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Replication of Influenza A and B Viruses in Human Diploid Cells

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SUMMARY

Under optimal conditions, of high multiplicities of infection and with trypsin included in the medium throughout the incubation period, high yields of infectious influenza A and B viruses ($10^{6.5}$ p.f.u./ml) and of antigenically active haemagglutinin (HA) (1 μ g/HA/ 10^6 cells) were produced in human diploid MRC-5 cells. Budding virus particles were seen as spherical or short rod-like protrusions on the surface of the infected cells, and also on cell filopodia. Virus-induced cytoplasmic and nuclear inclusions were present in infected cells. This virus-human cell system may be suitable for studies of influenza virus persistence and for production of immunologically active HA antigen.

Most studies on the replication of influenza A viruses in tissue culture have been carried out with the laboratory-adapted fowl plague A/FPV/27 or A/WSN/33 (H1N1) viruses in chick embryo fibroblast cells, both of which viruses have a relatively wide cell tropism (for review, see Dowdle & Schild, 1975). Although the biochemical mechanisms of virus RNA and polypeptide synthesis have been well defined (for reviews, see Barry & Mahy, 1979; Hay & Skehel, 1979), much less is known about the quantities of immunologically active virus antigens in the infected cell. In addition, only a few studies have been carried out using virus-infected human cells (Kilbourne *et al.*, 1964; Wilkinson & Borland, 1972; Golubev & Medvedeva, 1978). We have investigated the replication of several human influenza A and B viruses in cell cultures of human diploid fibroblasts and demonstrate that under suitable conditions of cell culture, productive infection with release of infective virions and intracellular accumulation of virus HA and nucleoprotein (NP) antigens occurs.

For most experiments an attenuated influenza A recombinant virus ('Alice') was used. This is an inhibitor-resistant variant of the MRC-2 recombinant between parental viruses A/PR/8/34 (H1N1) and A/England/42/72 (H3N2) (Lobmann *et al.*, 1976; Florent, 1980) and was cultivated in ten-day-old chick embryos by standard techniques (Dowdle & Schild, 1975). Human diploid MRC-5 cells were used between the 20th and 30th population doublings and stock cells were passaged weekly using the general procedures described by Hayflick & Moorehead (1961). For infection of diploid cells, virus was adsorbed to monolayers for 1 h at 35 °C and the monolayers were then overlaid with the production medium (medium 199 with 0.11% w/v NaHCO_3 , 0.025 M-HEPES buffer pH 8). Cell cultures were incubated at 35 °C in 5% CO_2 . Cells were harvested with medium and cells being frozen directly in the test plates. Virus HA and NP production was determined by the single radial immunodiffusion method described by Wood *et al.* (1977).

Highest virus yields were obtained at 72 h post-infection at 10^3 p.f.u./0.2 ml of virus input, in cultures with 1 μ g/ml trypsin in the medium. In cultures without protease in the medium, cell-associated Alice virus was found to be 1 \log_{10} p.f.u./ml higher than cell-free virus throughout the incubation time. Influenza A/NWS/33 was used as a control virus in these experiments since the virus, similar to A/WSN, would be expected to undergo productive infection with and without trypsin (Lazarowitz & Choppin 1975; Appleyard & Maber, 1974) and the distribution of cell-free and cell-associated A/NWS/33 virus followed the same pattern in diploid cell cultures with and without trypsin (Fig. 1).

Production of HA was also studied under different conditions of infection by analysing the quantity of antigenically active intracellular HA by single radial immunodiffusion. Highest yields of HA obtained after 48 and 72 h incubation of cells infected with Alice virus and following a low virus input (4 \log_{10} p.f.u. per flask) were 0.65 and 0.95 μ g HA per 10^6 cells

