Studies on Hemagglutination by NDV.* (30797)

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Certain past observations(1-5) are consistent with the hypothesis that the hemagglutinins of some strains of myxoviruses are not homogeneous, but instead represent a heterogeneous assembly of particles with differing activities. Furthermore, the exact nature of viral agglutinins in causing the agglutination of red blood cells (RBC) is as yet incompletely known(6).

The work reported herein investigates the specificity of the VIC strain of Newcastle disease virus (NDV) with regard to the demonstration of the presence of agglutinins with differing specificities. In addition, the nature of viral hemagglutination is investigated, using the mixed agglutination technique originally used by Topley *et al*(7) to demonstrate the bridging action of humoral antibodies in agglutination.

Materials and methods. NDV suspensions consisted of allantoic fluid from chicken embryos that had been inoculated with the VIC strain 48 hours previously; the dose was 0.1 ml of 1:100 diluted allantoic fluid from the last egg passage. For experiments involving elution, untreated virus was used; for all other experiments virus suspension which had previously been heated to 56°C for one-half hour to produce stable patterns related to reduction in neuraminidase activity(8) was employed. The material thus prepared had hemagglutinating activity for human and chicken RBC; it would not agglutinate rhesus RBC. The heated virus preparation produced agglutination patterns which were stable even after prolonged incubation.

The measles virus was a live preparation obtained commercially (measles HA antigen, Microbiological Associates). It was capable of agglutinating rhesus RBC but had no activity against either chicken or human RBC.

Before being used in mixed agglutination experiments, the NDV suspension was absorbed with one-fiftieth its volume of rhesus RBC. The measles virus was similarly absorbed using chicken RBC.

RBC were preserved either in Alsever's or ACD solution. Before use, they were washed 3 times and finally resuspended to a final concentration of 1% in phosphate buffered saline, pH 7.4. The same buffered saline was used for all dilutions in tests.

Absorptions of NDV with RBC were carried out at 0°C for 20 to 30 minutes during which time the tubes were frequently shaken. The RBC were then removed by centrifugation.

Hemagglutinin titrations were done at room temperature. Two-tenths ml volumes of virus suspension were prepared by serial 2-fold dilution. When parallel titrations against 2 RBC species were desired, a pilot row of dilutions was prepared from which aliquots were distributed. The tubes were incubated for one hour after the admixture of 0.1 ml of a 1% RBC suspension, and hemagglutination was then read by observation of the patterns. The highest dilution of virus suspension (prior to RBC addition) producing definite agglutination was taken as the hemagglutinating titer.

The formation of agglutinates for microscopic observation in mixed agglutination experiments was carried out using 0.2 ml aliquots of undiluted virus. To these were added 1% suspensions of 2 RBC species each in 0.1 ml aliquots. If necessary, enough buffered saline was added to produce a final volume of 0.6 ml. After one hour at room temperature, the sedimented cells were gently resuspended and poured onto a slide for microscopic reading.

Results. 1. Absorption of hemagglutinating activity by varying amounts of RBC. Aliquots of NDV were absorbed with varying

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TABLE I. Absorption of NDV with Different Volumes of RBC.

Species RBC used to absorb and volume as % of virus vol		Reciprocal of residual titer against: Chicken RBC	Human RBC
None (control virus)		128	64
Chicken	.6	64	32
	1.2	32	16
	2.5	16	8
	5.0	4	2
	10.0	<2	<2
Human	1.2	64	32
	2.5	64	16
	5.0	32	8
	10.0	32	4
	20.0	16	<2

volumes of packed RBC and the supernates were titrated for hemagglutinating activity against human and chicken erythrocytes. The results are shown in Table I. Absorption with progressively increasing amounts of chicken RBC removed agglutinins for chicken and human RBC in roughly parallel fashion; on the contrary, progressive increase in the amount of absorbing human RBC removed completely anti-human erythrocyte activity while the activity for chicken erythrocytes was diminished but not abolished.

2. Repeated absorptions with constant RBC volumes. Portions of NDV suspension were repeatedly absorbed with one-hundredth their volume of chicken RBC. At intervals, small aliquots were withdrawn and titrated for residual hemagglutinating activity. The experiment was repeated, using one-fiftieth volumes of human RBC to absorb. The results are displayed in Table II. Absorption

TABLE II. Serial Absorption of NDV with Constant Volumes of RBC.

