THE NATURE OF THE VIRUS RECEPTORS OF RED CELLS

IV. Effect of Sodium Periodate on the Elution of Influenza Virus from Red Cells

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(Received for publication, November 5, 1948)

It has previously been shown that some strains of influenza virus (1) are changed in respect to hemagglutination by heating. The Lee strain of influenza B, for example, when heated at 56°C. for an hour undergoes very little loss of hemagglutinin titer and although it is adsorbed well on red cells it is no longer eluted spontaneously. The fact that heated virus fails to be eluted from receptors has made it particularly useful for the detection and titration of receptor analogs in solution (2), and suggests that heated virus may also be useful in studying the forces which attract and fix the virus particle to the red cell. The present work is in part a study of conditions under which heated virus can be removed from cells.

In an earlier paper (3) it was shown that red cells treated with sodium periodate are incapable of adsorbing significant amounts of influenza virus. In the present paper the results of treating the *virus* with periodate are reported. It was found that periodate treatment of the virus had an effect similar to that of heating, in that the virus retained its properties of adsorption on red cells and hemagglutination but lost the capacity for self-elution. Differences were found between the two types of treated virus which were reflected in the manner in which they could be removed from cells.

Methods

The only virus employed was the Lee strain of influenza B, prepared by inoculation of seed virus into the allantoic sac of 11-day-old embryonated eggs, and harvesting after 2 days' incubation at 35.5°C. Virus suspensions were dialyzed against 0.11 M phosphate buffer of pH 7.2 in order to remove substances which precipitate from allantoic fluid on standing and an agent which preserves the virus from the action of sodium periodate.

Agglutination titrations were done with the aid of a photometer by methods previously described in detail (4). Titers given in a single experiment have been corrected for dilution of the virus so that the results are directly comparable. Agglutination inhibition tests were done by a method described previously (1).

Periodate solutions were prepared by dissolving $HIO_4 \cdot 2H_2O$ in distilled water and neutralizing with sodium hydroxide. Unless otherwise stated periodate concentrations are in terms of final molarity after mixing with virus but before addition of glucose. Whenever periodate was added to virus, cells, serum, or inhibitor, the excess of reagent was reduced by addition of glucose before further procedures were undertaken. The treatment of virus with sodium

periodate was always controlled by adding a mixture of glucose and periodate to an aliquot of the virus suspension. Since virus treated with glucose-reduced periodate behaved like untreated virus only the former control has been included in the protocols.

The term "heated virus" refers to suspensions which have been heated at 56°C. for 30 to 60 minutes.

Cholera vibrio filtrate was prepared using the Inaba strain (35A3) obtained from the National Institute of Health. The organisms were cultured in peptone water, being removed after 24 hours' incubation by centrifugation and Berkefeld filtration.

EXPERIMENTAL

Effect of Sodium Periodate on the Spontaneous Elution of Influenza Virus from Red Cells

Heating of the Lee strain of influenza B at 56°C. resulted in a slight drop in hemagglutinin titer, but there was no spontaneous elution of the adsorbed virus from the red cells (1). Influenza virus after periodate treatment also failed to elute from red cells.

One volume of virus suspension and 1 volume of sodium periodate solution (at four concentrations) were combined and after 30 minutes at room temperature were treated with 1 volume of 5 per cent glucose to reduce any excess reagent. A sample was taken for hemagglutinin titration. Control virus was treated with sodium periodate previously reduced with glucose. Equal volumes of treated virus and 2 per cent fowl red cells were combined after cooling each to 4°C. After 1 hour the red cells were removed by centrifugation, washed once with cold saline, and transferred to fresh buffer solution, equal in volume to that of the original virus suspension. The resuspended cells were incubated at 37°C. for 3 hours and the supernatant fluid was removed. The originally treated virus suspensions, the supernatants after red cell adsorption at 4°C. and after incubation at 37°C. were tested at one time for hemagglutinin titer. The effect of different concentrations of periodate on the viability of the virus was tested by inoculation of a 10^{-3} dilution of the mixtures into the allantoic sac of 11-day-old chick embryos. Results are shown in Table I.

