same was found to be true of formalinized Lee virus. Heated Lee virus, however, was not detectably eluted after adsorption on avian red cells. The experiments with ferret lung do not coincide therefore with the results of red cell adsorption. Whether or not ferret lung requires infectious PR8 virus for the occurrence of virus release cannot be answered at present. It seems quite possible that some less drastic method of virus inactivation may be found which will do away with the infectivity of the PR8 strain and leave the adsorption-release reaction with pulmonary cells intact.

The Adsorption of Influenza Virus in the Living Ferret.—After it was shown that influenza virus was adsorbed onto and released from the respiratory cells of excised lungs, it was decided to attempt to find out what happened when virus was inoculated into the lungs of living ferrets. For this purpose our methods had to be somewhat modified. It was necessary to use concentrated suspensions of virus since it was not feasible to inoculate living animals with large volumes of material. It was also necessary to kill the ferret in order to attempt to recover inoculated virus, so that each animal was used for only one period of exposure to the agent.

Allantoic fluid containing the PR8 strain was centrifuged for 1 hour at 12,000 R.P.M. in an angle centrifuge. The pellets containing virus were resuspended in 1/20 the original volume, using supernatant allantoic fluid as a diluent. 2 cc. of this concentrate, which had an agglutination titer of 4000, was inoculated intranasally into each of 5 ferrets under light anesthesia. After varying intervals the animals were reanesthetized, the lungs perfused, excised, and 10 cc. of dilute phosphate buffer injected intratracheally. 5 minutes after introducing the fluid a sample was taken. Thereafter the lung preparation was kept at 37°C., and a final sample was removed 5 hours after the lungs were excised. The animals were sacrificed after periods varying from 10 minutes to 24 hours. The perfusion took about 15 minutes to perform so that the first sample from the lung was taken 20 minutes after the death of the ferret. When all the samples were assembled, having been stored at 4°C., they were titrated simultaneously for agglutination titer, and the data are shown in Table III. For purposes of convenience the titers of the inoculum and samples were all corrected to a constant volume, thus making all the values directly comparable in terms of the amount of virus injected and recovered. Each ferret was utilized for two determinations: (*a*) a measure of the agglutinin adsorbed by the lung in the living animal in a given length of time, as shown by the titer of the initial sample (20 minutes after death); and (*b*) the amount of adsorbed virus which could be released from the excised lung, as shown by the titer of the sample taken 5 hours after excision. Since the virus in this experiment

TABLE III

Adsorption and Release of Influenza Virus by Lungs of Living Ferrets

Virus strain	Titer of inoculum	Time of sample after excision of lung	Time of exposure of virus to living lung				
			10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.
			Agglutination titer*				
PR8	4000	5 min.	<2	<2	<2	trace	120
		5 hrs.	512	72	<2	trace	120
Lee	632	5 min.	<2	<2	<2	<2	<2
		5 hrs.	256	trace	<2	<2	<2

* Figures indicate the agglutination titer which would have been found if all the free virus in the lung were contained in a volume of 2 cc., the same volume as the inoculum.

with the living ferrets was introduced by inhalation and not by injection, the results were not so easily reproduced as in the first experiment with the excised lungs, and many of the points in the experiment have been repeated.

As can be seen by the data in Table III, there was a rapid adsorption of virus by the lung of the living ferret, and unlike the behavior in the excised lung the virus was not significantly released in 4 or 8 hours. After 8 hours in the living ferret only a faint trace of free agglutinin was found but at 24 hours agglutinin was again free in appreciable quantity and was obtained by the simple infusion of fluid. The samples taken 5 hours after lung excision show that virus which had been in contact with the living lung for a brief period (10 minutes), even though it was completely adsorbed, still was capable of release from the excised lung. After 2 hours' contact between living lung and virus, much less agglutinin was released in the excised lung, and after 4 hours' contact there was no detectable release. Essentially the same results were obtained with influenza B virus in living ferrets.

This experiment shows that adsorption of virus in a viable lung takes place much as it did in the excised lung. However, after prolonged contact between the cells and the virus, the adsorbed material became in some way more firmly fixed so that the agglutinin was no longer released by simple methods. This change in degree of fixation was not found in excised lungs.

