A Delay in Maturation as a Cause of Small Plaque Size with the NM Strain of Influenza A Virus

By SUE MILLIKEN

Institute of Virology, University of Glasgow, Glasgow, Scotland

(Accepted 2 December 1966)

SUMMARY

Two influenza A viruses, neurotropic NWS and its recombinant NM are capable of forming plaques on BHK21 cells. NWS plaques are larger than NM plaques. The small plaque size of NM virus is related to a slower rate of increase of NM than NWS virus during many cycles of infection, not to inhibition by the non-cellular environment. The slow increase could be accounted for by a delay in the formation of infectious NM virus in each cycle accompanied by a simultaneous delay in release. The synthesis of NM virus is accompanied by a large quantity of unassembled or unstable non-infectious haemagglutinin; the synthesis of NWS virus yields assembled infectious haemagglutinin predominantly.

INTRODUCTION

There have been many recent reports of progressive influenza A infection in various types of mammalian cells (Hinz & Syverton, 1959; Wong & Kilbourne, 1961; Choppin, 1962; Grosberg, 1963; Lehmann-Grube, 1963). However, with the exception of Wong & Kilbourne (1961) and Sugiura & Kilbourne (1965), who used a human cell line, other studies were made in primary or secondary heterogeneous cell cultures which are unsatisfactory for studying precise relationships between virus and cell. Infection of BHK21 cells, a homogeneous continuous line of fibroblasts derived from baby hamster kidney (Macpherson & Stoker, 1962; Stoker & Macpherson, 1964), with two genetically related influenza A strains, NWS and NM showed plaques of different sizes for each virus. In this paper the plaque formation is described and investigated by studying the growth patterns of each virus strain.

METHODS

Infectivity titrations in chick embryo. The EID 50, or 50% embryo-infective dose was calculated by the method of Reed & Muench (1938) 3 days after allantoic inoculation of 11-day-old chick embryos. Haemagglutinin was demonstrated in the allantoic fluids of infected embryos.

Virus strains. The egg-adapted influenza A strains NWS (Stuart-Harris, 1939) and NM (Burnet & Lind, 1951) were used. NM is a recombinant strain derived from NWS and the MEL (Burnet, 1935) strain of influenza virus A. Virus stocks were prepared by high dilution passage in the allantoic cavities of 11-day old chick embryos. The fluids were harvested 44 hr after infection and stored at —70°. Only fluids with high infectivity:haemagglutinin ratios were used in experiments.
**Cells.** BHK 21 cells were maintained by continuous passage in Eagle's medium modified to contain twice the normal concentrations of amino acids and vitamins with 10% (v/v) tryptose phosphate broth and 10% (v/v) calf serum. Monolayers were prepared 18 to 24 hr before inoculation by seeding 5 x 10⁶ cells in 5 ml of modified Eagle's medium per 60 mm Petri dish. The cultures were incubated at 37° in a humid atmosphere containing 5% (v/v) CO₂.

**Plaque assay.** Monolayers of BHK 21 cells were washed once with phosphate buffered saline (Dulbecco & Vogt, 1954) and inoculated with 0.2 ml of virus diluted in saline buffered at pH 7.4 with 2-amino-2-hydroxymethylpropane-1,3-diol (tris) and containing 1% (v/v) unheated calf serum. Virus was adsorbed at 37° for 1 hr, the dishes being rocked every 10 to 15 min. Excess inoculum was then removed and each monolayer overlaid with 5 ml of modified Eagle's medium containing 1.3 times the concentration of constituents, with 5% calf serum, 0.9% (w/v) Difco Noble agar and 10 µg. of DEAE dextran per 100 ml. The last, while not essential, enhanced plaque definition and was therefore included routinely. Monolayers were stained at appropriate times by the addition of 0.012% (w/v) neutral red in 2.5 ml of overlay.