The virus in all preparations was adsorbed well on red cells at 0°C. At 37°C. the control virus and that treated with M/20,000 periodate eluted well, about 85 per cent of the adsorbed virus being recovered. Only partial elution occurred when the virus was treated with M/2000 periodate and there was no detectable elution of virus treated with larger amounts. In this respect virus treated with periodate at concentrations of M/200 or more behaved like heated virus. Periodate reduced with glucose had no effect and hence it is probable that the loss of ability to elute spontaneously was due directly to periodate and not to products following its reaction with glucose.

As shown in the first line of Table I, periodate added to influenza virus in a concentration of M/2000 or more resulted in an increase in hemagglutinin titer as great as twofold. No explanation of this increase is offered but it occurred consistently when enough periodate was used to affect elution. The permanence of union between periodate-treated virus and cells was also reflected in the type of agglutination seen. Red cells agglutinated by normal virus formed

clumps which were very delicate and were broken by the slightest shaking of the tube while clumps formed with periodate-treated virus were very firm and were broken up only with difficulty. As can be seen from the figures at the bottom of Table I there was a correlation between the effect of periodate on spontaneous elution and on the viability of the virus.

TABLE I

Effect of Sodium Periodate on the Spontaneous Elution of Influenza Virus from Red Cells

	Virus treated with $NaIO_4$ in concentration:				
	M/40*	M/200	M/2,000	M/20,000	Untreated
Titer before adsorption with cells	223	256	169	120	120
Titer after adsorption with cells	24	20	28	15	13
Virus units adsorbed	199	236	141	105	107
Virus units eluted	<2	<4	52	91	91
Virus eluted, *per cent*	<1	<2	37	86	85
Infectivity for chick embryos of virus after periodate treatment	0/4	0/4	1/4	4/4	4/4

Equal quantities of Lee virus suspension and periodate solution were combined and the periodate concentration given is the final concentration. After 30 minutes 1 volume of 5 per cent glucose was added to 2 volumes of the virus periodate mixture. To the control (untreated) was added a corresponding amount of periodate which had been previously inactivated with glucose. For the infectivity tests the allantoic fluid was diluted to 10^{-3} with phosphate buffer and combined with an equal amount of periodate. 11-day-old chick embryos were inoculated in the allantoic sac and the allantoic fluid tested for agglutinins after 2 days. The numerator shows the number of embryos showing positive evidence of infection over the number of embryos tested.

* Final concentration of periodate after mixture with virus.

Effect of Cholera Vibrio Filtrate on Elution of Periodate-Treated Virus

Briody (5) showed that heated virus which had been adsorbed on red cells could be eluted by means of a cholera vibrio filtrate. It had previously been found that such a filtrate removed the receptors of red cells (6) in a manner analogous to that of influenza virus. The following experiment was performed to determine whether cholera vibrio filtrate could remove periodate-treated virus from red cells.

Lee virus treated in four ways was used in this experiment: (1) Two volumes of virus were combined with 1 volume of M/20 sodium periodate and after 30 minutes with 1 volume of 5 per cent glucose. (2) Virus was heated at 56°C. and diluted with 1 volume of buffer. (3) Heated virus was treated with periodate as in (1). (4) One volume of control virus was combined with 1 volume of a mixture consisting of equal parts M/20 sodium periodate and 5 per cent glucose. All suspensions were chilled to 0°C. and sufficient packed fowl red cells

were added to make a final concentration of 1 per cent. After 30 minutes the cells were removed by centrifugation, washed once in cold saline, and divided into portions which were suspended in either phosphate buffer or cholera vibrio filtrate, in a volume equal to half that of the original virus suspension. The resuspended cells were incubated 3 hours at 37°C. and the supernatant fluids recovered. Samples of the originally treated virus, the supernatants after adsorption at 0°C. and after incubation at 37°C. were tested for hemagglutinin titer. The results are given in Table II.

