# Antiviral Immune Hemolysis[1] (37275)

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Recently is was shown that mouse ascites tumor cells treated with a paramyxovirus at 37° became sensitized within an hour to lysis by antiviral antiserum and rabbit complement (1). Analogous studies with erythrocytes were suggested by the observation of Burnet and his associates (2, 3) that Newcastle disease virus and mumps virus became irreversibly bound to red cells at 37°. The virus sensitized cells after reversal of hemagglutination and resuspension became agglutinable by antiviral antibody to a high titer and clumped with untreated erythrocytes. The viral sensitizing agent was not removable from cells by antibody or neuraminidase. Sendai virus and NDV are by themselves hemolytic but we have found that virus/cell multiplicities below the hemolytic level will sensitize to lysis by antiviral antibody and rabbit or guinea pig complement. Comparative studies with two strains of influenza virus are reported.

*Material and Methods.* Blood was obtained from 16–18 day chick embryos and from $C_3H/Bi$ mice. Erythrocytes were washed free of serum and used the same day or after storage for not longer than 24 hr at 4°. Some experiments were done with erythrocytes from adult chickens but there was increased nonspecific hemolysis when these were kept in Alsever's solution more than 2 weeks.

Newcastle disease virus (NDV) strains Cal and Isl, parainfluenza I Sendai (SiV) and influenza strains $PR_8$ and NWS were grown in the allantoic sac of chick embryos. Hemagglutinin titers were determined with chicken erythrocytes by the method of Salk. Sera were obtained from rabbits immunized with these viruses and were titered for hemagglutination inhibition against 8 HA units/ml of specific antigen. Antisera (ab) were inactivated at 56° and those to be used with chicken erythrocytes were absorbed to remove antibodies against the cells. Rabbit complement (C′) was used at dilutions of 1:30–1:40 and guinea pig C′ at 1:10–1:20. Since the paramyxoviruses are hemolytic by themselves at higher virus cell multiplicities, and this is somewhat enhanced by C′ it was necessary to adjust the concentrations of virus, cells, and C′ so that no hemolysis was seen in the controls without antiserum. With 1% cells in phosphate-buffered saline hemolysis by NDV and SiV occurred at about 100 HA units/ml after incubation for 2 hr at 37°. In most experiments cells at a concentration of 10% were added to an equal volume of virus dilutions containing 200 HA/ml or less. Stabilization of the suspensions was then done according to the method of Lind (3) by incubation for 2 hr at 37° with frequent mixing. The initial viral agglutination was reversed as some virus eluted but a portion remained attached. After centrifugation the virus-sensitized cells were diluted to 1% and incubated for an additional hour at 37°. In some experiments ab was added at this step.

The diluted sensitized erythrocytes, or erythrocytes similarly incubated without virus were then added to dilutions of ab plus C′, placed at 37° for 2 hr. The final volume was 1 ml consisting of equal parts of 1% cells and ab + C′ dilutions all in phosphate-buffered saline pH 7.4. Additional controls consisted of sensitized cells with ab or C′ alone. Hemolysis was not observed with inactivated antiserum by itself. If hemolysis occurred in the C′ control tests were repeated with more dilute complement. The end-

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points of titrations were taken as the last tube showing a definite red color in the supernatant after cells had settled. The next higher dilution often showed a very faint or indefinite tinge which was disregarded. Cells were resuspended and placed at 4° overnight when additional hemolysis often became evident. There was no significant hemolysis with heterologous antisera (NDV ab and cells sensitized with SiV or vice versa) indicating the absence of hemolytic reaction attributable to antibody against components of infected allantoic fluid other than the virus.

*Results.* Figure 1 summarizes the results of several hemolysis titrations of SiV antiserum against various sensitizing doses of virus in HA units/ml as measured with chick embryo cells. With low virus/cell multiplicities a large amount of ab was required for hemolysis. With increasing virus/cell the serum titer rose about proportionately reaching a maximum of 1:20,000 at 160 HA units/ml. There appears to be a slight upward projection of the line at the higher virus concentrations. A similar curve located lower on the graph was obtained with SiV inactivated with β-propriolactone. Lower serum titers were observed with guinea pig C′ and chick embryo cells, and with mouse cells and rabbit or guinea pig C′ as shown by single points which are averages of several measurements. Variations in titer are thought to be due in part to differences in cell fragility.

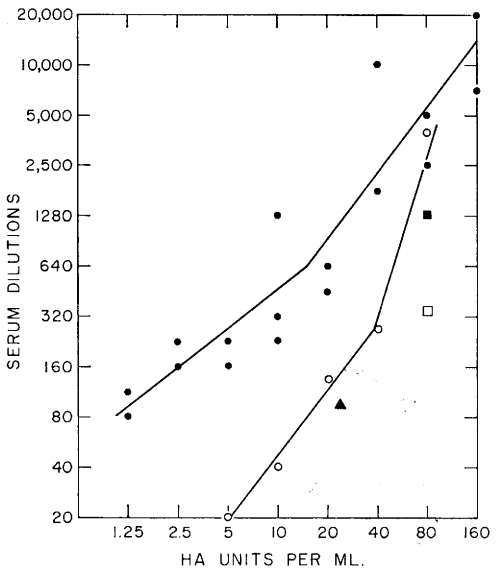


FIG. 1. Hemolytic titer of SiV antiserum (HAI titer 2500–5000) and rabbit complement 1:40 with various sensitizing doses of virus on 5% chick embryo erythrocytes. (●) active virus, (○) virus inactivated with β proprioluctone. Comparative measurements with mouse erythrocytes and (▲) rabbit complement or (□) guinea pig complement; (■) guinea pig complement and chick embryo cells. A single SiV antiserum was used.

