THE RELATIONSHIP OF THE RECEPTORS OF A NEW STRAIN OF VIRUS TO THOSE OF THE MUMPS-NDV-INFLUENZA GROUP

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In a previous paper (1) a number of experiments were shown illustrating the relationships among a group of strains consisting of several examples of influenza virus and one strain each of Newcastle disease virus (NDV) and mumps. One of the most striking aspects of this relationship is that agents of such diverse pathological properties have a number of receptors in common and that each agent is able to inactivate the entire receptor complex. The present paper concerns the receptor relationships of a new strain to the rest of this group of viruses. This strain, *1233*, was isolated from man by Dr. R. M. Taylor and has the property of adsorbing on and eluting from fowl red cells. Taylor found (2) strain 1233 to be serologically unrelated to members of the mumps-NDV-influenza (MNI) group and the evidence presented below, in conjunction with serological data may be sufficient to classify this agent as a new species. From the standpoint of the receptor complex this virus is of unusual interest in that strain 1233 receptors and inhibitors appear to be almost completely distinct from those of the MNI group and this is the first example of such a strain.

EXPERIMENTAL

With the exception of strain 1233, the methods and materials were the same as those employed in a previous paper (1). Strain 1233 was obtained from Dr. R. M. Taylor, of the New York Laboratories of the International Health Division of The Rockefeller Foundation, in February, 1949. Taylor (2) isolated the strain from an individual suffering from a mild upper respiratory infection at a time when a mild epidemic of influenza A was beginning in New York City. The agent was isolated from throat washings on serial passage in eggs *via* the amniotic route and its presence was detected by the hemagglutinin reaction. During a number of early passages the behavior of the strain was erratic but when received in this laboratory it had been through fifty amniotic passages and was quite stable. All the material used in subsequent tests was prepared by inoculating the amniotic sac of 11 day old embryos and harvesting the amniotic fluid after 24 hours' incubation at 35°C. The pooled amniotic fluids were dialyzed against saline. All tests with this virus were done by the pattern method at 4°C. The usual hemagglutinin titer of amniotic fluid was 1:4000.

In Vitro Behavior of Strain 1233

The hemagglutinin of strain 1233 behaves like that of NDV in that it can be demonstrated at refrigerator temperature much better than at room tem-

perature. Titration of an amniotic fluid containing the virus with cells of different fowl yielded values varying from less than 1: 2 to 1: 256 at 25°C. while all titers were about 1: 4000 when tested at 4°C. Cells which were agglutinated at room temperature usually did not reagglutinate when redispersed. The treatment of strain 1233 with M/40 sodium periodate for 15 minutes altered the hemagglutinin so that the reaction was equally good at both temperatures and redispersed cells reagglutinated quite well. These results suggest that the 1233 receptor is one which is rapidly split off, even by small concentrations of virus and that the receptor-destroying power of the virus is lost on periodate treatment, as is true of viruses of the MNI group (1).

While investigating the capacity of cholera vibrio filtrate to remove strain 1233 receptors from fowl red cells, it was repeatedly observed that control cells in the experiment failed to agglutinate and this change was traced to the addition of sodium citrate to the filtrate-cell mixture. When it was found that the presence of oxalate or calgon had similar effects it was supposed that the calcium ion was necessary for agglutination. However, cells exposed to citrate and then thoroughly washed agglutinated normally, while the addition of calcium had a depressant effect on agglutination. This led to the testing of the effect of other electrolytes on the agglutination of red cells by strain 1233.

A number of salts were made up in a series of concentrations, —0.4M, 0.2M, etc. The pH of the various solutions was brought to near neutrality and the salt concentrations were brought up to physiological strength with sodium chloride. Dilutions of four different viruses were made with these solutions and the titer of the virus in each determined by the pattern method. The results are given in Table I.

Of the salts tested, six had a definite depressant effect, at 0.4 M concentration, on agglutination of fowl cells by strain 1233, while Mu virus agglutination was influenced much less and PR8 and NDV were significantly depressed only by KI. On repeated tests the effect of calcium compounds was found to be even more marked on strain 1233 than that shown. The explanation of the sensitivity of strain 1233 agglutination to these salts is not known but is important to appreciate in studying this reaction.

Adaptation to the Allantoic Sac and Interference with Strain 1233.—Unlike most influenza strains, 1233 was adapted to the allantoic sac with considerable difficulty. On primary inoculation of amniotic virus into the allantoic sac the results were very erratic and some embryos developed hemagglutinin titers as high as 1:64 but most were negative. On subsequent allantoic passage the results were also uncertain. Even after twenty passages, virus did not demonstrably grow in all embryos, and the average hemagglutinin titer was low (1:64). The *in ovo* titer of passage material was about 10^{-6}. The optimum period for maximum growth in the allantoic sac was 4 days as contrasted with 24 to 36 hours in the amniotic sac.