Free agglutinin was found in the first sample from the PR8 ferret killed 24 hours after inoculation. This may well have been due to multiplication of the virus within the cells, with subsequent breakdown of the cell walls and release of the infective agent.[2] The free virus at 24 hours may be analogous to the eluted virus obtained with excised pulmonary tissue in a shorter interval. The presence of free agglutinin and virus at 24 hours suggests that the cell receptors normally present may have been destroyed.

One side observation during the above tests seems worthy of note. When ferrets were given 2 cc. of unconcentrated allantoic fluid intranasally very little gross pulmonary change was visible for 24 hours. When ferrets were given 2 cc. of the concentrated preparations, however, they were found to develop quite marked hemorrhagic lesions in the lower lobes in as short a time as 2 hours. The hemorrhagic areas frequently did not perfuse well, and on several occasions such lungs yielded fluid which contained red cells. The presence of even a small amount of blood may have masked the elution of hemagglutinins in some preparations, if the eluted virus were small in amount.

The Reaction between Influenza Virus and Ground Tissue Suspensions.—We have attempted to demonstrate the adsorption and release of influenza virus from suspensions of ground tissue, prepared from perfused lungs and livers. Such reactions with ground cells did not yield conclusive results. One of the

[2] In mice maximum multiplication of virus may take place in 24 hours.

difficulties was the high content of agglutination inhibitor present in certain tissues, as *e.g.* ferret lung.

A perfused ferret lung was ground in saline (20 per cent wet weight suspension) and the larger particles were discarded. The remainder of the suspension was diluted in twofold steps of 0.5 cc. each. To each dilution of lung suspension enough PR8 virus was added to make a final concentration of 4 agglutination units. After mixing, avian red cells were added, and the 50 per cent inhibition titer was measured. It was found that the lung extract inhibited 4 units of agglutinin to a titer of 10,000 (dilution factor in terms of wet weight of ferret lung). Washing the lung particles with saline removed this inhibitor.

TABLE IV

Adsorption of Influenza Hemagglutinins by Ground Cell Suspensions of Lung and Liver

Cell suspension	Virus strain	Time of combination of virus and cell suspension, *min.*						
		0	1	15	30	60	120	300
		Agglutination titer						
Ferret lung	A	538	182	147	97	97	120	84
	B	52	28	23	21	24	37	42
Ferret liver	A	362	39	12	7	4	3.5	4
	B	632	315	158	97	79	112	169
Rabbit liver	A	164	<2	<2	<2	<2	<2	<2
	B	338	84	60	56	60	64	97

Washed suspensions of ferret and rabbit lungs and livers were tested for their capacity to adsorb virus. A suspension of fine tissue sediment (approximately 5 per cent by volume) was mixed with an equal quantity of allantoic fluid. Samples of the mixtures were taken at intervals, and the sediment was removed by centrifugation. All of the samples were then titered for agglutinin content. An experiment with ferret lung and ferret and rabbit liver is recorded in Table IV. Both liver tissues and ferret lungs showed a marked adsorption of PR8 virus without evidence of release, while B virus was adsorbed less well but was released to a slight extent. Some tissues such as washed human red cell stroma showed marked adsorption of PR8 and very little or no adsorption of Lee with no detectable release of either virus.

These experiments were very inconclusive except to suggest that in perfused liver, as well as in pulmonary cells, some analogue of the red cell receptor substance may have been present, giving the slight degree of virus release. They also suggest that with fragmented cells the adsorption of virus may resemble that occurring with protamine or aluminum hydroxide more closely than that occurring with intact red blood and respiratory cells. The efficiency of

ferret pulmonary cells in adsorbing virus was markedly reduced by grinding and washing.

DISCUSSION

In the first experiment described, in which the reaction between perfused ferret lung and the Lee and PR8 strains was tested, it was pointed out that the rate of adsorption and release of these two strains was quite different and also that a strikingly similar difference was found in the reaction between these two strains and avian red cells (5). This similarity furnishes some evidence that the two phenomena (virus reaction with red cells and respiratory cells) are fundamentally similar.