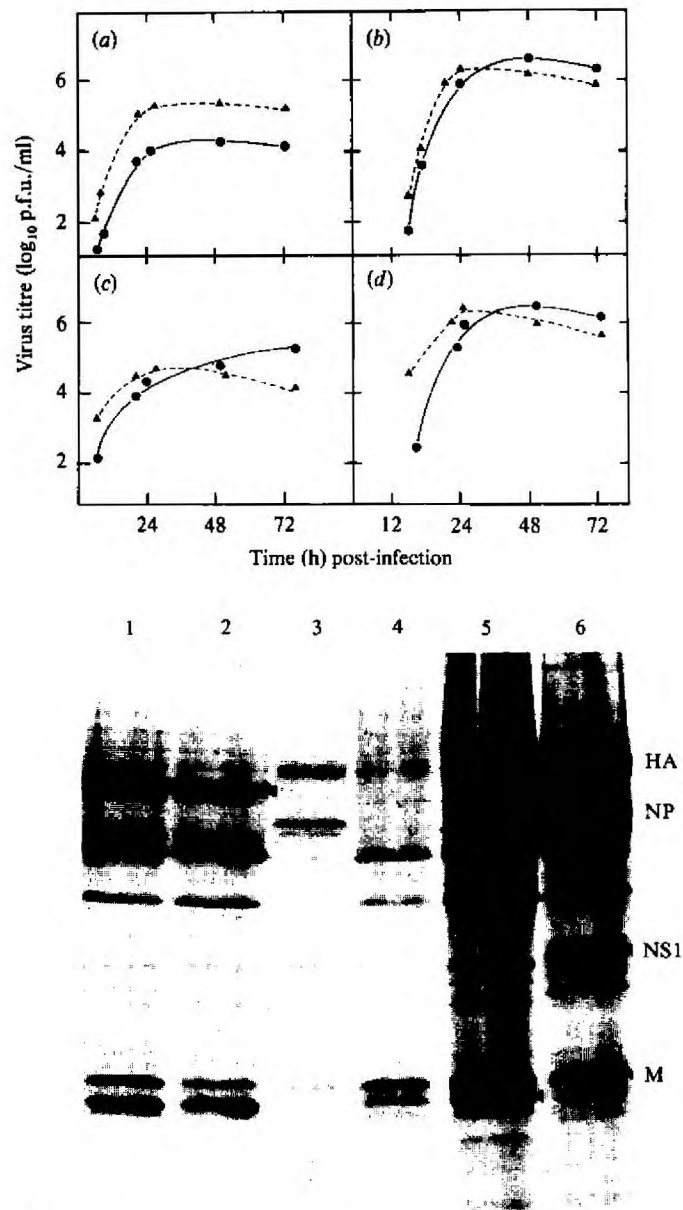


Fig. 1. Productive infection of human diploid MRC-5 cells and analysis of virus-induced polypeptides. Top section, *a* to *d*: replication of influenza A viruses in trypsin-treated (*b*, *d*) and untreated (*a*, *c*) cells. Procedure as in the text, using a virus input of 10^4 p.f.u./0.2 ml. Trypsin concentration was $0.3 \mu\text{g/ml}$, the highest concentration at which monolayers keep integrity. (*a*, *b*) Alice strain of influenza A virus, (*c*, *d*) A/NWS/33 (H1N1); assayed as cell-free (●) and cell-associated (▲) virus. Lower section: influenza A and B virus-induced structural and non-structural polypeptides in MRC-5 cells. Cells in 60 mm Petri dishes were infected with 5 p.f.u. per cell and incubated in maintenance medium for 7 or 24 h before being pulse-labelled with [^{35}S]methionine (Oxford *et al.*, 1981*a*). Polypeptides were separated using 20% polyacrylamide gels and the buffer systems described by Laemmli (1970) but with a lower bisacrylamide concentration, of 0.066%, and a twofold increase in the concentration of running buffer (Oxford *et al.*, 1981*b*). The samples were: 1, Alice virus, 199 medium + trypsin; 2, Alice virus, Gey's medium; 3, B/HK/73 virus, 199 medium; 4, A/USSR/92/77 virus, 199 medium; 5, A/USSR/92/77 virus, 199 medium + trypsin; 6, B/HK/73, 199 medium + trypsin. The bands indicated are HA, haemagglutinin, NP, nucleoprotein, NS1, non-structural protein 1, and M, matrix protein. Note that NS1 of influenza B/HK/73 migrates slowly between the NP and M polypeptides whilst NS1 of the influenza A viruses migrates close to, but more slowly than the M polypeptide (Oxford *et al.*, 1981*a*). The identity of virus-induced polypeptides was established in preliminary experiments by comigration with Coomassie Brilliant Blue-stained proteins from purified virus and by comparison of infected pulse-labelled cells with uninfected pulse-labelled cells (*d*: ... not shown).