Antiviral immune hemolysis (AVIH) with two strains of NDV was compared with the results for SiV. Chick embryo cells sensitized with 128 HA units/ml of NDV-Isl gave serum hemolytic titer of 1:1200 or more with rabbit C′ (first line of Table I). As with SiV, lower titers were observed with guinea pig C′ and chick embryo or mouse cells.

It has been reported that formalin inactivation of NDV abolishes its ability to sensitize ascites cells to antiviral immune cytolysis (1) even though there is irreversible adsorption to cells. Failure to elute indicates destruction of neuraminidase activity but hemagglutination is unchanged. Similar findings for AVIH are reported in Table I. Chick embryo or mouse erythrocytes incubated with large sensitizing doses of formalinized NDV gave serum hemolytic titers one tenth or less of those with active virus.

Other measurements of AVIH were done with two influenza strains. The strain NWS showed no AVIH at high virus/cell multiplicity and high concentrations of antibody. $PR_8$, on the other hand, gave some AVIH but titers were much lower than with the paramyxoviruses even with maximum sensitizing concentration of virus.

The effect of heating to destroy neuraminidase activity was studied with influenza and paramyxoviruses. In the case of $PR_8$ heating at 56° had little effect on hemagglutination and resulted in slightly higher titers by AV IH. Strain NWS could be heated only to 52° without destroying HA but this did not produce detectable sensitization for AVIH (not shown in table). With NDV strain Isl, in contrast to influenza $PR_8$, a temperature of 56° diminished sensitizing activity and gave lower titer. Neither HA nor AVIH activity of SiV survived a temperature of 52°.

In previous studies with mouse ascites tu-

TABLE I. Comparison of AVIH with NDV and Influenza Strains. Effect of Cell and Complement Species, Formalin and Heat Inactivation.

| Virus | Cells[a] | Comp.[a] | Serum[b] dil. 1: | Minimum HA units/ml[c] |
|---|---|---|---|---|
| NDV-Is | CE | R | 1200 | 128 |
| NDV-Is | CE | GP | 600 | 80 |
| NDV-Is | M | GP | 150 | 250 |
| NDV-Cal | CE | GP | 300 | 128 |
| NDV-Cal | M | GP | 120 | 250 |
| NDV-C(F)[d] | CE | GP | <30 | >250 |
| NDV-C(F) | M | GP | <30 | >250 |
| NWS | CE | GP | <10 | >128 |
| $PR_8$ | CE | GP | 30 | 500 |
| $PR_8$ | M | GP | 10 | 250 |
| $PR_8$ | CE | R | 60 | 500 |
| $PR_8$ (56°)[e] | CE | R | 120 | 250 |

[a] GP = guinea pig, R = rabbit, CE = chick embryo, M = mouse.

[b] HAI titer of NDV antiserum 1200–2500, $PR_8$ antiserum 1200–2500, NWS antiserum 600–1200.

[c] Number of HA units/ml incubated with 5% erythrocyte suspension at 37° for 2 hr and centrifuged. Lowest concentration of virus that gives hemolysis with indicated dilution of antiserum and complement.

[d] (F): Virus inactivated with 0.05% formaldehyde 2 weeks at 4°. HA same as active virus.

[e] Allantoic fluid virus heated to 56° for 30 min.

mor cells (1), it was found that SiV adsorbed at 4° was removed by treatment of the cells with antiviral ab so that on subsequent additions of C′ no cytolysis occurred. Virus adsorbed at 37° was not removed by ab. These experiments were repeated with erythrocytes sensitized with NDV or SiV. Results are presented in Table II in terms of the highest dilution of sensitizing virus giving hemolysis when cells were treated with ab and later with C′ or both together. When ab was added before C′ chick cells treated with NDV gave clear evidence of a temperature effect in only one experiment where more virus was required for sensitization at 4° than at 37° (first line of Table II). With SiV there was a reduction in the hemolytic titer of the virus at 4° with antibody added either before C′ or at the same time. Over all, little difference was seen between pretreatment with antibody and the usual procedure of adding virus sensitized cells to ab + C′.

These results suggest irreversible attachment of some viral antigen to erythrocytes even at 4°. Quantitative variations with cell species and virus strain occur but apparently ab, instead of removing virus, becomes fixed to cell-associated virus, subsequently reacting with complement to give hemolysis in the same manner as ab + C′ together.