In view of the questionable classification of this organism it was of interest to see how it behaved in interference tests with other agents of the MNI group. These tests are as yet incomplete but preliminary experiments indicate fairly clearly that there is an interference between a primary inoculum of 1233 and challenge inocula of influenza or NDV. Table II shows such a preliminary experiment in which several dilutions of strain 1233 were inoculated into the allantoic sac and after 24 hours the eggs were given a small challenge dose of Lee virus. The fluids were harvested after a further 48 hours. Since Lee virus

TABLE I

Effect of Various Salts on Agglutination of Fowl Cells by 1233 Virus

Virus tested	Molar concentration	Dilutions of virus made in:								
		Na_2HPO_4	NH_4Cl	KCl	$CaCl_2$	K oxalate	Na citrate	Na_2SO_4	KI	Control
1233	0.4	1024	512	64	8	64	4	32	32	1024
	0.2	1024	1024	1024	64	256	64	64	128	
	0.1	1024	1024	1024	256	512	128	256	512	
	0.05	1024	1024	1024	512	512	512	250	1024	
PR8	0.4	1024	1024	1024	512	1024	1024	1024	4	1024
Mu	0.4	200	256	256	128	128	256	128	128	256
NDV	0.4	128	128	64	64	64	256	128	8	128

The salt solutions were brought to neutrality before use and in the lower molarities NaCl was used to bring the diluent up to physiological strength. Twofold virus dilutions were made with solutions and the figures given are the hemagglutinin titers.

in this dosage induces hemagglutinin titers of 1:1000 to 1:4000 and 1233 (amniotic strain) titers of less than 1: 2 to 1: 256, interference by strain 1233 will result in low titers and lack of interference in high titers. As seen in Table II inocula of 1233 at 10^{-1} and 10^{-3} were effective in blocking the development of high titer fluids and hence probably interfered with the growth of the Lee strain. Titrations at two temperatures are also given since agglutination by 1233 is usually absent at room temperature while the titer of Lee virus was only slightly diminished. Similar evidence has been obtained of interference between 1233 and NDV but tests with Mu virus have not been completed. Strain 1233, when given intracerebrally to mice, also interfered with the lethal

action of a challenge dose of WEE virus. In this respect it resembles other members of the MNI group (3).

Reciprocal Cross-Tests for Exhaustion of Red Cell Receptors.—Reciprocal cross-tests for the exhaustion of fowl red cell receptors were carried out as in the experiment previously reported. (1).

TABLE II

Interference Test between Strain 1233 and Strain Lee

Primary inoculum 1233	Challenge inoculum Lee	Tested at	Hemagglutinin titer of individual eggs				
10^{-1}	Saline	4°	16	4	4		
		25°	<2	<2	<2		
10^{-3}	Saline	4°	32	4	<2	<2	
		25°	<2	<2	<2	<2	
10^{-5}	Saline	4°	256	32	2	2	<2
		25°	2	2	<2	<2	<2
Saline	10^{-6}	4°	8000	4000	4000	2000	
		25°	2000	2000	1000	1000	
10^{-1}	10^{-6}	4°	256	32	8	4	<2
		25°	<2	8	<2	<2	<2
10^{-3}	10^{-6}	4°	8	8	8	<2	<2
		25°	8	<2	<2	<2	<2
10^{-5}	10^{-6}	4°	4000	2000	2000	512	256
		25°	1000	1000	1000	256	125

The initial inoculum was injected in the allantoic sac of 10 day old chick embryos. After 24 hours the eggs were given the second inoculation by the same route. After 48 hours' further incubation the allantoic fluids were withdrawn and titered with fowl cells by the pattern method, both at refrigerator and room temperature. The titers given are the reciprocal of virus dilution at the end point.

Allantoic fluid virus of strains Mu, NDV, Lee, and Melb was concentrated 10 times by centrifugation and was mixed with fowl red cells at 0.5 per cent final concentration. Dialyzed amniotic fluid containing 1233 (pattern titer 4000) was also used.[1] These mixtures were incubated (with penicillin 200 u./cc.) for 8 hours, after which the cells were washed and resuspended at 1 per cent concentration in saline. Each cell suspension was then stable. Stability with strain 1233 was actually achieved in a much shorter time than this. Each suspension was mixed with 8 units of nine different viruses of the MNI group. The results are given in Table III.