TABLE II

Effect of Cholera Vibrio Filtrate on the Elution of Heated and Periodate-Treated Virus Which Has Been Adsorbed on Red Cells

	Virus treated:			
	Untreated	Heated 56°C. 1 hr.	$NaIO_4$ M/60*	Heated 56°C. and $NaIO_4$ M/60
Titer before adding cells	97	74	194	128
Titer after adsorption with cells	9	<8	18	<8
Units virus adsorbed	88	>66	176	>120
Units eluted into buffer	74	<2	<2	<2
Units eluted into cholera vibrio filtrate	84	>60	<2	<2

To 2 volumes of virus was added 1 volume of periodate (columns 3 and 4) followed after 30 minutes by 1 volume of 5 per cent glucose. A corresponding amount of periodate previously mixed with glucose was added to the untreated control (column 1) and of buffer to the heated virus. Packed cells were then added to a final concentration of 0.5 per cent. After adsorption for 30 minutes at 0°C. the cells were removed and added to a volume of phosphate buffer equal to the original volume of virus. Elution was carried out at 37°C. for 3 hours. The titers were all corrected for volume changes and may be compared directly.

* Final concentration of periodate after mixture with virus.

The virus from all four preparations was adsorbed well on red cells. As in previous experiments only the control virus eluted at 37°C. In confirmation of Briody (5), virus which had been heated at 56°C. failed to elute spontaneously from cells suspended in buffer but eluted almost completely from cells in cholera vibrio filtrate. Virus which had been treated with periodate (either with or without heating) failed to elute either in buffer or vibrio filtrate. This was the first of several differences found between the behavior of heated and periodate-treated influenza virus. Similarly a difference was found when native influenza virus was used to promote the elution of heated or periodate-treated viruses.

The Elution of Heated Virus in the Presence of Normal Virus

Since both cholera vibrio filtrate and normal influenza virus have the property of destroying cell receptors, it was of interest to determine whether normal virus, like cholera vibrio filtrate, had an eluting effect on heated virus.

After heating a suspension of Lee virus for 1 hour, packed fowl cells were added to give a final concentration of 1 per cent. The suspension stood at room temperature for 30 minutes, after which the cells were removed by centrifugation and washed once with saline. Five-tenths cc. of packed cells was suspended in 20 cc. each of unheated Lee virus, phosphate buffer, and heated Lee virus. Samples were removed after incubation at 37°C. for 1, 2, and 20 hours, and were clarified and tested for hemagglutinin titer.

The titer of heated and unheated virus before the addition of cells is shown in the first line of Table III. The cells added to each suspension had adsorbed

TABLE III

The Effect of Unheated Influenza Virus on Heated Virus (56°C.) Which Had Been Absorbed on Red Cells

Agglutination titer	Suspension of:		
	Unheated Lee virus	Buffer only	Lee virus heated 56°C. 1 hr.
Before addition of cells	84	—	76
1 hr. after addition of cells	169	<4	74
2 hrs. after addition of cells	256	<4	74
20 hrs. after addition of cells	362	39	158
Excess agglutinins present after subtraction of controls*			
After 1 hr	85		0
After 2 hrs.	172		0
After 20 hrs	239		45

Red cells adsorbed heated (56°C.) Lee virus. After washing aliquots of cells were suspended in unheated Lee virus, in buffer, and in heated (56°C.) Lee virus. At 1, 2, and 20 hours the cells were removed from samples and tested for agglutinin titer.

* These are figures from the upper part of the table after subtraction of the agglutinins present in the virus preparations before the addition of cells and of the agglutinins spontaneously eluted in buffer. Had all of the adsorbed agglutinins been released the titers here would be 325.

sufficient virus, that were it all eluted, the titer in the test would have increased by 325. After 2 hours there was no significant elution of virus from the cells suspended in buffer, or from cells treated with heated virus. However after exposure of the cells to unheated virus the titer rose from an initial level of 84 to 256. On standing overnight the titer was still further increased but during this period there was some elution of virus in buffer and in the heated virus preparation. The figures in the lower part of Table III show the net titer of hemagglutinin eluted from the cells following incubation with virus, after subtraction of the initial agglutinin and that eluted spontaneously in the buffer blank. About three-fourths of the heated virus adsorbed on the red cells was eluted through the action of untreated virus.