*Discussion.* In general the characteristics of AVIH parallel the findings for early antiviral immune cytolysis with ascites tumor cells previously reported. In both irreversible binding of viruses, or possibly envelope antigens, at 37° leads to sensitization for lysis by antiviral antibody and complement. For immune cytolysis or hemolysis with the influenza viruses, much higher concentrations of ab and virus are required than with paramyxoviruses. Burnet (2) noted differences between paramyxoviruses and the influenza group in that the latter were almost completely eluted from red cells. Evidence for irreversible binding at 4° of paramyxoviruses to erythrocytes was found and in contrast to ascites cells, Sendai virus was only partially removed by treatment with antibody at that temperature. Rabies virus is apparently irreversibly bound to sheep cells since such adsorbed virus has been used for antiviral antibody production *in vitro* (7). As shown in Fig. 1, AVIH was obtained at a level of 1 HA unit/ml with a 5% cell suspension, an

TABLE II. Effect of Pretreatment with Ab of Erythrocytes Sensitized with Virus at 37° or 4°.

| Virus | Cells (%) | Serum dil. 1: | Highest dilution of virus giving hemolysis | | | |
|---|---|---|---|---|---|---|
| | | | antibody before C′[a] | | antibody + C′ together[b] | |
| | | | 37° | 4° | 37° | 4° |
| NDV-C | C 10% | 60 | 160 | 40 | 40 | 40 |
| NDV-C | C 10% | 240 | 80 | 40 | <40 | <40 |
| NDV-C | M 10% | 120 | <10 | 20 | 10 | 20 |
| NDV-C | M 1% | 40 | >160 | >160 | 80 | 80 |
| NDV-1 | C 10% | 120 | 40 | 80 | 40 | 40 |
| NDV-1 | M 1% | 160 | — | — | >40 | <20 |
| Si | C 1% | 40 | 160 | 40 | >160 | 40 |
| Si | M 1% | 40 | 20 | <10 | 40 | <10 |

[a] Erythrocytes (10% or 1%) were incubated with virus dilutions for 2 hr at 37° or 4° and centrifuged. Cells were resuspended to make a 1% suspension, antibody at the indicated final dilution was added, and tubes placed at 37° or 4° for an additional hour. To centrifuged and resuspended cells, guinea pig C′ was added and all tubes incubated at 37°.

[b] As above except that cells were resuspended in PBS for second incubation period. Sensitized cells added to a mixture of antibody and complement.

estimated multiplicity of between 1 and 10 virus particles per cell. With ascites tumor cells, guinea pig complement showed very little lytic activity (6) either with antiviral or anticell ab but in AVIH it was only slightly less active than rabbit complement.

Studies with the electron microscope of the interaction of Sendai virus (at very high multiplicity) with the red cell membrane have revealed breakdown of both virus and cell structure (4). Additional observations with ferritin-labeled antibody indicated persistence of viral antigen on cell membrane fragments. The question may be raised as to whether the cell-sensitizing agent in AVIH is the intact virion or an antigenic fragment. Lind (3) was unable to separate the cell sensitizing agent from mumps virus by ultracentrifugation. However, intact virions may undergo dissolution after attachment but still retain reactivity with ab and C′. The envelopes of Sendai virus solubilized in detergent (5) can be reassembled by removal of the detergent on resin columns. The small reassembled particles retain hemolytic and fusing capacities and it is conceivable that they might sensitize erythrocytes to AVIH.

Viral infectivity and hemagglutination do not correlate with ability to produce AVIH. Virus inactivated by β-propriolactone still has the sensitizing property. Also there is no evidence that viruses replicate in erythrocytes. The potential of NDV for AVIH is diminished or abolished by heat or formaldehyde treatment which destroys neuraminidase activity and increases irreversible binding of hemagglutinin. However, the $PR_8$ strain of influenza virus when heated to 56° shows slightly increased AVIH although neuraminidase is inactivated (2). Paramyxoviruses almost uniformly have cell fusing and hemolytic activity and when contrasted with the influenza strains there is some correlation of these properties with AVIH. In the present study virus sensitized cells did not show hemolysis with C′ and antibody to a different virus. This suggests that virus-specific antibody takes part and that AVIH is not a nonspecific enhancement of natural viral hemolysis by unknown serum factors.

The high sensitivity of AVIH with parainfluenza I virus suggests that this reaction might occur *in vivo*. With viremia and irreversible binding of viral antigens to erythrocytes, hemolysis would be possible when antibody subsequently appeared. Reaction with antibody and C′ of persistent viral antigens on tissues or red cells might also result in a form of immune complex disease. Other human viruses biologically similar to the parainfluenza group include mumps, measles, respiratory syncytial and corona viruses. There

is increasing evidence for immunopathologic reactions following infection with some of these.

*Summary.* Newcastle and parainfluenza I viruses when incubated with mouse or chick embryo erythrocytes at low multiplicity sensitized the red cells to hemolysis by antiviral antibody and complement. Two strains of influenza virus showed little or no sensitizing activity to influenza antibody. As determined by hemolytic titer paramyxovirus bound to erythrocytes at 37° could not be removed by pretreatment with antibody alone and virus absorbed at 4° was only partially removed by antibody at this temperature. Effects of inactivating infectivity and viral neuraminidase on sensitizing ability of the viruses were examined.

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