		Reciprocal of residual titer against: Chicken RBC	Human RBC
Sequential absorption by chicken RBC	Unabsorbed	128	32
	×1	16	4
	×2	2	<1
	×3	1	<1
Sequential absorption by human RBC	Unabsorbed	64	16
	×1	32	16
	×2	32	8
	×3	32	8
	×5	32	2
	×7	32	<1

with chicken RBC removed agglutinating activity for both chicken and human erythrocytes in essentially parallel fashion. Absorption using human RBC finally resulted in complete disappearance of agglutinins for human erythrocytes, without considerably affecting those for chicken RBC.

3. Repeated absorptions of eluted virus with human RBC. Human RBC were used to absorb agglutinins from an NDV suspension at 0°C. The virus was then eluted from the washed cells into buffered saline by incubation at 37°C. The resulting eluate was then repeatedly absorbed with one-fiftieth its volume of human RBC. Titration of aliquots after various numbers of absorptions revealed a roughly parallel removal of agglutinating activities for chicken and human erythrocytes (Table III) in contrast to the

TABLE III. NDV Eluted from Human RBC: Residual Hemagglutinins on Serial Absorption by Human RBC.

No. of absorptions	Reciprocal of residual titer against: Chicken RBC	Human RBC
0	16	8
1	8	4
2	8	4
3	4	2
5	4	1
7	4	1
9	2	1

distinct lack of such parallelism seen when whole virus was absorbed in this manner.

4. Effect of blockage of chicken RBC receptor sites with absorbed virus. After preliminary experiments showed that thorough pre-treatment of RBC with previously heated NDV abolished their ability to remove hemagglutinating activity from a viral suspension, the effect of blockage of receptor sites using absorbed NDV was investigated. An aliquot of NDV was absorbed with packed human RBC until no agglutinating activity against human RBC remained. Chicken RBC were then subjected to receptor blockage by 3 incubations in 12 times their volume of this aliquot of absorbed NDV. After blocking, the cells were washed with buffered saline to remove unabsorbed agglutinins. As controls, other aliquots of chicken RBC were treated

TABLE IV. Selective Blockage of Chicken RBC Receptors.

	Reciprocal of residual titer against:	
	Chicken RBC	Human RBC
Unabsorbed virus	128	32
Virus absorbed by unaltered chicken RBC	2	<2
Virus absorbed by chicken RBC blocked with untreated NDV	64	16
Virus absorbed with chicken RBC blocked with human RBC-absorbed NDV	32	<2

similarly, either with buffered saline or with unabsorbed NDV.

Finally, 1 ml aliquots of a fresh suspension of NDV were absorbed with 0.25 ml of packed erythrocytes treated in the manners described. After absorption, the supernatant fluids were titrated for agglutinating activity against human and chicken RBC. The results are shown in Table IV. The blocking effect of previous incubation of the chicken RBC with NDV is shown by the observation that erythrocytes so treated absorbed very little agglutinating activity from the test virus suspension, in comparison with the control RBC. What is noteworthy, however, is that chicken RBC blocked with NDV which had been previously absorbed by human RBC behaved in an anomalous fashion. They absorbed very little anti-chicken agglutinins from suspension, behaving in this regard as blocked RBC. On the other hand, they absorbed anti-human agglutinins well, being comparable in this regard to untreated RBC.

5. Mixed agglutination. NDV, measles virus, or a mixture of both were added to erythrocyte mixtures consisting of a suspension of chicken RBC and a suspension of mammalian RBC, either human or rhesus. The presence and character of any resulting agglutinates was observed microscopically. The results given in Table V show that RBC of 2 species form mixed agglutinates in the presence of a virus capable of agglutinating each type separately (NDV against chicken RBC and human RBC). In contrast, when RBC of 2 species are agglutinated by a mixture of 2 viruses, neither of them capable of agglutinating both RBC species (NDV and measles against chicken RBC and rhesus RBC), separate homologous agglutinates are formed.

Discussion. The data presented show that human RBC are incapable of removing all agglutinating activity for chicken RBC from NDV suspensions. Instead, as absorption is done more thoroughly the anti-chicken RBC activity remains fairly stable after an initial drop. On the contrary, when chicken RBC are used for absorption, residual titers against both human and chicken RBC diminish in roughly parallel fashion as absorption proceeds.