In a similar experiment heated PR8 virus, adsorbed on cells, was successfully eluted by the action of unheated Lee virus. However, when periodate-treated Lee virus was adsorbed on red cells, and dispersed in native Lee virus, no detectable elution took place. The similarity of these results to those reported in the previous experiment suggests that the mechanism of elution of heated virus with vibrio filtrate and native virus may be basically the same.

TABLE IV

Effect of Salt Concentration on Elution of Heated and Periodate-Treated Virus Which Had Been Adsorbed on Red Cells

Salt used	Concentration of salt	Adsorption of cells with:	
		Periodate-treated virus	Heated 56°C. virus
		Titer of eluate:	
	per cent		
	0.85	5	<2
	Control		
NaCl	3.5	14	
	5.0	158	<4
	7.5*	182	
	10.0*	138	<4
$(NH_4)_2SO_4$	2.5	24	
	5.0	52	
	7.5	147	<4
	10.0	8	
KCl	10.0	8	<4
Na_2SO_4	10.0	20	<4

Red cells were suspended in virus which had been treated with $NaIO_4$ M/100 and in heated (56°C.) virus, strain Lee. After 30 minutes they were removed and washed. Two-tenths of a cc. of packed cells was suspended in 1.8 cc. of salt solution. After 30 minutes the cells were spun out. The expected titer if all adsorbed virus had eluted would have been 500 for periodate-treated virus and 690 for heated virus.

* Three volumes of distilled water were added to the suspension of cells in salt solution in order to facilitate removal of the gel which formed.

Elution of Influenza Virus by Salt and Heat

A number of procedures were tested to find methods of removing virus from cells when elution was not spontaneous. Cells on which periodate or heat-treated virus was adsorbed were dispersed in hypotonic saline and in distilled water without obtaining evidence of virus release. Cells were exposed to solutions of pH between 5.5 and 9.0 without any release of adsorbed virus.

When cells were suspended in sodium chloride solutions of 5 to 10 per cent concentration there was evidence of considerable elution of periodate-treated virus.

A Lee virus suspension was treated with sodium periodate (final concentration M/60) for 30 minutes after which the excess reagent was reduced by addition of glucose. Another aliquot of virus was heated at 56°C. for 30 minutes. Packed red cells were added to the suspensions, sufficient to make a 2 per cent concentration. After 1 hour at room temperature the cells were removed and washed and 0.2 cc. of packed cells was added to 2 cc. of each of a number of salt solutions of different concentrations. After 30 minutes the cells were removed by centrifugation and the supernatants tested for hemagglutinin titer.

The amount of periodate-treated virus adsorbed by the cells, had it all eluted, was sufficient to yield a titer of 500 (Table IV). In 5 to 10 per cent sodium chloride about one-third of the virus was set free. In ammonium sulfate somewhat less was liberated and in other salts still less. Heated virus adsorbed on red cells in comparable amounts did not elute significantly with any of the salts used. Sodium chloride solutions of 5 to 10 per cent concentration caused disintegration of the cells but the loss of cell structure *per se* was not the reason for virus release since it had no effect on elution of heated virus.

Red cells which had adsorbed heated virus, eluted small but significant amounts of agglutinin (2 to 3 per cent) when the combination was held at 56°C. for 15 to 30 minutes. Periodate-treated virus was not liberated under these conditions.

Destruction of Receptors for Periodate-Treated Virus

Since cholera vibrio filtrate and native influenza virus both removed heated but not periodate-treated virus from cells, it seemed possible that the virus-cell linkage might be different in the two cases. It might be possible that periodate changed the virus so that it attached to the cell at a new site, which was not affected by vibrio filtrate or native virus. To investigate this possibility the following experiment was performed.

Virus receptors were removed from fowl red cells in three ways:

1. Red cells (0.5 per cent concentration) were treated with sodium periodate (M/100 final concentration) for 30 minutes. The cells were removed by centrifugation, washed once with 5 per cent glucose, and twice with saline.

2. Two cc. of packed red cells was suspended in 150 cc. of cholera vibrio filtrate and incubated at 37°C. for 3 hours, after which the cells were removed and washed.

