Enhancement of Haemolysis by Newcastle Disease Virus (NDV) after Pre-treatment with Heterophile Antibody and Complement

By K. APOSTOLOV AND M. I. SAWA

Department of Virology, Royal Postgraduate Medical School, London, England

(Accepted 12 July 1976)

SUMMARY

Pre-treatment of Newcastle disease virus (NDV) with fresh human plasma enhances its haemolytic (HL) capacity by several factors. The effect is due to complement activation by the heterophile anti-chick antibody present in human plasma. All the adult human plasmas tested were effective, also 91/100 human cord blood sera. The antibody was mainly of the IgM class. The enhanced HL was due to integration and transference of the complement 'holed' virus envelope membrane and subsequent leakage of haemoglobin. High concentration of activated complement destroys the integrity of the virus envelope. Treatment of chick erythrocytes and fibroblasts with human plasma also produced lysis of the cells.

INTRODUCTION

All the viruses of the paramyxovirus group, including measles, have been shown to lyse the erythrocytes which they haemagglutinate (HA). HL can be increased by several factors as well as being completely abolished after pre-treatment of the virus by various chemical and physical agents. These changes in HL are not paralleled by changes in HA titre (Apostolov & Waterson, 1975). Electron microscopic studies by ultrathin sectioning (Howe & Morgan, 1969; Apostolov & Almeida, 1972), negative staining (Apostolov & Almeida, 1972; Apostolov & Poste, 1972), and freeze-etching (Apostolov & Short, 1974) have shown that the process of HL involves the attachment of the virus to the erythrocyte and the fusion and integration of the nanogranular layer (the basal membrane) of the virus envelope into the cell membrane. The HL capacity of a virus population depends largely on the physical condition of the virus membrane before fusion and on the composition of the suspending medium (Apostolov & Waterson, 1975). In a previous paper it was also shown that after the completion of the primary (virus) haemolysis additional HL can be obtained with virus-specific and host antibody in the presence of complement (Apostolov & Waterson, 1974).

In this paper we present the results of a study on the effect of pre-treatment of Newcastle disease virus (NDV) with heterophile antibody in the presence of complement (C').

METHODS

Viruses. The Queensland strain of NDV was obtained from Dr D. J. Alexander, Central Veterinary Laboratory, Weybridge. The virus was grown, harvested, concentrated and purified by the same method as previously described for Sendai virus (Apostolov & Almeida, 1972).

Erythrocytes. Human 'O' type blood, kept up to 21 days after collection, was obtained
from the Blood Transfusion Bank. The plasma was separated for the studies and the erythro-
cytes were washed four times in phosphate buffered saline (PBS) and used as a 10 % suspension
in PBS. Also, 10 % suspension for haemolysis (HL) and 0.5 % for haemagglutination (HA)
of fresh chick cells were prepared by the same method.

**Human plasma and animal serum.** Most of the human plasma and the serum from different
animal species was obtained from freshly drawn blood, except in a few instances when
plasma or serum stored at -70 °C was used.

**Haemolysis (HL).** A standard method using 4 ml of 10 % suspension of erythrocytes was
used throughout in order to avoid (HL) values near 100 % lysis. After various pre-treatments
0.25 ml of virus was added at a concentration of 1024 HAU/0.25 ml. After pre-incubation
for 30 min at 4 °C the cells were centrifuged at 3000 rev/min for 10 min, the supernatant
fluid was discarded and the volume reconstituted to 4 ml with PBS. HL was performed in a
water bath at 37 °C for 30 min. Mixing of the reagents in a shaker was performed twice,
before and after incubation. The haemolysis was then stopped by cooling in a water bath at
4 °C and the tubes were centrifuged using an MSE refrigerated centrifuge at 2000 rev/min
for 5 min. Haemolysis was determined by reading the supernatant fluid at 540 nm against
distilled water in a Unicam SP 600 spectrophotometer.

**Titration of haemagglutination activity (HA).** HA was measured in standard, plastic plates
using 0.25 ml as unit volume. Twofold dilutions of the sample to be titrated were made in
PBS and 0.25 ml of 0.5 % chicken red blood cells was added to each cup.

**Titration of infectivity.** Infectivity for chick embryos was estimated by inoculating 0.1 ml
of serially diluted virus suspension into the allantoic cavity of 11-day-old chick embryos. The
50 % egg infection dose (EID$_{50}$) was estimated by the Reed & Muench method after testing
for HA 72 h after inoculation (Reed & Muench, 1938).