**Haemagglutinin assay.** Twofold dilutions of virus were made in 0.25 ml volumes of physiological saline. An equal volume of 1% guinea pig red blood cells was added and mixed. The cells were allowed to settle for 1 hr before end points were read. The highest dilution giving complete haemagglutination was taken as the end point.

**Haemadsorption.** Infected monolayers were treated with trypsin long enough for cells to round up. The trypsin was then removed and the cells suspended in 0.5% guinea pig red blood cells in saline containing 0.1% (v/v) unheated calf serum. After 10 min the proportion of haemadsorbing cells was determined. When long experiments were made, cells which had detached from the glass plus trypsinized cells were centrifuged then resuspended in 0.5% (v/v) guinea pig red blood cells and counted. This ensured that all cells were sampled, an important factor during the later hours of infection.

**Growth curves.** Monolayers were infected using the plaque assay procedure. After adsorption each cell sheet was washed three times with PBS then fed with 4.5 ml. modified Eagle's medium and incubated. Samples were taken at appropriate intervals, zero time being that at which the cells were fed. At low multiplicities of infection virus reproduction was confined to one cycle by dispersing the cells of newly infected monolayers with trypsin and seeding 10⁴-6 cells per Petri dish at which concentration no significant second cycle of infection occurred. When total virus was measured, cells and supernatant medium were harvested and stored at −70°. Each sample was subsequently frozen and thawed three times and the cellular debris removed by centrifugation before assaying. If released virus only was being measured, supernatant medium was harvested, centrifuged to sediment any debris and stored at −70° until assayed.

**Density gradients.** Potassium d-tartrate gradients were prepared with 1.8 ml. 45% (w/w) and 2.2 ml. 2% (w/w) solutions of the reagent using a gradient mixer. Virus was layered on top and then centrifuged for 60 min. at 35,000 rev./min. in the Spinco SW 39 rotor. Consecutively numbered fractions of five drops were collected from the bottom of each tube.

**Elution rates.** Virus elution rates were measured as follows: cell fragments were prepared by freezing and thawing 4 x 10⁷ BHK 21 cells in 2 ml of Ca-acetate buffer,
Influenza virus plaque formation

pH 6·0 (Ada & Stone, 1950) and sedimented by centrifugation. They were then resuspended in equivalent amounts of each virus diluted in chilled buffer and held at 4° for 60 min., by when over 90% of the virus had been adsorbed. The virus + BHK 21 complex was next washed three times at 4° then placed at 37°. At hourly intervals samples were centrifuged and the supernatants assayed for freed virus by haemagglutination titration. The washings were also titrated in case this step caused reversal of the binding, but no haemagglutinin was detected.

Immunofluorescence. The production of S antigen in NWS- and NM-infected BHK 21 cells was demonstrated by the indirect method with a human convalescent influenza serum (which did not react with the V antigen of these strains), and sheep anti-human globulin conjugated with fluorescein isothiocyanate. Cells were grown on coverslips and fixed for 10 min. in acetone at room temperature before being stained. They were examined by u.v.-blue illumination in a Reichert fluorescence microscope.

RESULTS

Plaque formation and dose response

Both NWS and NM produced round, turbid plaques which were visible even without staining when held against strong indirect light. However, NWS plaques were larger and could be detected sooner than those of NM (Pl. 1, fig. 1). Seventy-two hr after infection, NWS plaques ranged from 1·5 to 2·5 mm. in diameter with a mean of 2·1 mm.; NM plaques were not yet visible. At 96 hr, NWS plaques had increased to a mean diameter of 2·5 mm., whereas NM plaques varied from 0·5 to 2·0 mm., the mean being 1·4 mm. The plaques of both strains increased in size with further incubation. Both viruses gave a linear dose-response curve when titrated on BHK 21 monolayers, the number of plaques formed being proportional to the amount of virus present. To compare the infectivity for chick embryos with that by plaque count, tenfold dilutions of virus were assayed on BHK 21 monolayers and by allantoic inoculation of 11-day-old chick embryos. The embryonic fluids were harvested on the third day and the EID 50's determined. Both viruses gave an EID 50:plaque ratio of 0·95, indicating that plaque formation was highly efficient.