The results are explainable by assuming that 2 agglutinin types, *"a"* and *"b"* are present in the NDV strain used. Additionally, 2 erythrocyte receptor types, *"A"* and *"B,"* corresponding to their respective agglutinins, may be postulated. If it is assumed that human RBC possess only type *A* receptors and that chicken RBC possess both *A* and *B* receptors, the relative absorbing abilities of the 2 erythrocyte species would be explainable.

This hypothesis also elucidates the behavior on absorption of agglutinins eluted from human RBC. Here, only type *a* agglutinins would be expected to be present in the eluate; absorption with human RBC would then remove agglutinating activity against both species in parallel fashion.

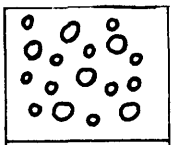
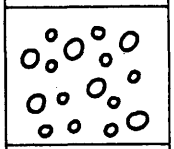
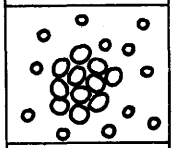
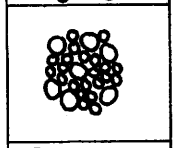
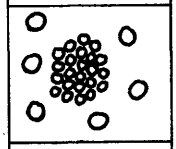
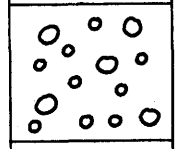
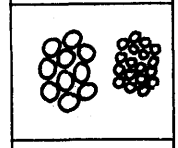
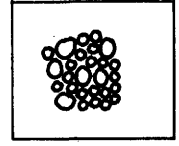
The results of partial blocking of chicken RBC receptors tend to confirm this hypothesis by demonstrating the possibility of selective blocking of receptors. Here, absorption of NDV with human RBC may be thought of as removing the *a* agglutinins, leaving only the *b* type. Treatment of chicken RBC with this material selectively blocked the *B* receptors. The unblocked receptors were able to absorb *a* agglutinins from a fresh aliquot of the virus, producing an absorbed suspension with the properties predictable by this hypothesis: it agglutinated chicken but not human RBC.

It may be anticipated that a variety of myxovirus receptor types does exist. Burnet (1) postulated a series of receptor types differing in accessibility to viral receptor-destroying activity by virtue of their position

with regard to the cell surface. Less active viruses were considered to remove only the superficial receptors, leaving the RBC susceptible to agglutination by the more active members of the group.

This concept, based on receptor sites differing in location alone, is somewhat difficult to apply when RBC agglutination by measles virus is considered. This virus, now classified as a myxovirus(9), agglutinates rhesus RBC only(10) and is inactive against RBC of species ordinarily agglutinable by other

Table V

Virus	RBC Species	Agglutinates Observed	Schematic Appearance of Agglutinates
None	Chicken + Rhesus	None	
None	Chicken + Human	None	
NDV	Chicken + Rhesus	Homologous Chicken Only	
NDV	Chicken + Human	Mixed Human and Chicken	
Measles	Chicken + Rhesus	Homologous Rhesus Only	
Measles	Chicken + Human	None	
NDV and Measles	Chicken + Rhesus	Separate Homologous of Each Type	
NDV and Measles	Chicken + Human	Mixed Human and Chicken	

viruses of the group. Since influenza strains, NDV, and other classical myxoviruses do not agglutinate rhesus RBC, the concept of a graded series(11) of similar receptors cannot be applied and it becomes necessary to consider the possibility of receptors differing from one another not only in accessibility but also in actual configuration.

Additionally, differences in elution behavior of virus particles in certain strains of myxoviruses(2) as well as changes in elution behavior of suspensions of these viruses on heating have led to the hypothesis that there may be several types of receptors, possibly varying in structural configuration(3).

Haff and Stewart(12) have supplied additional evidence for the presence of more types of myxovirus receptors than the N-acetyl neuraminic acid receptor already known. Working with tissue cells, they found evidence for a neuraminidase-resistant receptor, and postulated a variety of receptors differing in spatial configuration or chemical composition.

It seems likely that the agglutinins present in any one myxovirus strain may also be inhomogeneous with regard to receptor specificity. Burnet and Bull(4) have reported that certain influenza virus strains have a low titer against chicken RBC when first isolated; after repeated passage in the chicken embryo they acquire the ability to agglutinate chicken RBC to high titer. The phenomenon (called "O to D variation") seems explainable on the basis of the acquisition of a new agglutinin type with specificity directed against a receptor present on chicken RBC. Hemagglutinating fluids resembling pure O or pure D types could be obtained by absorption with mouse RBC followed by elution.

