THE AGGLUTINATION OF HUMAN ERYTHROCYTES MODIFIED BY TREATMENT WITH NEWCASTLE DISEASE AND INFLUENZA VIRUS¹

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Burnet and Anderson (1946) reported that human erythrocytes which had been modified by adsorption and elution of Newcastle disease virus (NDV) could be agglutinated by anti-NDV animal sera and by the diluted serum of some patients with infectious mononucleosis. This observation led to speculation concerning a connection between the NDV and the etiology of infectious mononucleosis.

Our interest was aroused when it was possible for us to confirm Burnet and Anderson's work by demonstrating that serum from a patient convalescing from infectious mononucleosis agglutinated NDV-modified human erythrocytes as did that from a rabbit vaccinated with NDV. A number of questions remained unanswered, however, and the present communication is concerned with some of them. Is the agglutination reaction specific? Would NDV-treated cells be agglutinated by other human and antiviral rabbit sera? Would sera which agglutinated human erythrocytes treated by NDV also agglutinate cells treated by normal allantoic fluid (NAF), PR-8, or Lee viruses? Is the factor in serum that is necessary for this agglutination identical with anti-NDV antibody?

MATERIALS AND METHODS

Viruses. The PR-8 strain of influenza A virus, the Lee strain of influenza B, and a strain of NDV originally recovered by Dr. Beaudette were used in this study. Pools of allantoic fluid from 12- and 13-day-old chick embryos which had been infected 2 days before were employed as the source of virus. Uninfected allantoic fluid from embryos of a similar age was used as a control and termed normal allantoic fluid (NAF).

Cells. Human group O red blood cells were collected in an excess of 2 per cent sodium citrate and washed several times with saline; the desired concentration was prepared from the sediment remaining after centrifugation at 1,500 rpm for 10 minutes.

"Virus-modified" human red blood cells were prepared by mixing a suspension of cells with an equal volume of undiluted infected allantoic fluid. The mixtures were shaken and left at 37 C for several hours. After a number of washings with normal saline, the cells were tested for agglutinability in saline. When a sta-

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bilized preparation was obtained, a suspension of the desired concentration was prepared. Details are given in the text. Between tests the cells were kept at 4 C. They were used for as long as 12 days.

Sera. PR-8-immune serum was prepared by giving rabbits a single intravenous injection of 10 ml from a pool of allantoic fluid infected with PR-8. Anti-NAF serum was prepared in a similar fashion using uninfected allantoic fluid. The Lee and NDV antisera were also made in this manner by using 10 ml of allantoic fluid infected with Lee or ND viruses. The rabbits used for the latter sera received an additional or second injection after 6 weeks. In each instance serum was collected approximately 2 weeks after the last injection.

Human sera were obtained from various sources. The sera from patients with infectious mononucleosis and from those with serum sickness were collected by Dr. Peter Vogel and Dr. Samuel Karelitz of the Mount Sinai Hospital. The sera from patients with influenza and from normal individuals were from the collection of Dr. Frank L. Horsfall, Jr., and those from patients with hepatitis were from Dr. Henry Kunkel, both of the hospital of the Rockefeller Institute. All sera were kept at 4 C without any added preservatives.

Saline. An 0.85 per cent solution of sodium chloride containing 0.01 M phosphate buffer at pH 7.2 was used throughout.

Agglutination tests. The sera were inactivated at 56 C for 30 minutes, and twofold dilutions in saline were prepared. Then, to 0.2 ml of each serum dilution, 0.2 ml of a 1.5 per cent suspension of modified cells were added. The tubes were shaken and permitted to remain undisturbed at room temperature for 1½ hours. The pattern of cells at the bottom of each tube was then noted. If no agglutination occurred, there was a small well-defined button. If agglutination occurred, the sediment pattern was different and varied from a thin layer over the entire bowl of the tube to a broad sediment with irregular edges. This pattern was arbitrarily graded from +1 to +4. The last tube showing a +2 or greater reaction was considered to be positive. As a control for each serum, a similar test was made at the same time with untreated human cells.