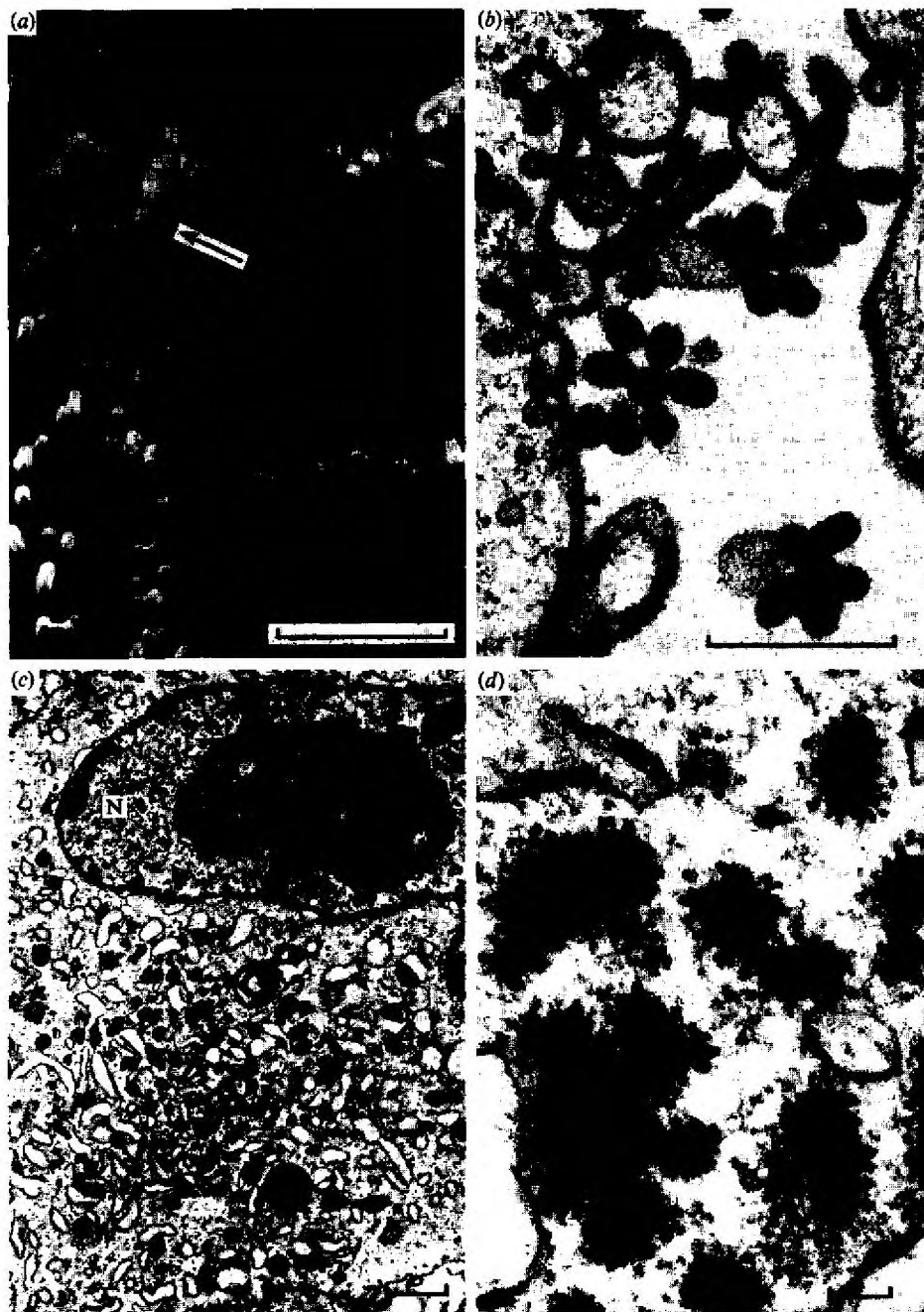


Fig. 2. Scanning and transmission electron micrographs of human diploid cells infected with influenza A viruses. Cells were infected with 5 p.f.u./cell of Alice or A/NWS/33 (H1N1) viruses and examined by electron microscopy 24 h later. (a) SEM showing budding of NWS on filopodia and some filamentous particles (arrow), bar marker represents 1 μm ; (b) TEM showing budding of Alice on cell processes and a single filamentous particle (arrow), bar marker represents 0.5 μm ; (c) sectioned cell infected with NWS and showing dense inclusions in the nucleus (N) and cytoplasm, bar marker represents 1 μm ; (d) higher magnification of the crystalline cytoplasmic inclusions and associated ribosomes, bar marker represents 0.1 μm . Specimens for both SEM and TEM were fixed in glutaraldehyde, osmium tetroxide and uranyl acetate followed by dehydration in ethanol. TEM specimens were embedded in Araldite and sections were stained with uranyl acetate and lead citrate. SEM specimens were critical-point dried in carbon dioxide and sputter-coated with a layer of gold 8 nm in thickness.

respectively. Haemagglutinin production was not significantly affected intracellularly by the presence of trypsin in the overlay medium, since concentrations of HA in the presence of 1 µg/ml trypsin at the above times were 0.7 and 1.0 µg HA per 10⁶ cells. Following infection of embryonated hens' eggs with corresponding doses of virus 0.7, 0.9 and 1.5 µg/HA in 0.1 ml was detected in the allantoic fluid at 24, 48 and 72 h post-infection. Thus, with an estimated 1.8 × 10⁸ chorioallantoic membrane cells per egg (Cairns & Edney, 1952) and a mean value (from five experiments) of 138.7 µg HA produced per egg, the estimated influenza HA yield in embryonated hens' eggs is 0.77 µg HA per 10⁶ cells. At present we have not established whether the intracellular HA which is detected is glycosylated or non-glycosylated or present as monomers or as single HA1 and HA2 polypeptides. Nucleoprotein antigen was also detected at approximately the same concentration by using single radial immunodiffusion and monospecific antiserum to NP incorporated into the agarose.