Sokol(5), working with a strain of Newcastle disease virus, demonstrated heterogeneity of hemagglutinins on the basis of chromatography and density gradient centrifugation. This heterogeneity did not seem to be due to the presence of fragmented viral particles.

The experiments cited herein do not unequivocally prove a dichotomy of the NDV hemagglutinins. An alternative explanation could be formulated that a spectrum of particle types exists, ranging from those with great activity for *A* receptors and little for *B* receptors to the opposite extreme. In any case, a diversity of receptors involving at least two types is indicated by these experiments.

The mixed agglutination experiments were designed to test the hypothesis that agglutination occurs as the result of bridging of RBC one to another by the attachment of at least bivalent viral particles to specific receptors on each cell, as opposed to the hypothesis that an agglutinable state is created by more adherence of viral particles to the RBC surfaces.

The mixed agglutination of chicken and human RBC by NDV may be explained by either hypothesis. On the other hand, if the second hypothesis were true, one would expect the appearance of mixed agglutinates when chicken and rhesus RBC were exposed to the mixture of NDV and measles virus. This, however, was not the case. The conclusion may be drawn that agglutination results from the specific bridging of erythrocytes one to another by multivalent virus particles.

Summary and conclusions. By absorption experiments, a strain of NDV was shown to have at least 2 agglutinins with different specificities. Human RBC were shown to possess receptors for only one type, in contrast to chicken RBC which had receptors for both types. This conclusion was further supported by the results of experiments using NDV eluted from human RBC as well as by the absorption of NDV with chicken RBC with selectively blocked receptors. Further experiments using mixed agglutination showed that hemagglutination by the myxoviruses is brought about by the bridging of cells having similar receptors by multivalent viral particles.

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Cholesterol Flux into Aorta in Experimental Nephrosis.* (30798)

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Hyperlipemia and hypercholesterolemia are characteristic features of the nephrotic syndrome. In nephrosis in man, the lipemia is due largely to an increase in the s_f 10-20 lipoproteins, the class associated with atherogenesis(1). Though there are few studies of the incidence and severity of atherosclerosis in nephrosis, the available evidence suggests that the prolonged hyperlipemia of this disease is associated with increased atherosclerosis(1,2). Experimental nephrosis in the rat, however, was not found to be associated with increased atherosclerosis(3,4), perhaps because of the relatively short duration of nephrosis in the animals studied. The purpose of the present work was to determine whether the hypercholesterolemia of experimental nephrosis is associated with increased deposition of cholesterol in the aorta and with an increased flux of cholesterol from plasma into aortic wall, a situation which might be expected to lead over a long period to increased atherosclerosis.

Methods. Male Sprague-Dawley rats weighing 200-225 g were divided into 4 groups: (i) Normal (non-nephrotic) rats fed the standard laboratory diet (Purina laboratory chow). (ii) Normal rats fed a diet based on that described by Hartroft *et al* (6). It contained 1% cholesterol. Initially the content of butter fat was 3%, but this was increased to 5% after 2 weeks and to 6% after 4 weeks, in order to maintain a relatively constant level of plasma cholesterol. This diet will be referred to as "medium fat." (iii) Normal rats fed a diet similar to that of Group (ii), but containing 5% cholesterol and 20% butter (referred to as "high fat" diet). Both medium and high fat diets contained 0.1% thiouracil rather than 0.3% as originally used(6). (iv) Nephrotic rats fed the standard laboratory diet. Nephrosis was induced by injection of rabbit anti-rat kidney serum(5); animals were considered to be nephrotic if urinary protein excretion was 300 mg or more per day, 12-14 days after injection.

Plasma cholesterol concentrations were measured at weekly intervals. After 8 weeks, the animals were sacrificed. Flux rate of cholesterol from plasma to aorta was calculated by the method of Newman and Zilversmit(7) as previously described(8). Plasma lipoproteins were separated in the ultracentrifuge(9). The adventitia was stripped from the aorta. Cholesterol(10) and phospholipid (11) were measured in chloroform:methanol (2:1) extracts of the residual media and intima, in whole plasma and in the plasma lipoprotein fractions.

Results. The results are shown in Tables I and II. It is evident that the increase in plasma cholesterol in nephrosis was due primarily to an increase in the "chylomicron" fraction (density <1.006). With a moderate

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