[1]It has not been feasible so far to concentrate this virus by centrifugation since the losses in pellet resuspension are so great.

The exhaustion of receptors from mumps-treated cells was slightly incomplete but apart from this the MNI viruses had entirely removed the MNI receptors, at the same time leaving the 1233 receptors intact. Conversely, the exhaustion of cells with 1233 removed 1233 receptors completely without producing any visible effect on the agglutination of these cells by any of the MNI strains. The significance of the negative effect of 1233 on MNI receptors is somewhat obscured by the fact that this virus was not used in concentrated form, as were the others. Nevertheless there was sufficient virus to remove homologous receptors in less than 2 hours and further incubation for more than four times this period had no further effect.

TABLE III

Cross-Test for Receptor Exhaustion between Strain 1233 and Viruses of the Mumps-NDV-Influenza Group

Treated cells tested with strain:	Cells exhausted with strain:					
	Mu	NDV	Lee	Melb	1233	Control
Mu	0	0	0	0	++	++
NDV	0	0	0	0	++	++
PR8	0	0	0	0	++	++
WS	±	0	0	0	++	++
Melb	0	0	0	0	++	++
Lee	±	0	0	0	++	++
NY-47-1	++	0	0	0	++	++
1233	++	++	++	++	0	++
Saline control	0	0	0	0	0	0

A mixture of 0.5 per cent cells and virus was incubated at 37°C. for 8 hours. The cells were thoroughly washed in saline, resuspended in saline at 1 per cent concentration, and added to 8 agglutinating units of a number of viruses. NDV and 1233 tests were carried out at 4°C. ++ indicates full agglutination.

The action of vibrio filtrate on 1233 receptors is also of interest but is not so crucial in regard to their specificity. In previous tests (1) this filtrate removed all MNI receptors from fowl cells in 3 hours or less at a dilution of 1:32. Similar experiments were carried out here with filtrate at 1:10 dilution. This removed MNI receptors very rapidly as shown in Fig. 1. In this test the cells were no longer agglutinated by Mu after 80 minutes whereas 1233 agglutinated to full titer after 360 minutes. Cells from several fowl were tested in the same way and no evidence was found that cholera vibrio filtrate inactivated the 1233 receptor.

Destruction of VHI by Strain 1233.—Since human plasma did not contain measurable amounts of 1233-VHI all tests were carried out with egg white inhibitor which had a titer of 1:1200. Destruction of EW inhibitor was carried

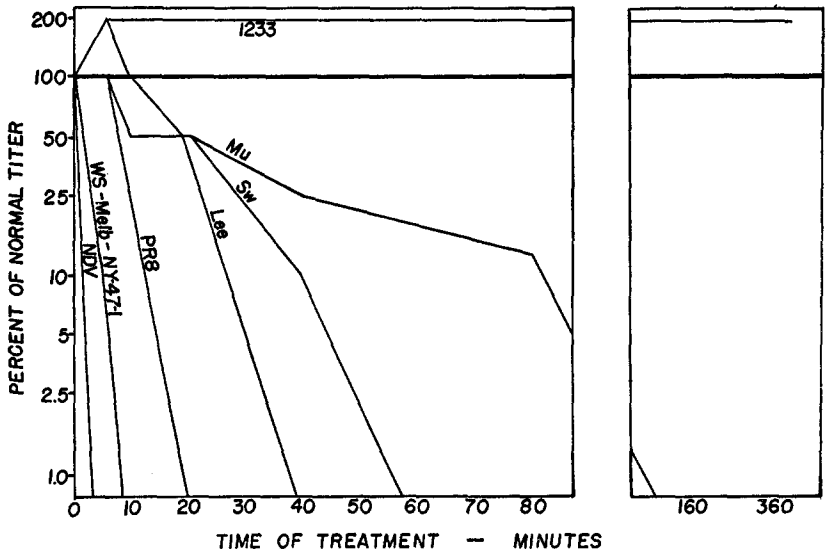

FIG. 1. Red cells from a single fowl were treated with cholera vibrio filtrate (1:10 dilution) at 37°. At various intervals cells were removed, sodium citrate added to a final concentration of 2 per cent, and the cells thoroughly washed with saline. The cells were made up to 1 per cent concentration in saline and added to dilutions of a number of viruses. The hemagglutinin titers obtained with treated cells are plotted in terms of per cent of titer with normal cells (on a logarithmic scale).