Multicycle growth

Effect of multiplicity of yield

The effect of multiplicity on the yield of infectious virus (von Magnus, 1954) was tested at various multiplicities from 10 to 0·001 plaque forming units (p.f.u.) per cell. Six successive passages were made each of 44 hr incubation and the same input multiplicity was used throughout by assaying for infectivity between passages. Good yields of each virus could be maintained only at less that 0·1 p.f.u. per cell.

The supernatant fluid from the third passage of NWS at a multiplicity of 0·001 was also assayed for infectivity in chick embryos, the EID 50:plaque ratio being 0·87. In addition, a particle count made by electron microscopy of this same sample gave a particle:plaque ratio of 6·6 ± 1·8. These findings were taken to mean that highly infective virus was produced in BHK 21 cells and that compared with NWS, NM was not adversely affected by the high multiplicity reached during plaque formation.

The final titre of NM was always 10 to 100 times less than that of NWS. The yield of each virus and the rate of extension of infection were therefore examined next.
effect of incubation temperature was studied at the same time because of its known effect on growth of influenza A (Burnet, 1936) and plaque size of RNA viruses (Dukes & Wenner, 1957).

**Yield and spread of virus**

Several cycles of growth of NWS and NM were followed in order to see whether there was any difference related to the plaque size in fluid medium as opposed to under agar. Monolayers were infected at a multiplicity of 0.01. Following the addition of modified Eagle’s medium the group of cultures was divided so that one third of each was incubated at 37°, 34° and 32°. At 4 hr intervals the supernatant fluids were harvested from duplicate cultures in each series and the cells tested for haemadsorption. NWS grew best at the highest temperature, reaching a maximum 28 hr after infection while NM took 56 hr to do so, the difference being more marked at the lower temperature (Fig. 1). With NM it took 48 hr for all the cells to become haemadsorbing; with NWS, this occurred within 24 hr (Fig. 2). A cytopathic effect with both NWS and NM was first seen microscopically when 70% of the cells were haemadsorbing. Cells rounded up and eventually came off the glass. The small plaque was, therefore, caused by an infection which spread slowly in liquid media as well as under agar.

**Single cycle growth**

To determine the cause of the slow growth of NM the one cycle growth patterns of the two viruses were compared at multiplicities of 10.0, 1.0 and 0.01. The monolayers were held at 37° or 32° from zero hour onwards. The same relative input of particles was maintained by using virus preparations having identical particle:infectivity ratios, the number of particles being calculated from the haemagglutinin titres. The
Influenza virus plaque formation

results from experiments at 37° are shown in Fig. 3, which refers to total virus production, cell associated plus released. Results at the lower temperature followed the same pattern.

Both viruses produced haemagglutinin simultaneously but there was a delay in the production of infective NM. High multiplicity of infection increased the delay with NM and influenced the total amount of infective virus produced. Low multiplicity of infection resulted in a relatively shorter delay although delay still occurred. In an attempt to establish at what point(s) the delay occurred with NM, individual stages of the growth cycle were studied.

Stages of single cycle growth

Adsorption. Monolayers infected with either NWS or NM were incubated at 37°. At 20 min. intervals the excess inoculum was removed from four cultures of each set and the monolayers overlaid. Both viruses adsorbed at similar rates, reaching a maximum at 60 min.

Synthesis. The time of synthesis and the location of soluble antigen was studied by specific fluorescent staining. Examination at half-hourly intervals of cells which had been infected with 20 p.f.u./cell revealed that the times of synthesis and of transport of
soluble antigen coincided in both virus infections. Faint, but specific, nuclear staining could first be detected 1½ hr after infection. This increased in intensity up to 2½ hr, when specific staining began in the perinuclear area. By 3 hr cytoplasmic staining was strong and it masked cellular details at 4 hr, except that most nuclei were now unstained. These immunofluorescent findings for NWS and NM are similar to those of Fraser (1967) on abortive infection with influenza A virus, except that the time intervals are shorter at a multiplicity of 20 p.f.u./cell. The delay in the production of infectious NM which occurs at high and low multiplicities of infection cannot, on this evidence, be attributed to failure to produce or transport soluble antigen (Franklin & Breitenfeld, 1959).