**Chick embryo fibroblast (CEF) monolayers.** A standard method was used. The wings, legs
and head were removed from 10-day-old chick embryos and the tissue was finely minced.
Tissue pieces were washed twice in PBS and then trypsinized (0.25 % trypsin) for 30 min at
37 °C. Calf serum 10 % (v/v) was added after trypsinization to stop it, and cells were filtered
through sintered glass No. 1. Filtrated cells were centrifuged for 5 min at 1000 rev/min in an
MSE refrigerated centrifuge. Cells were diluted with growth medium to a concentration of
1 x 10$^7$ cells/ml and dispensed into 5 cm diam. plastic Petri dishes. A confluent monolayer
was obtained after 24 h.

**Electron microscopy.** The negative staining technique was applied as described previously

**RESULTS**

The incubation of fresh or frozen plasma from some animal sources with NDV virus
increases the haemolytic activity by several factors (Fig. 1). This effect could not be obtained
by homologous chick plasma. The degree of HL enhancement for chick-grown virus varies
from species to species, from nil for calf to maximum for human plasma (Fig. 1). Heating of
haemolytically active plasma at 56 °C for 30 min abolishes the enhancing effect but does not
prevent primary (virus) haemolysis. The enhancing effect is due to complement activation by
the heterophile antibody. The complement ‘holes’ produced in the virus membrane after
incubation of the virus with haemolytically active plasma are illustrated in Fig. 2 and 3. We
have studied 100 human plasmas obtained from the Blood Transfusion Bank and have found
that they vary in their ability to enhance the haemolytic property of the virus, but all of them
produced a degree of enhancement after pre-treatment of the virus. Also, 100 cord blood
sera were examined and only nine were found that did not produce enhanced haemolysis.
Complement enhancement of NDV haemolysis

Fig. 1. Enhanced haemolysis after pre-treatment of egg-grown NDV with various plasmas from animals. 0.1 ml of neat plasma was incubated for 5 min with 0.1 ml of virus (1024 HAU/0.25 ml) and after adsorption to the red cells for 30 min at 4 °C, haemolysis was performed at 37 °C for 30 min. The values in the columns are based on minimum of three samples per species. Columns: F = PBS, 2 = chick plasma, 3 = rat plasma, 4 = rabbit plasma, 5 = human plasma, 6 = guinea pig plasma, 7 = sheep plasma, 8 = foetal calf plasma, 9 = mouse plasma.

These were pooled and used as a source of complement. The heterophile antibody responsible for complement activation could be adsorbed by the virus after incubation in the cold. After washing the virus and adding negative cord blood serum, incubation at 37 °C for 30 min produced enhanced haemolysis (Fig. 4). However, it is evident that antibody alone did not inhibit primary HL. The fractionation of the highly active human plasma on Sephadex G200 column (Fireman et al. 1974) and subsequent addition of negative cord blood serum to the fraction as complement source revealed that the active antibody is predominantly of IgM class (Fig. 5).

The amount of enhanced haemolysis with any active plasma depends on the concentration of antibody and complement and on the time of pre-incubation of the virus with antibody and complement. At a high concentration there is at first a sharp increase in the haemolytic property followed by a decrease down to or below the level of primary or virus haemolysis. This effect is ameliorated on dilution of the plasma (Fig. 6). Electron microscopy of the virus after prolonged incubation with plasma reveals that most virus particles have a partially or completely destroyed nanogranular layer (basal membrane; Fig. 2 and 3). Therefore, it could be presumed that in this experiment (Fig. 6) there is at first an increase in the haemolytic property due to increase in the number of virus particles with complement ‘holes’ in them, and afterwards progressively more particles have their membrane destroyed and fail to fuse with the cells.

This interpretation is also supported by the effect of this treatment on the other parameters of virus activity associated with the envelope, as shown in Fig. 7. Pre-treatment of the virus also affects the infectivity and the HA titre, presumably by gradual destruction of the integrity of the virus envelope through destruction of the membrane. The treatment of chick
Fig. 2. Pre-treated virus particles with complement holes. Virus particles after negative staining from a preparation where virus was pre-treated with neat human plasma for 30 min. Complement holes are clearly visible. At the site of highest concentration of complement holes the nanogranular layer (basal membrane) was partially destroyed (open arrows). Note the small size (50 nm) of the spikes (closed arrow).

Fig. 3. A particle from the same preparation as Fig. 2. Note the absence of a clear membrane profile, the presence of complement holes (arrow) and the disarray of spikes.
Complement enhancement of NDV haemolysis

Fig. 4. Adsorption of heterophile antibody and complement activation. Heated neat human plasma with an equal amount of virus was incubated for 2 h at 4 °C and then washed three times as for concentration of the virus (see Methods), then fresh negative cord blood serum was added and the mixture tested for haemolysis. Columns: 1 = virus plus heated plasma plus negative cord serum, 2 = virus plus heated plasma, 3 = virus plus negative cord serum, 4 = virus plus PBS.