EXPERIMENTAL RESULTS

In the following experiments, human erythrocytes were treated with normal allantoic fluid (NAF), PR-8, and Lee viruses as well as with Newcastle disease virus (NDV). Selected human and rabbit sera were then tested with each variety of treated cell. Finally, sera which gave comparable titers in the agglutination test were studied for their concentration of anti-NDV agglutination-inhibition antibody.

I. The Interaction between Human Sera and Human Erythrocytes Treated with NAF, Influenza, and ND Viruses

It is known that sera from some individuals are more reactive than others in a variety of nonspecific immunological systems (Havens *et al.*, 1948; Young, 1946; Thomas *et al.*, 1943; Kaufman, 1944; Davis, 1944). These include those with hepatitis, primary atypical pneumonia, infectious mononucleosis, and serum

sickness. It was therefore of interest to determine whether a serum that agglutinated NDV-treated human red blood cells would also agglutinate similar cells that had been modified by treatment with other viruses.

Seven sera were selected for study. They were from a normal individual and from patients convalescing from influenza A, influenza B, serum sickness, and infectious mononucleosis. The lowest dilution of serum tested was 1:40. Cells were treated with normal allantoic fluid (NAF) and with fluid infected with Newcastle disease virus (NDV), PR-8, or Lee viruses. This was done by incubating a mixture of equal volumes of allantoic fluid and a 15 per cent suspension of erythrocytes for 90 minutes at 37 C, 3 hours at 20 C, and 18 hours at 4 C before washing the cells with saline. The virus was then eluted from the cells over a total period of 3 days with frequent washings. The agglutination tests were made with the techniques already described.

TABLE 1

Agglutination of human cells treated with NDV and influenza viruses by human sera

SERA	RESULTS* WITH CELLS TREATED WITH				
	NDV	PR-8	Lee	NAF	Saline
No. 17 Normal.....	0†	0	0	0	0
No. 15 Influenza A.....	0	0	0	0	0
No. 16 Influenza B.....	0	0	0	0	0
No. 10 Serum sickness.....	0	0	0	0	0
No. 11 Serum sickness.....	80	0	0	0	0
No. 19 Infectious mononucleosis.....	320	40	0	0	0
No. 12 Infectious mononucleosis.....	640	0	0	0	0

* Results given as reciprocal of highest dilution giving a +2 or greater agglutination.

† 0 = <40.

None of these sera when diluted beyond 1:40 agglutinated the cells treated with PR-8, Lee, or NAF. This is noteworthy since serum from one of the patients with serum sickness and from both of those with infectious mononucleosis agglutinated the NDV-treated cells (table 1). The titers were 1:80, 1:320, and 1:640.

The results of this experiment, therefore, demonstrate that the factor present in these sera that agglutinates NDV-modified cells does not agglutinate cells treated with NAF nor with influenza viruses.

This experiment also suggests that the agglutination of NDV-treated cells is not specific. It may be brought about by human sera from other than convalescent infectious mononucleosis patients. Indeed, just as with the specimen from the patient with serum sickness in this experiment, we were able to demonstrate that, in moderately high dilution, the serum of some individuals with hepatitis may also agglutinate these cells. In table 2 the results are given of agglutination tests with NDV-treated cells and 27 human sera. Although titers of 1:120 or greater were obtained only with specimens from patients with infectious mono-

nucleosis, three of those from patients with hepatitis and one with serum sickness had titers as high as 1:80. Evans and Curnen (1948) have also reported several similar instances of agglutination of NDV-treated cells by sera from patients with a variety of illnesses.

TABLE 2

Results of NDV agglutination tests with human sera and NDV-modified human cells

PATIENT	CONDITION	END POINT*	PATIENT	CONDITION	END POINT
1	Normal	10	12	Infectious mononucleosis	480
17	Normal	<10	19	Infectious mononucleosis	240
229	Normal	15	23	Infectious mononucleosis	120
235	Normal	<10	28	Infectious mononucleosis	20
241	Normal	<10	30	Infectious mononucleosis	<10
243	Normal	<10	29	Infectious mononucleosis	<10
248	Normal	<10			
257	Normal	<10	2	Hepatitis	<10
			G	Hepatitis	<10
15	Influenza A	<10	P	Hepatitis	<10
16	Influenza B	<10	R	Hepatitis	80
			S	Hepatitis	80
3	Serum sickness	20	N	Hepatitis	80
9	Serum sickness	20			
10	Serum sickness	15	27	Infectious lymphocytosis	60
11	Serum sickness	80			

* End point given is the average of two or more determinations and is presented as the reciprocal of the highest serum dilution giving a +2 or greater agglutination.