Fig. 4. Single cycle growth of NWS and NM in BHK21 cells infected at a multiplicity of 1-0 p.f.u./cell. Released virus only. P.f.u.; NWS, +—+; NM, ——; Haemagglutinin; NWS, +——+; NM, ——.

Release. Influenza particles are formed at the cell surface; the rate at which, they are released is mainly determined by their enzymic activity (Cairns & Mason, 1954). If the delay in the appearance of infective NM were due to rate of release, then one would expect this virus to be less enzymically active, i.e. to elute more slowly than NWS, although previous work with this virus suggested otherwise (Burnet & Edney, 1951). However, in order to examine this, replication was studied at a multiplicity of 1-0 but only extracellular, released virus was measured. At the same time the elution rate of each virus from fragmented BHK 21 cells was determined. NM eluted more rapidly than NWS. On the other hand, in growth curves, NWS could always be detected extracellularly earlier than NM (Fig. 4). In each infection, the ratios of infectivity to haemagglutinin remained similar indicating that the early non-infectious NM haemagglutinin, demonstrable when intracellular as well as extracellular virus was harvested, was not released as such. Since, at the multiplicity of infection used, both viruses eventually produced in one cycle the same amounts of infective virus, with the more enzymically active one accomplishing this later in the growth cycle than the less active, slow release after maturation could be effectively ruled out as the cause of the delay.
Influenza virus plaque formation

Assembly. The experimental data presented thus far suggested that delay involved either the RNA component, since infectivity was low, or some stage of assembly. The early difference in the infectivity:haemagglutinin ratios of total NM as opposed to released NM could be accounted for by initial synthesis of faulty RNA, delayed synthesis of infectious RNA or initial inaccessibility of this component. However, it is also possible that even in the presence of adequate amounts of RNA, soluble antigen and haemagglutinin NM particles were neither properly assembled, nor released for the first hour of two of production because of the absence of factors not yet identified. Such inadequately assembled virus could be physically different from mature virus.

Fig. 5

Fig. 6

Fig. 5. Haemagglutinin in fractions collected from density gradient centrifugation of concentrated total yields from BHK21 cells 4 hr after infection with NWS or NM. Relative densities and the percentage of total haemagglutinin comprised by each peak are indicated.

Fig. 6. As in fig. 5. Haemagglutinin present in fractions collected from density gradient centrifugation of released virus 6 hr after infection of BHK21 cells with NWS or NM.

In order to investigate this, single cycle growth experiments were made at an initial multiplicity of 1.0 and total virus was harvested from replicate cultures at the following times: 4 hr, i.e. just before the point at which new infective NM could first be detected; 6 hr, just after the first demonstration of a rise in infectivity with NM. The haemagglutinin from all of an equal number of replicate cultures of each virus was centrifuged each harvest in succession in the same tube through 45\% (w/w) potassium D-tartrate at 22,500 rev./min. in the Spinco SW 25. rotor. After each single centrifugation the top 80\% of supernatant fluid, which did not contain haemagglutinin, was removed and replaced by more potassium D-tartrate. When the 4 and 6 hr harvests of each virus had been concentrated in this way, the total yields of haemagglutinin were dialysed against phosphate buffered saline containing 1\% unheated calf serum and the concentrated sample centrifuged in a gradient of potassium D-tartrate. The fractions of the gradient were then assayed for haemagglutinin.