3. Lee virus in allantoic fluid was concentrated 30 times by high speed centrifugation. Enough packed red cells were added to make a 3 per cent suspension which was incubated at 37°C. for 3 hours to permit elution of the virus. At the end of this time there was no clumping of the cells which were washed thoroughly, until no virus eluted from them on further incubation.

To test the capacity of these cells to adsorb virus, three types of virus suspension were prepared: (1) Normal virus, (2) virus treated with a final concentration of M/60 sodium

periodate and reduced with glucose, and (3) virus heated at 56°C. for 30 minutes. Aliquots of each of the packed cell preparations were added to each of the virus suspensions to give a final cell concentration of 2 per cent. The mixtures stood at 0°C. for 30 minutes after which the cells were removed by centrifugation and the supernatants tested for residual hemagglutinins.

The control (untreated) cells in the above experiment (Table V) adsorbed virus of all types very well, reducing the supernatant titers to less than four. Red cells treated with vibrio filtrate or with periodate adsorbed practically no virus of any type. Cells treated with Lee virus were incompletely exhausted

TABLE V

Absorption of Periodate- and Heat-Treated Virus on Red Cells Exhausted of Receptors by Cholera Vibrio Filtrate, Periodate, and Influenza Virus

Adsorbed with cells exhausted by:	Treatment of virus			
	Periodate M/60	Heating 56°C.	Periodate and heating	Control No treatment
Control titer. No adsorption	138	56	104	112
Cholera vibrio filtrate	106	52	91	104
Sodium periodate M/100	112	56	84	97
Lee virus	74	32	69	52
Normal cells	<4	<4	<4	<4

The treatment of the virus with periodate was the same as described under Table II. The red cells were exhausted of receptors by exposure of 2 cc. of packed cells to 150 cc. of cholera vibrio filtrate for 3 hours at 37°C. Another lot of cells was exposed to M. 100 sodium periodate for 30 minutes, washed with an excess of glucose and then saline. For exhaustion of receptors with virus 1 cc. of cells was exposed to 30 cc. of Lee virus (concentrated from 900 cc. of allantoic fluid). After 3 hours' incubation (37°C.) the cells were thoroughly washed and in a control tube eluted no detectable agglutinin. The adsorptions were done at 0°C. with a final cell concentration of 2 per cent and allantoic fluid concentration of 1:2, except for the periodate cells where the concentration was 4 per cent.

of receptors and adsorbed a little normal virus and about the same proportion of each of the treated viruses.

From this experiment it seems clear that the removal of receptors for normal virus, by any of three means, also removed the receptors for periodate- and heat-treated virus. Since there was no indication of a special cell receptor for periodate-treated virus the failure of vibrio filtrate or normal virus to liberate periodate-treated virus from the cells must be explained in some other way.

Inhibition of Periodate-Treated Virus Agglutination

Since periodate-treated virus failed to destroy red cell receptors its hemagglutinating activity should be inhibited by the factor in normal plasma which inhibits heated virus (1). In the following experiment the effect of a plasma

fraction, containing a potent virus hemagglutinin inhibitor (VHI) for heated virus was tested on periodate-treated virus. The effect of a specific immune serum was also tested.

Three preparations of virus were used: untreated, periodate-treated, and virus heated at 56°C. The details of treatment were the same as those in the preceding experiment. Four inhibitor preparations were used: (1) The IV-4 fraction of human plasma which was shown to contain large amounts of VHI (2), (2) the same preparation as in (1) after boiling 30 minutes, (3) normal rabbit serum, and (4) immune Lee rabbit serum. The VHI was removed from the last two preparations by treating a 1:2 dilution with an equal volume of M/20 sodium periodate for 30 minutes, and reducing the excess reagent with glucose. Inhibition titrations were done with 4 agglutinating units of each of the three virus suspensions. The activity of the IV-4 preparation was expressed in units activity per milligram (2). The titer of the rabbit serum was calculated in the usual manner (4).