II. *Interaction between Rabbit Sera and Human Erythrocytes Treated with NAF, Influenza, and ND Viruses*

In order to investigate further the nature of the agglutinating factor, rabbit sera were employed, since their antibody content could be more readily ascertained.

Sera were collected from a normal rabbit and from rabbits immunized with normal allantoic fluid and with allantoic fluid infected with PR-8, Lee, or NDV. Because the immune sera were made with allantoic fluid, each was absorbed with minced chick embryo and tested both prior and following absorption. Human erythrocytes were treated with PR-8, Lee, NDV, or NAF by incubating at 37 C equal volumes of a 6 per cent suspension of human cells and allantoic fluid for 4 hours. The cells were then washed and the virus was eluted over a total of 3 days, with frequent washings. Each batch of cells was tested against each of the sera in the agglutination test described under "Materials and Methods."

None of the rabbit sera agglutinated the cells treated with PR-8, Lee, nor NAF. The results with the NDV-treated cells were quite different and are presented in figure 1. It will be seen that the normal rabbit serum contained agglutinins that were removed when the serum was absorbed with chick tissue. The anti-NAF, anti-PR-8, and anti-Lee sera also contained agglutinins that were removed by

absorption with chick tissue. The anti-NDV sera, which were made in a fashion identical to the anti-Lee, contained an even greater concentration of agglutinins. However, these were not completely removed by absorption.

The agglutinins present in normal rabbit sera may be "T" agglutinin, as suggested by Burnet and Anderson (1946). This is a factor which is present in varying titers in practically all human and many mammalian sera and is responsible for the nonspecific agglutination of human blood cells by sera contaminated with certain bacteria. This phenomenon was first described by Thomsen in 1926, and shown to be due to a bacterial enzyme by Friedenrich in 1927. The presence of antichick agglutinins in the rabbit sera prepared by injecting these animals with chick allantoic fluid is readily understood. The agglutinating factor (or factors) for NDV-treated cells remaining in the anti-NDV sera after absorption with chick tissue indicates that, in addition to the agglu-

Figure 1. Agglutination by rabbit sera of human cells treated with NDV.

tinins present in the normal and other rabbit sera, these anti-NDV sera contain a specific factor for some component of NDV-treated cells.

In one experiment, group A and O human cells were treated with NDV and tested against the same rabbit sera. It was found that after absorption with chick tissue, anti-NDV sera gave identical titers with either the O- or A-treated cells. Consequently, some factor other than the group substance present in these cells is modified by treatment with NDV and is acted upon by these sera.

III. Comparison of Human Infectious Mononucleosis Serum and Anti-NDV Rabbit Serum

Anti-NDV rabbit serum not only contains agglutinins for NDV-modified human cells, but also specific agglutination-inhibition antibodies for NDV. It was, therefore, of interest to determine whether human sera that contain agglutinins for these modified cells also have NDV agglutination-inhibition antibodies.

For this experiment three sera were selected; one from a normal man; one

from a patient with infectious mononucleosis and a high NDV-modified cell agglutination titer, and one from a rabbit immunized against NDV. Agglutination-inhibition titrations were performed as described in detail elsewhere (Florman, 1947). Serial dilutions of each serum were made in 0.2 ml of a virus preparation containing 32 agglutinating units. The mixture was incubated in a 37 C water bath for 60 minutes. The tubes were then chilled and to each were added 0.4 ml of a 1.5 per cent suspension of chicken cells. After 60 minutes at 4 C, the tests were read by observing the nature of the sediment. The highest serum dilution showing less than +2 agglutination was considered as the end point. The NDV-treated cell agglutinations were conducted as in the preceding experiments.