In further experiments the polypeptides induced by the Alice virus and other influenza viruses in these human diploid cells were analysed. Since diploid cells are more culturally fastidious than MDCK or Vero cells, which are commonly used in pulse-label experiments and incubated with a depleted Gey's medium, both Gey's and medium 199 were used in these experiments. Similar results were obtained with both media. All virus-induced structural and non-structural polypeptides were present 7 h post-infection (Fig. 1*b*); the relative amounts of the different virus-induced polypeptides and their migration characteristics closely resembled those described in previous reports of influenza A or B virus infected non-human cells (Inglis *et al.*, 1976; Skehel, 1972; Ritchey *et al.*, 1977, Palese *et al.*, 1977; Oxford *et al.*, 1981*a*; Hugentobler *et al.*, 1981).

Monolayers of MRC-5 cells were examined by electron microscopy 24 h after infection with either influenza A/NWS/33 or the Alice strain of influenza. Scanning electron microscopy (SEM) showed budding particles on nearly all cells when a high multiplicity of infection of either virus strain was used (Fig. 2*a*), although a greater number of particles was present on cells infected with the A/NWS/33 strain than on cells infected with the Alice strain. Transmission electron microscopy (TEM) of sectioned cells confirmed the quantitative interpretations of SEM and the tendency for budding to occur on the cell filopodia (Fig. 2*b*). The cytoplasm of A/NWS/33-infected cells contained numerous electron-dense inclusions which appeared to have a crystalline structure (Fig. 2*c, d*). The nuclei of the NWS virus-infected cells also contained large aggregates of dense material (Fig. 2*c*). Comparable cytoplasmic structures have been tentatively identified as NS1 protein in A/WSN virus-infected CEF cells (Compans & Dimmock 1969; Petri *et al.*, 1982). No such cytoplasmic inclusions were seen in Alice-infected cells and only small granular inclusions were observed in the cell nuclei.

At present, influenza viruses for use as both inactivated or live attenuated vaccines are cultivated in embryonated hens' eggs (for reviews, see Selby 1976; Voller & Friedman, 1978). The well-characterized diploid cell strain MRC-5 is considered a suitable substrate for the production of certain other virus vaccines (Jacobs, 1970) and this could now be extended to include influenza A and B viruses. Most biochemical studies on influenza virus infection of cells (Barry & Mahy, 1979; Barrett *et al.*, 1979; Hay & Skehel, 1979) have been carried out with A/FPV/27 or A/NWS/33 virus-infected chick cells. The human diploid cell system described here may therefore present a novel virus-cell system more relevant to the study of human infection by influenza A and B viruses.

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