Fig. 5. Human plasma fractionation and HL. Human plasma was fractionated on a Sephadex column. The fractions had an effective dilution of the original plasma of 1:4, after addition of negative cord blood serum (see Fig. 4) haemolysis was performed for the IgM and IgG fractions. ---, represents percentage transmission of fractions, ----, represents the values of haemolysis for the fractions.
red cells with the same plasmas in the absence of the virus also produced a marked degree of direct haemolysis, but the virus membrane is at least four times more sensitive to the action of activated complement (Fig. 8). The 'toxic' effect of human plasma on chicken fibroblasts is illustrated in Fig. 9. The activated complement lyses a large percentage of the fibroblasts, probably in the same way as direct haemolysis. The heterophile antibody responsible for enhanced HL can be completely removed after repeated adsorptions of human plasmas with chicken erythrocytes at 4 °C.

**DISCUSSION**

The results presented in this paper support the theory that the nanogranular layer (basal membrane) of the virus is essential for the process of virus-to-cell fusion and subsequent haemolysis (Apostolov & Almeida, 1972). The paramyxoviruses mature by budding from the host cytoplasmic membrane (Morgan & Howe, 1968). The resulting virus envelope is bound on the outside by the projections (spikes) which contain the virus-specific glycoproteins, VP1 responsible for HA and the neuraminidase activity (Tozawa, Watanabe & Ishida, 1973; Scheid & Choppin, 1973) and VP2 which is probably involved in HL and cell fusion (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). These virus glycoproteins probably correspond morphologically to the large and small spikes seen by electron microscopy (Apostolov & Almeida, 1972; Fig. 2). Virus-specific haemagglutination-inhibiting antibodies also inhibit HL by preventing attachment (Howe & Morgan, 1969; Apostolov & Waterson, 1975). Recently it has been shown that antibody to VP1 inhibits HA, HL and cell fusion, but antibody to VP2 inhibits only HL and cell fusion (Seto, Becht & Rott, 1974). It
Complement enhancement of NDV haemolysis

Fig. 7. The effect of incubation of the virus on haemolysis, infectivity and haemagglutination. The virus was incubated with plasma (▲, ●, ■), and PBS as a control (△, ○, □) for various times, and samples taken for determination of haemolysis (●, ○), infectivity (■, □), and haemagglutination (▲, △) as described in Methods.

could be postulated that VP2 (the small projection) could have a role in bridging the gap between the virus and the cell membrane to allow fusion of the two. Antibody to VP2 would prevent the contact of the two membranes.

The inner layer of the virus envelope is the nanogranular layer of the virus membrane, seen clearly by negative staining (Apostolov & Almeida, 1972). Chemical analysis has shown that the lipids of the envelope are closely related quantitatively, and qualitatively, to the lipids of the host (Klenk & Choppin, 1969). It was also shown that the proteins of the envelope are virus-specific (Choppin et al., 1972). From the results in this paper and previous studies it may be assumed that during the process of conversion of cell membrane to virus membrane, the host membrane proteins are left out but the glycolipid antigenic moieties are transposed in toto (Rott et al. 1966; Apostolov & Waterson, 1974). Thus, the nanogranular layer which is essential for fusion and HL is entirely derived from the host and should be similar, if not identical, for all myxoviruses grown in the same host cell. The evidence that the virus membrane is a semi-permeable biological membrane comes from biological (Apostolov & Waterson, 1974) and physicochemical studies (Lenard et al. 1975).
Fig. 8. The effect of human plasma on chick erythrocytes in the absence (○—○) and in the presence (■—■) of NDV. ——, primary HL. Conditions of experiment as in Fig. 6.

Fig. 9. The effect of undiluted human plasma on chick fibroblast monolayer. The culture was incubated for 30 min with (a) heated and (b) unheated fresh plasma, phase contrast photography taken after incubation for 18 h.
Complement enhancement of NDV haemolysis

In previous work it has been clearly demonstrated that various physical and chemical pre-treatments which affect the nanogranular layer of the membrane (the basal membrane) of NDV or Sendai virus result in increase of HL (Apostolov & Waterson, 1975). In this paper we have demonstrated that activated complement also affects the nanogranular layer and enhances haemolysis. The concept that the capacity for HL depends on the physical state of the nanogranular layer (Apostolov & Damjanovic, 1973) was strengthened by the recent demonstration that single cycle harvested Sendai virus was not haemolytic but did induce cell fusion (Homma et al. 1976). This finding is not surprising; in a previous paper it was suggested that HL is subsequent to virus-to-cell membrane fusion and that cell-to-cell fusion is a result of cell-virus-cell bridging (Apostolov & Almeida, 1972; Apostolov & Waterson, 1974). So when the nanogranular layer is stable, as it is when harvested early, virus-to-cell membrane fusion and cell-to-cell fusion occur but there is no HL. On the other hand, when the nanogranular layer is made unstable, or broken, by physical treatment, a rise in HL and a drop in cell fusion index could be demonstrated (Hosaka, 1975). This dissociation in these properties of the virus is probably a result of the fragility and disintegration of the nanogranular layer prior to bridging of the cells.