In investigating the red cell-virus reaction we observed that after adsorbed virus had been completely eluted, the cells were incapable of adsorbing further virus. While we did not make an analogous test with ferret lung, it seems certain that the respiratory receptor substance was exhausted at the end of 5 hours' exposure to B virus, for example, since after this time the cells within the lung were bathed in fluid containing virus in even higher concentration than the original inoculum. Furthermore, the virus at the end of the experiment behaved like untreated virus in its capacity to agglutinate red cells and infect mice. Hence we may assume that, as with avian red cells, the receptor substance on the surface of the respiratory cell was exhausted or destroyed. This phenomenon presents an even stronger analogy between the red cell and respiratory cell reaction.

One of the most interesting aspects of the adsorption-elution reaction with respiratory cells is speculation concerning the possible rôle of this reaction in natural infection with the virus. We have already shown that in the living ferret the elution phase does not take place, at least not within the same period as in the excised lung. The most obvious explanation for the difference in behavior of virus in intact and excised lungs is that in the former the virus, once it has been adsorbed, goes on and becomes even more firmly fixed, while in the latter the destruction of the receptor substance is the only stage that takes place and hence the virus is released. It seems quite likely that destruction of the receptor substance takes place in the living ferret also, since 24 hours after infection free agglutinin for chicken red blood cells was present in the lung. Incidentally the finding of free agglutinin and virus in the lung 24 hours after infection corroborates Taylor's finding (9) that mice given a sublethal dose of virus died of overwhelming infection when broth or horse serum was instilled intranasally 24 hours after the original infection. The additional fluid could have provided transport for readily elutable virus.

While the data are meager as yet, it is tempting to formulate a tentative hypothesis as to the possible mechanism of the early stages of influenza virus infection of the respiratory cell. It may not be sufficient merely for an inhaled

virus particle to come in contact with any point on a susceptible cell, and it may be necessary for it to become attached to a specific receptor substance to gain entrance. Before the virus can infect, it may also have to alter or destroy this receptor substance by means of an enzyme in order to pave the way for penetration and parasitism of that cell. Once the receptor substance is destroyed the virus becomes more firmly bound, parasitizes the cell, multiplies, and again appears free in the lung making possible spread of infection by contiguity. Since a close correlation has been demonstrated between the neutralizing and agglutination-inhibiting power of various human sera, it may be that neutralization (in mice) consists mainly of covering over that portion of the virus which ordinarily attaches itself to the receptor substance.

The experiments with ground tissues suggest that a substance, similar to the red cell receptor, may be present in tissues other than red cells and respiratory epithelium. Such a situation has been firmly established in respect to other substances, for example, the blood group A substance, where their presence has been demonstrated in many types of mammalian cell besides the red cell.

SUMMARY

A study of the reaction between influenza virus and the cells of the excised and perfused ferret lung has yielded the following results: (1) The cells of the lung rapidly adsorbed large amounts of intratracheally inoculated virus. (2) After a short interval the pulmonary cells began spontaneously to release the adsorbed virus, and in the case of influenza B the release was 75 per cent complete after 5 hours. (3) The Lee strain was more completely released from pulmonary cells after 5 hours than was the PR8 strain. (4) After the cells released the adsorbed virus they appeared incapable of adsorbing virus as before. (5) When the mouse-infecting capacity of the virus had been done away with by heat or formalin, the virus was adsorbed by the pulmonary cells but was not released.

In all except the last of the characteristics listed the interaction between influenza virus and the pulmonary cells closely resembles that between influenza virus and avian red blood cells.

In the living ferret inhaled influenza virus was also rapidly adsorbed by the lung, but in a very short time the adsorbed virus which at first could be readily eluted (after perfusion and excision of the lung) became so much more firmly fixed as not to be released by this method. Free virus could not be demonstrated in the living ferret until 24 hours after the animal had been exposed to the inoculum.

On the basis of these and previous experiments it is postulated that the destruction of a specific receptor substance,—which may involve an enzymatic reaction,—may be a necessary preliminary event in the parasitism of susceptible cells by influenza virus.

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