TABLE IV

Inactivation of Egg White Inhibitor by a Number of Viruses

EW treated with strain:	Per cent of original VHI remaining as tested against strain:								
	Lee	WS	Sw	NY-47-1	Melb	Mu	NDV	PR8	1233
PR8 (50)	0.1>	0.2>	0.02>	0.02>	0.02>	0.1	83	0.1>	100
Lee (20)	0.4>	0.4>	1.3>	0.05>	1.9>	0.8	100	6.4	100
Mu (80)	0.6	2.1	44	38	59	0.4	100	63	100
NDV (100)	0.1>	0.2>	0.1>	2.8	1.6	0.1>	6.4	1.3	50
1233 (64)	60	96	89	100	100	73	100	96	1.6

An extract of egg white containing 0.5 per cent non-diffusible solids was combined with an equal volume of each of the viruses shown in the left hand column. The mixtures were incubated for 24 hours at 37°C., heated at 65°C. for 30 minutes to inactivate the treating virus, and then tested for residual VHI with a number of periodate-inactivated viruses. The figures given represent the residual percentage of the original VHI content. The figures in parentheses in the first column are the number of units of virus used to treat the egg white.

out with a number of strains including Mu, NDV, PR8, Lee, and 1233. The technique of measuring destruction was the same as that used in a previous paper (1). For these tests fairly large amounts of virus were used. The destruc-

tion of inhibitor was tested by periodate-treated viruses of nine diffierent strains. The results of 24 hours' contact between EW and various viruses are shown in Table IV. From this it may be seen that there was considerable destruction of VHI for all the viruses except NDV and 1233, which remained virtually intact. Strain 1233 on the other hand destroyed its own inhibitor without significant destruction of any of the other VHI's. Some slight relationship between NDV- and 1233-VHI is perhaps indicated in that NDV had a moderate (50 per cent) effect on 1233-VHI which was duplicated in several experiments though not of the same order of magnitude as the loss of NDV-VHI by NDV.

Treatment of EW with vibrio filtrate gave different results than red cells in that an amount which destroyed MNI inhibitors completely had a moderate but definite effect on 1233-VHI.

DISCUSSION

One of the main problems with strain 1233 is that of classifying the agent from the standpoint of its pathological, serological, and physiological aspects. From the work of Taylor it is reasonably clear that the agent came from a human throat washing and was not picked up on propagation and from data on the production of antibodies following intranasal instillation in mice, ferrets, and monkeys it may be said that the virus has pneumotropic tendencies. The relationship of the virus to human infection is not so clear since the investigation of a large number of paired sera from respiratory infections in Taylor's laboratory (personal communication) revealed only two instances in which a definite rise in 1233 antibody was found. A similar investigation in this laboratory with 50 pair of sera gave no instance of a significant antibody increase.

We have not attempted to confirm Taylor's results on the serological identity of strain 1233, which he found to be distinct from mumps, Newcastle disease virus, and a number of influenza strains including A strains of 1947. Inasmuch as strain 1233 agglutination is not significantly inhibited by normal serum factors, cross-tests of specificity by agglutination inhibition are relatively clear and easy to interpret. The question raised by the serological tests is whether this strain is a separate type of one of the MNI viruses (say influenza C) or whether it is an example of a fourth virus of this group.

Taken alone, the data on the receptor relationships of strain 1233 would favor the view that this is a new species of virus making a fourth member of the MNI group. The behavior of 1233 in straight agglutination resembles that of NDV most markedly but the mutual exhaustion tests of receptors indicate that this strain stands rather alone in its characteristics since there was no reciprocal exhaustion of receptors, indicating that the receptors for MNI viruses and 1233 were mutually exclusive. This seems to be a qualitatively dif-

ferent relationship from that which exists between the members of the MNI group. At the present time not enough is known of the basic significance of the receptor reaction to say how important this data may be in virus classification but it is conceivable that it may be a very fundamental difference. It is possible that on further investigation of the problem using other species of red cells or inhibitors from other sources, the exclusive receptor-inhibitor relationships found here may break down. Until this is shown, however, one may take the view that strain 1233 is a single example of a new virus species, related to other members of the MNI group, the pathogenic qualities of which have not yet been well defined.

SUMMARY

The interrelationships of the cellular receptors and the hemagglutinin inhibitors of a new strain of virus (1233) to members of the mumps–Newcastle disease-influenza group have been investigated. It was found that strain 1233 does not destroy the receptors or inhibitors of the other group, nor does the latter destroy 1233 receptors or inhibitor. The sole exception to this statement was a moderate destruction of 1233 inhibitor in egg white by Newcastle disease virus. The classification of strain 1233 was discussed in the light of this evidence, evidence which tends to place strain 1233 in a different category from that of any other strain of the MNI group.

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