Effect of Input Multiplicity on Infection of Cells with Myxoviruses as Studied by Hemadsorption (33484)

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The adsorption of certain erythrocytes to the surface of cells infected with a myxovirus has been shown to be a highly specific phenomenon occurring only on cells infected with virus (1–3). In monolayer cell cultures infected with virus, standard hemadsorption procedures provide a relatively simple and rapid means of detecting infection but it is impossible to make an accurate estimate of the number of individual cells infected (4).

Two approaches have been developed to adapt the hemadsorption test for quantitative use. White *et al.* (5) made quantitative measurements of hemadsorption following the infection of single cells with the MEL strain of influenza virus and more recently have followed the progress of viral multiplication (6). In their system secondary infection of cells did not occur because influenza virus undergoes an abortive cycle when infecting HeLa cells (7). Marcus (8) defined the sequence of events occurring at the cell surface of single cells infected with a myxovirus and more recently Marcus and Carver (9–10) detected and studied a new type of viral interference. In their work infected cells were plated at high dilution so that progeny virus could not infect uninfected cells.

In our experiments Fowl plague virus (FPV) and Newcastle disease virus (NDV) were studied in a fully competent cell system which was maintained in monolayer culture in the presence of specific antiviral antibody until just prior to hemadsorption assay. This was undertaken to quantitate the relationship between input multiplicity, the number of cells showing hemadsorption and the number of cells producing infectious virus in monolayer culture.

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Materials and Methods. Cell cultures. Chicken embryo fibroblast cultures were prepared from 10-day-old embryos by treatment with 0.25% trypsin in phosphate buffered saline (PBS). The cell suspension was filtered twice through a double layer of sterile gauze, centrifuged at 600*g* for 5 min and the cell pellet was resuspended in medium 199 containing 5% calf serum, to give a final concentration of 1.5×10^6 cells/ml. Cells were dispensed into plastic petri dishes in 4-ml amounts and incubated at 37° in an atmosphere of 5% CO_2 in air.

Viruses. Newcastle disease virus California 11914, and the Rostock strain of Fowl plague virus were grown in 10-day-old fertile eggs, and the allantoic fluid was harvested following incubation at 37° for 36 hr. The virus was concentrated by centrifuging the allantoic fluid at 30,000*g* for 1 hr and resuspending the pellet in 1/50 volume of PBS containing 0.1% gelatin.

All virus stocks were stored in sealed glass ampules at —70°.

Infection of cell monolayers. Monolayer cultures were washed once with warm PBS, and 0.5 ml of virus dilution was added. Virus adsorption was allowed to proceed for 30 min at 37°. The cells were then washed twice with warm PBS to remove free virus, 2 ml of a dilution of specific antiserum were added to each dish and the dishes were incubated at 37° for a further 10 min. The cells were washed with warm PBS and 2 ml of growth medium were added to each dish. The cells were then incubated for a further 7 hr at 37° in an atmosphere of 5% CO_2 in air. In certain experiments the cells were not washed so that antibody was allowed to remain throughout the incubation period.

Hemadsorption. Cell monolayers were washed once with PBS and 2 ml of 0.02% trypsin in PBS containing 0.002% EDTA

were added to each dish; and the dishes were incubated at 37° for 10 min. Cells were removed from the petri dishes by gentle pipetting and sedimented by centrifugation at 600*g* for 5 min. The supernatant was discarded; the cell pellet was resuspended in 3 ml of PBS; and 0.04 ml of a 25% suspension of fresh washed guinea pig red blood cells in PBS was added to each tube. After gentle agitation the tubes were allowed to stand at room temperature for 15 min prior to samples being removed for counting under phase contrast microscopy. The percentage of hemadsorbing cells was scored by counting 100 cells selected at random; duplicate preparations were made and counted in each case.