We chose the system NDV-human, plasma-human erythrocytes because there are no HA inhibitory antibodies to NDV, which are seldom encountered outside birds. However, it can be predicted that similar results in experiments using heterophile antibody would be obtained with all the paramyxoviruses grown in chick chorioallantoic membrane. In fact, we have conducted most of the experiments presented in this paper with Sendai virus and obtained comparable results (to be published).

It is interesting that although influenza viruses have architecture which is similar in some respects to paramyxoviruses (Apostolov & Flewett, 1969) they do not produce haemolysis and do not fuse cells. It may be speculated that the difference is in the projections (spikes). As discussed above, the paramyxoviruses have small and tall spikes, influenza virions have only tall (10 nm) spikes. The tall spikes could by interposition separate the virus and cell membranes so that they fail to fuse. Another possibility is that the influenza spikes may have lipophobic properties inhibiting contact of the membranes. The concept that the spikes are an obstacle to virus-to-cell fusion is supported by the finding that reconstituted virus envelopes which have influenza spikes and Sendai virus membranes do not haemolyse while the reversed combination does (Hosaka, 1975).

From the results, it is clear that the heterophile antibody attaches to the nanogranular layer and activates complement without affecting the capacity of the virus to attach to erythrocytes (HA) and produce HL. It may be postulated that after the attachment the interposed antibody does not constitute a sufficient barrier to the fusing attraction of the two membranes. The activation of complement is documented in Fig. 2 and 3 as complement ‘holes’ (Humphrey & Dourmashkin, 1965). The nanogranular layer, together with the complement holes, is fused and integrated into the erythrocyte membrane, with the resulting effect of ‘lysis by proxy’. An alternative way of producing the complement holes is by allowing integration of the nanogranular layer first and then treating with antibody and complement (Apostolov & Waterson, 1974). The amount of enhanced HL can be changed by varying the amount of activated complement in two ways: (1) by changing the concentration of plasma or (2) changing the incubation time (Fig. 7). A high concentration of complement leads to the paradoxical effect of reducing HL, HA and infectivity, presumably by destroying the nanogranular layer and leading to the collapse of the virus envelope (Fig. 7).

Heterophile, haemagglutinating antibody can be demonstrated after fusion and integration of the nanogranular layer. This phenomenon was first described, but not explained, by
Burnet & Anderson (1946) and extended by Rott et al. (1966). It is clear that, for haemagglutination by heterophile antibody to occur, the spikes must float off, exposing the integrated virus membrane. This contention is supported by the fact that concentrated virus preparations are not agglutinated by the same antibody. Although the antibody is adsorbed (Fig. 4), the spikes prevent bridging of the virus membranes and agglutination.

Heterophile antibody is common to various animal species (Tanaka & Leduc, 1956). Therefore, it is not surprising that antibody to the nanogranular layer of chick-grown virus is found in some animal species including humans (Fig. 1). It is interesting that the heterophile antibody is predominantly of the IgM class and is probably identical to the heterophile IgM antibody involved in the agglutination of NDV-treated erythrocytes (Wilkinson & Carmichael, 1964). The heterophile antibody found in cord blood suggests that the sensitising antigen passes the placental barrier and the antibody is produced de novo.

On the basis of the results in this paper it could also be postulated that all the viruses that mature by budding from cell membranes contain similar glycolipid host membrane antigens. We have found by electron microscopy that infectious bronchitis virus (IBV) grown in eggs is also susceptible to the lytic effect of human plasma (unpublished data). Therefore, we are of the opinion that the recent results on the inactivation of oncornaviruses by human plasma could be due to the same mechanism (Welsh et al. 1975).

Recently, it was found that Sendai antibody raised in rabbits, at high dilutions and in the presence of complement can also induce increased HL (Shimizu et al. 1976). However, there are no data on whether the serum was adsorbed with chick cells, as the effect could be due to host antibody (Apostolov & Waterson, 1974).

We thank Mr S. Sidhu for excellent technical assistance and Professor A. P. Waterson for discussing and commenting on the manuscript.

REFERENCES


Complement enhancement of NDV haemolysis


(Received 28 April 1976)