TABLE VI

Inhibition by Specific Antibody and by Serum Inhibitor of Agglutionation Due to Periodate- and Heat-Treated Virus

Tested with virus	Type of inhibitor			
	IV-4 unheated	IV-4 heated 100°C.	Normal rabbit serum	Lee immune rabbit serum
Untreated	<1	7	<16	5400
Heated 56°C	23	228	<16	958
Periodate M/60	86	632	<16	7650

Both normal and immune rabbit sera were treated with periodate (see text) to remove inhibitor. Four agglutinating units of each virus preparation were used and the 50 per cent end points calculated for the sera. The end points given for the IV-4 material are in terms of units of inhibitor per milligram.

As was to be expected from previous work, normal virus agglutination was inhibited in high degree by rabbit antibody only (Table VI). Both heated and periodate-treated virus behaved in similar fashion in that the IV-4 fraction caused significant inhibition, which increased markedly after this fraction was heated. Since, as noted earlier, periodate treatment of virus doubled the agglutination titer of the suspension, while heating lowered it slightly, much less of the former preparation was needed to obtain 4 agglutinating units. Possibly this may account for the fact that periodate-treated virus required less of either the IV-4 fraction or of antibody than heated virus for the same degree of inhibition.

DISCUSSION

Although heated virus does not spontaneously elute from red cells it was almost completely removed by exposing cells to unheated virus. Since unheated virus particles are undoubtedly very large in relation to their point of

attachment to cells, it is difficult to see how an unheated particle could destroy the cell receptor because of the physical obstruction offered by the attached heated particle. It seems likely, therefore, that the union between heated virus and cell receptors is somewhat dissociable, although in a cell virus suspension the number of free particles at any one time may be too small to measure. Even a small degree of dissociation of heated virus however, would give unheated particles a chance to substitute for them, following which the receptor would be destroyed in the usual manner. Eventually all the receptors would be destroyed, freeing the virus, both heated and unheated. A similar explanation could apply to the release of heated virus by cholera vibrio filtrate. Following this same approach one could assume that the union between periodate-treated virus and receptor had much less tendency to dissociate, so that the cell receptors never became accessible to either active virus or cholera vibrio filtrate. The difference in linkage between heated and periodate-treated virus with the cell receptor was illustrated by the removal of a large percentage of periodate-treated but not of heated virus from cells by high concentrations of salt.

Anderson (7) recently reported that "red cells treated with suitable concentrations of periodate" adsorb influenza virus and that the virus adsorbed on such treated cells did not elute either spontaneously or after treatment with cholera vibrio filtrate. The net results of treatment of cells with periodate, reported by Anderson, are very similar to those reported above in which only the virus was treated with periodate. There was adsorption of virus, but no spontaneous elution nor elution by vibrio filtrate. Anderson's experiments led him to postulate a theory that the forces of attraction between virus and cell receptor were not entirely identical with the forces destroyed by the virus when once attached. On the basis of the evidence presented above there seems to be no necessity for modifying the simple theory originally set forward (8) that a receptor point on the red cell is responsible for virus adsorption and is destroyed by the virus, presumably by enzymatic action.

The nature of the effect of periodate on the virus is not known. If as has been suggested (8) the active group on the virus particle is an enzyme, then periodate, like heat, alters the enzyme sufficiently to render it incapable of destroying the red cell receptor, but leaves intact the property of combining with substrate.

SUMMARY

Influenza virus, treated with sodium periodate, was adsorbed well on red cells but lacked the capacity for spontaneous elution. Heated virus was eluted from red cells by the action of cholera vibrio filtrate, unheated influenza virus, and to a small extent by heating at 56°C. Periodate-treated virus was not elutable by these methods but was liberated by exposure of the adsorbing cells to concentrations of sodium chloride of 5 to 10 per cent. This treatment had no effect on elution of heated virus.

BIBLIOGRAPHY

1. Hirst, G. K., *J. Exp. Med.*, 1948, **87,** 315.
2. Hirst, G. K., *J. Exp. Med.*, 1949, **89,** 223.
3. Hirst, G. K., *J. Exp. Med.*, 1948, **87,** 301.
4. Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45,** 273.
5. Briody, B. A., *J. Immunol.*, 1948, **59,** 115.
6. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exp. Path.*, 1946, **27,** 228.
7. Anderson, S. G., *Australian J. Sc.*, 1947, **10,** 83.
8. Hirst, G. K., *J. Exp. Med.*, 1942, **76,** 195.