Infected cell complexes. For detection of FPV and NDV cells producing infectious virus, a modification of the plaque procedure, as described by Drake and Lay (11), was used. After incubation the infected monolayer cells were suspended by trypsinization as described for hemadsorption. Even through the cells had been treated with antiviral antibody, the procedure was carried out no later than 3-hr postinfection to completely exclude the possibility of newly formed virus being scored as an infected cell complex. Cells were washed once with PBS, suspended in growth medium, and counted. Appropriate dilutions were made in medium, 0.1 ml of the diluted cell suspension being added to 0.9 ml of overlay medium (medium 199 containing 5% calf serum and 0.9% agar); and 0.2 ml of the cell dilution in overlay medium was added to the monolayers in each of four 60-mm plastic petri dishes and the agar was allowed to solidify. Four ml of overlay medium were added carefully to each dish and the dishes were incubated for 48–60 hr at 37° in an atmosphere of 5% CO_2 in air. Plaques were counted following the addition of a neutral red solution to each plate.

Results. In preliminary experiments maximum numbers of positive hemadsorbing cells were reached within 8 hr for both FPV and NDV. Therefore all hemadsorption assays were done at 8-hr postinfection.

Effect of antiserum on percentage of hemadsorbing cells. No significant differences in percentage of hemadsorbing cells was detected between cultures treated postinfection with antiserum for 10 min and those treated throughout the 8-hr incubation period (Table I). If fresh antiserum was added to the cell suspension following trypsinization, hemadsorption was blocked completely. In addition, when the incubation medium containing antiserum was added back to the culture after trysinization, hemadsorption was inhibited, showing that viral specific antibody was in excess throughout the experiment.

TABLE I. Effect of Antiserum Treatment on the Number of Positive Hemadsorbing Cells.

Antiserum exposure postinfection[a]	Suspension medium after trypsinization[b]	Av percentage of positive hemadsorbing cells
30–40 min	No antiserum	53.0
30 min–8 hr	No antiserum	51.3
0	No antiserum	63.6
30 min–8 hr	Antiserum	0
30 min–8 hr	Antiserum[c]	0
0	Antiserum	0

[a] Cells were incubated with or without antiserum then washed three times and incubated in medium + 5% calf serum for the remainder of the incubation.

[b] Cells were washed three times with Ca^{2+}- and Mg^{2+}-free PBS and suspended with trypsin–EDTA. After one wash they were suspended in medium with or without antiserum.

[c] Cells were suspended after trypsinization in the medium in which they had been incubated for the 8-hr period.

Effect of multiplicity on the percentage of hemadsorbing cells. Monolayer cultures of chicken cells were exposed to NDV or FPV at various multiplicities; and 8-hr postinfection the percentages of hemadsorbing cells were determined. Figures 1 and 2 show that both viruses exhibit curves relating hemadsorption to multiplicity with a very rapid increase in the percentage of hemadsoring cells between multiplicities of 0.75 and 3/cell, there being a leveling off between 3 and 24 virus particles/cell. It would thus appear that the percentage of hemadsorbing cells is directly related to the input multiplicty of virus.

The relationship between hemadsorbing cells and the number of cells producing infec-

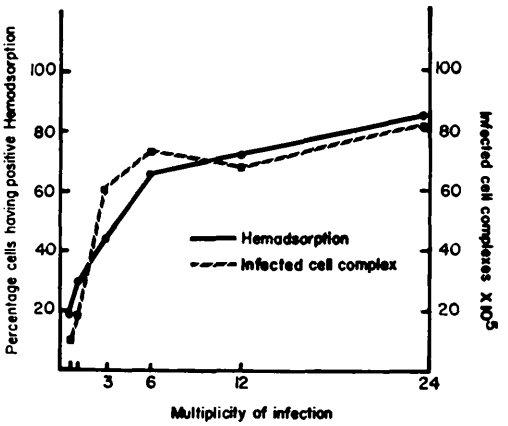

FIG. 1. The relationship between hemadsorbing cells and the number of cells producing infectious virus in NDV infected chicken embryo cells at various multiplicities of infection. The number of cells showing hemadsorption and the number of cells producing infectious virus were determined as described in "Materials and Methods."

tious virus. To compare the phenomenon of hemadsorption with the production of infectious virus it was necessary to compare the results obtained for infectivity of the preparations using the infectious cell complex method with the results of hemadsorption studies carried out on the same preparations. Cells were infected at various multiplicities and Fig. 1 shows that for NDV the percentage of cells producing infectious virus rises with the percentage of cells showing hemadsorption at the various multiplicities. However for FPV (Fig. 2) it is clear that the percentage of hemadsorbing cells producing infectious virus falls rapidly with increasing multiplicity having a equilibrium point at an input multiplicity between 1.5 and 3 infectious virus particles/cell. Thus, although cells infected with FPV at a multiplicity greater than one may exhibit the phenomenon of hemadsorption, their ability to produce infectious virus is markedly reduced. Also there was observed an overall 10-fold reduction in the number of cells capable of producing infectious FPV although the number of hemadsorbing cells for FPV and NDV were the same.