NWS gave one main peak, NM two (Fig. 5). The virus harvested at 4 hr was indistinguishable from that at 6 hr. The fractions under each peak were pooled, dialysed as before and tested for both infectivity and the presence of assembled particles.
Infectivity was found to be associated solely with the fast NWS and NM peaks. Electron micrographs of these samples show typical, fully assembled particles (Pl. 1, fig. 2a, b). No recognizable particles could be found in material from the slow NM bands. In addition, when virus released at 6 hr was examined in the same way each strain banded as a main peak of assembled particles (Fig. 6).

DISCUSSION

In a system where adsorption rate, yield of infectious virus per cycle and depression of yield by the high multiplicity of infection that would take place in plaque formation are the same for the NM and NWS virus strains, it is reasonable to ascribe the small size of NM plaques to the slow growth of that strain. At the time when plaques were measured, the NM strain would have gone through about 80% of the number of cycles accomplished by the large plaque former, NWS, if a point 8 hr after infection is taken as the time of maximum release for NM, and 6½ hr for NWS. This does not allow for the prolongation of the NM cycle which is seen at higher multiplicities.

The reasons for concluding that delayed maturation explains the retarded growth of NM virus are that each virus, under the same conditions, made the same amount of haemagglutinin at the same stages of the cycle yet NWS had optimal infectivity throughout; infectious NM was not found until 1½ to 2 hr later at the time of release. Soluble antigen, the second main antigenic component of the influenza virion, seems also to have been available in adequate amounts so far as fluorescent antibody can indicate. At the limit of visibility the amounts of antigen revealed by the same conjugated serum should have been equal and intranuclear synthesis of the soluble antigen the same in the early stages of the cycle at least; and similar concentrations were seen outside the nucleus at the same time in both infections.

The action of neuraminidase of influenza virus need not be considered since release of virus in this system is not related to enzymic activity. It might be needed as a structural unit.

The presence of NM haemagglutinin of lesser buoyant density could be due to a precursor of infectious haemagglutinin which has accumulated because of the postulated delay or to instability of the haemagglutinin of normal density, but there is no evidence from the centrifugation analysis that released NWS virus is unstable in potassium D-tartrate.

Choppin's (1963) findings with an influenza A2 system are very similar to the observations in this paper, but he recorded a slower growth for the small plaque virus throughout the cycle and did not distinguish between total virus and released virus. He attributed retarded growth to faulty assembly because of incompatibility of virus antigens. The behaviour of NM virus is more easily explained as a delay which, once overcome, is followed by normal rates of maturation and release. There is no decrease of slope of the curve of virus release as one might expect from the constant presence of faulty or incompatible subunits. It is rather as though a missing factor had been suddenly made available or had at length reached a concentration necessary for maturation to proceed. Such a hypothesis is consistent with the fact that, at the end of the infection cycle, all NM virus, released and cell-associated, had a haemagglutinin: infectivity ratio equal to NWS.

It should be possible to examine the genetic and synthetic relationships between
Influenza virus plaque formation

NM and NWS by comparing them with MEL virus, the other parent of the NM recombinant. It produces non-infectious haemagglutinin in BHK 21 cells and it is very likely that the delay in the growth of NM virus is derived from the MEL genome.

I wish to thank Drs E. Follett and D. H. Watson for the examination and counting of samples by electron microscopy and Dr L. V. Crawford for his guidance in density gradient techniques and interpretation. I am especially grateful to Dr K. B. Fraser for his interest and encouragement throughout the work and to Professor M. Stoker and Dr V. Thorne for advice and suggestions in reporting it.

REFERENCES


(Received 11 August 1966)
EXPLANATION OF PLATE

Fig. 1. Influenza A plaques in BHK21 monolayers; (a) NWS 72 hr after inoculation, (b) NWS 96 hr after inoculation, (c) NM 96 hr after inoculation.

Fig. 2. Electron micrographs of material from the fast-moving haemagglutinin fraction in a potassium d-tartrate density gradient. (a) NWS, (b) NM. Phosphotungstic acid negative staining. Bars = 1000 Å.