The results are given in table 3. It is seen that, although both the rabbit anti-NDV serum and the human infectious mononucleosis serum contained comparable agglutinins, only the rabbit anti-NDV serum had the NDV agglutination-inhibition antibody. Similar experiments have since been published by others

TABLE 3
Comparison of NDV agglutination-inhibition antibody and agglutinin titers for NDV-modified human cells

SERA	NDV A-1 TITER	NDV-MODIFIED CELL AGGLUTININ TITER
No. 17, Normal human.....	1:16	<1:20
No. 19, Infectious mononucleosis.....	<1:4	1:320
R-413, Rabbit anti-NDV.....	1:1,024	1:640

(Anderson, 1947; Evans and Curnen, 1948). They, too, indicate that NDV agglutination-inhibition antibodies are not present in the sera of patients with infectious mononucleosis.

DISCUSSION

It has already been indicated that sera from individuals other than those convalescing from infectious mononucleosis occasionally may agglutinate NDV-treated human red blood cells. It should also be noted that not all sera from individuals with this clinical syndrome exhibit this phenomenon. Our personal experience has been limited to a study of sera from only 6 such patients (table 2). However, the results in the 13 cases reported by Burnet and Anderson (1946) and in the 23 patients of Evans and Curnen (1948), which represent the only other published studies, may be added to our 6 cases and summarized. It is thus found that only 15 of 42 patients (35 per cent) gave reactions with sera diluted 1:120 or higher. This may be compared with the fact that in this dilution the sera of 29 of these individuals (70 per cent) gave positive sheep cell heterophile reactions.

A satisfactory explanation for the agglutination of NDV-treated human erythrocytes is not yet available. The possibility that this agglutination is but another manifestation of the heterophile antibody content of the sera is readily

excluded. Human sera that in high dilution agglutinate the NDV-modified human cells may fail even in low dilution to agglutinate sheep cells. Similarly sera with high heterophile titers may not contain any appreciable concentration of the agglutinating factor for the NDV-modified cells. Direct proof of the difference between these two factors was obtained by us and by others (Anderson, 1947; Evans and Curnen, 1948) by absorption. From a patient who had recovered from infectious mononucleosis a serum was obtained that had a high heterophile titer as well as a high titer of agglutinating factor for NDV-treated cells. When this serum was absorbed with sheep cells so that the heterophile antibody was completely removed, the titer of the agglutinating factor for NDV was unaffected. Similarly, if the serum was first absorbed with NDV-treated human cells, the heterophile antibody titer for sheep cells remained unchanged.

It has been demonstrated with various rabbit sera that the agglutination of NDV-treated human cells may be brought about by a variety of serum factors. These may include antichick tissue agglutinins, "T" agglutinins, and specific anti-NDV antibody. It would therefore seem logical to assume that human sera may also contain a number of factors each capable of inducing the reaction. The failure of the postinfectious mononucleosis sera to react with human erythrocytes that have been treated with NAF, or with such fluid containing influenza virus, may be taken to mean that they do not contain any considerable amount of antichick agglutinin. We did not investigate their "T" agglutinin content. However, Lind and McArthur (1947) found that most adult sera contain some.

The observation that a serum from a patient with infectious mononucleosis with a high titer of agglutinating factor for NDV-treated human cells differs from a rabbit anti-NDV serum with a comparable titer in not containing any anti-NDV agglutination-inhibiting antibody is significant. It limits the degree of similarity between the etiologic agents of these two diseases. However, it does not exclude the possibility of an antigenic relationship.

SUMMARY

Sera from patients with infectious mononucleosis, which agglutinate human red cells modified with Newcastle disease virus (NDV), do not agglutinate human cells modified by PR-8 or Lee viruses or by normal allantoic fluid (NAF).

Not all patients with infectious mononucleosis develop the capacity to agglutinate NDV-treated cells. Serum from an occasional patient with hepatitis or serum sickness may also agglutinate these cells.

Anti-NDV rabbit sera contain several agglutinating factors for NDV-modified erythrocytes, at least one of which appears to be specific. The agglutinating factors in anti-PR-8, anti-Lee, and anti-NAF rabbit sera are all removed by absorption with normal chick tissue.

Serum from a patient convalescing from infectious mononucleosis that has a titer for NDV-modified cells comparable to that of an anti-NDV rabbit serum differs from the latter in that it does not contain any NDV agglutination-inhibition antibody.

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