Discussion. Employing a technique of hemadsorption it was possible to measure accurately the number of myxovirus infected cells in monolayer cultures. Secondary infection of cells by virus progeny was completely blocked with the use of antiserum (Table I). It appeared that treatment with trypsin–EDTA during the cell suspension procedure removed antibody from the virus-modified cell membrane allowing adsorption of guinea pig erythrocytes to take place on the surface of the infected cells. This enabled us to work with a cell system that produced infectious virus and did not require replating at high cell delution (8–10) to reduce secondary infection by newly formed virus.

Newcastle disease virus and Fowl plague virus were used in the above experiments to infect chicken embryo cells in monolayer cultures at various multiplicities of infection. The NDV exhibited a rapid increase in the percentage of hemadsorbing cells with increasing multiplicity of infection up to an input of six virus particles per cell with a marked leveling off reaching saturation at the higher multiplicities. The number of cells

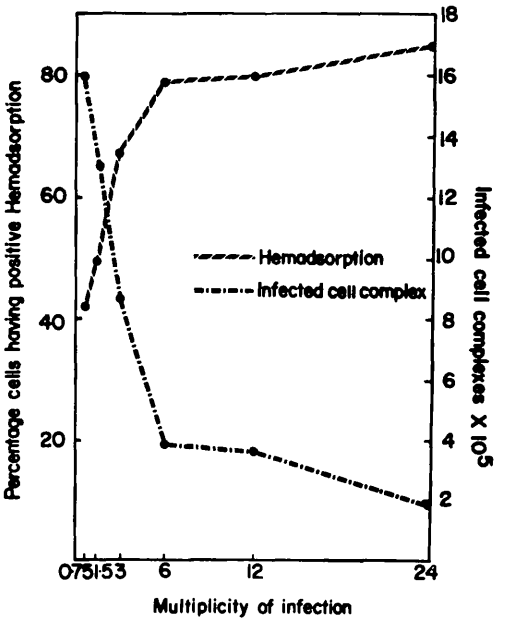

FIG. 2. The relationship between hemadsorbing cells and the number of cells producing infectious virus in FPV infected chicken embryo cells at various multiplicities of infection. The number of cells showing hemadsorption and the number of cells producing infectious virus were determined as described in "Materials and Methods."

producing infectious virus also increased at the same rate and was proportional to the number of cells exhibiting positive hemadsorption (Fig. 1).

The results of FPV were similar to those obtained for NDV as measured by hemadsorption but the number of cells producing infectious virus decreased with increasing percentage hemadsorption. Essentially, for FPV, the results agree with those of White *et al.* (6) who found that in HeLa cells infected with the BEL strain of influenza A virus, at multiplicities greater than one, the number of hemadsorbing cells increased with time the higher the multiplicity. However, in addition, in the FPV infected chicken embryo cell system it was found that increasing numbers of hemadsorbing cells led to a corresponding decrease in the number of cells producing infectious virus as would be expected from previous studies on the formation of incomplete virus (12). This again stresses the problems faced in any biochemical study with the influenza viruses. At multiplicities sufficient to infect the majority of cultured cells incomplete virus are formed leading to what may be spurious results.

Such a system, of rapidly and accurately measuring cells infected in monolayer culture with myxoviruses, could be applied to many experimental situations requireing an accurate assessment of the numbers of monolayer cells infected and producing virus at any given time.

Summary. A method is described whereby it was possible to rapidly and quantitatively determine the number of competent host cells infected in monolayer culture with myxoviruses. Fowl plague virus and Newcastle disease virus were compared with respect to thier ability to form infectious virus at various multiplicities of infection. It was also shown, that specific antiserum, if present throughout the multiplication period, did not interfere with the final test results.

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