Immunofluorescence of Abortive and Complete Infections by Influenza A Virus in Hamster BHK21 cells and Mouse L cells

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SUMMARY

The synthesis and transport of the soluble antigen of influenza A virus were traced by immunofluorescent staining with human convalescent sera during abortive and complete infection of hamster BHK21 cells and mouse L cells. Times of synthesis in the nucleus, transport to the cytoplasm and extrusion by the cell were the same in complete and in abortive infection.

The product of abortive infection contained soluble antigen, went through the same stages of adsorption, penetration and disposal as infectious virus but differed from it in being non-infectious, non-interfering and in not producing phenotypic mixing. These deficiencies suggest that RNA is either absent from or fails to function in abortive virus.

Ring-like structures were detected in the nucleus at late stages in the synthetic cycle. Their antigenic nature was not ascertained but in L cells soluble antigen appeared first round a nucleolar halo.

INTRODUCTION

Influenza A strains that have been adapted to the chick embryo produce in certain mammalian tissues one cycle of infection accompanied by cytopathic effects and limited by the production of noninfectious virus (Schlesinger, 1950; Henle, Girardi & Henle, 1955). A similar result follows the infection of L cells with fowl plague virus (Franklin & Breitenfeld, 1959) which like influenza A (Liu, 1955) is synthesized in the nucleus and cytoplasm (Breitenfeld & Schäfer, 1957). Infection of L cells with Newcastle disease virus (NDV), which reproduces in the cytoplasm only (Prince & Ginsberg, 1957; Traver, Northrop & Walker, 1960) is also abortive (Wilcox, 1959). The intracellular distributions of the soluble and particle antigens of these two viruses have been traced separately. Abortive infection of fowl plague virus in L cells is accompanied, and perhaps caused, by the retention of soluble antigen in the nucleus of the infected cell, with consequent failure of the two antigens to integrate and form infectious virus (Franklin & Breitenfeld, 1959). No such effect could account for the production of noninfectious NDV in L cells because, as Reda, Rott & Schäfer (1964) have recently demonstrated, NDV, in abortive as in successful synthesis, does not produce its soluble antigen within the nucleus.

Holtermann and his colleagues (Holtermann, Hillis & Moffat, 1960; Moffat, Holtermann & Hillis, 1960; Hillis, Moffat & Holtermann, 1960) have shown that the

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soluble and particle antigens of influenza A virus in abortive infection of HeLa cells are situated in the nucleus and cytoplasm respectively, and migration of the soluble antigen was noted, but not studied particularly. The system which is now described is especially advantageous for studying abortive and complete infection, since both types of infection may be compared in one genetically homogeneous line of mammalian cells and each is produced by serologically related, as well as unrelated, mutants of influenza A virus. The experiments are based on a finding (Mülliken, personal communication) that the neurotropic variant of the ws strain of influenza A and its genetic recombinant with the non-neurotropic mel strain produce plaques on monolayers of BHK21 cells; the ws, mel and other strains of influenza virus produce noninfectious virus only, and consequently no plaques. Abortive influenzal infection could be expected to simulate infection of L cells with fowl plague virus which is avian influenza A.

**METHODS**

*Virus strains.* The mel (Burnet, 1936), wse (Burnet & Lush, 1938) and nws (Stuart-Harris, 1939) strains of influenza A virus and also the Lee (Francis, 1940) strain of influenza B virus were grown suballantoically in 11-day chick embryos from inocula at limiting infectivity, harvested after 42 hr incubation at 35° and stored at -60°. wse and nws are serologically almost identical. mel is distinct. All three are antigenically unlike influenza A2. mel-C13 virus refers to the one-cycle product of mel in BHK21-C13 cells which were infected with mel at a multiplicity of about 10 plaque forming units (p.f.u.)/cell. After excess inoculum had been washed off, the infected cells were incubated overnight in 5 ml. of ETC. The supernatant fluid, clarified by low speed centrifugation, was mel-C13 virus and usually had a haemagglutinin titre of 64 to 128. As will be seen later, about 1 particle in every 10^5 or 10^6 was infectious and represented residual inoculum.

*Virus titrations.* Virus infectivity was titrated suballantoically in 11-day-old chick embryos which were tested 3 days later for the production of haemagglutinin. Titres are expressed in egg-infective doses (EID 50), calculated by the method of Reed & Muench (1938). Haemagglutination and anti-haemagglutinin tests were carried out in plastic plates against a 1 % suspension of guinea pig red blood cells in saline, using 0.25 ml. of each reagent and reading the test by the pattern of agglutination 1 hr after the addition of red cells. Titres are expressed in agglutinating doses.

*Cells.* BHK21 cells are a continuous line of hamster kidney cells (Stoker & Macpherson, 1964). They were grown from a stock line of the original clone C13 which was preserved in ETC with 5 % (v/v) glycerol at -80°. L cells were similarly cultivated from stored cells that had been re-cloned in this laboratory (Breeze, 1964).

*Media.* ETC, the growth medium, consists of double strength Eagle's medium, 10 % (v/v) tryptose-phosphate broth and 10 % (v/v) calf serum with streptomycin 100 μg. and penicillin, 100 units per ml. (Stoker & Macpherson, 1964). After infection, growth medium was replaced with ETC in which the concentration of calf serum was reduced to 2 % (v/v).

* Diluting fluid.* Virus dilutions and inocula were made in saline buffered with 2-amino 2-hydroxymethyl propane 1:3 diol (tris) (pH 7·1) with calf serum added to 1 % (v/v).

*Monolayers.* Cultures for fluorescence microscopy were prepared by plating 2 × 10^6–0
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cells in 5 ml. ETC over glass coverslips in 50 mm. dishes, and incubating at 37° in a CO₂ incubator. Cells were infected in two ways: (1) 2 × 10⁶ to 10⁶ cells in suspension at a ratio of 5 EID 50 per cell in 1 ml. volumes. Adsorption was 90% complete. (2) in monolayers at a ratio of 30 EID 50 per cell in 1 ml. volumes, enough to submerge all coverslips. Adsorption was 15 to 30% effective in different tests. Other ratios were used when necessary, as stated in the test. Excess virus was removed by washing the cells in 1% calf serum in saline, then adding maintenance medium. Suspended cells were washed, plated in 5 ml. ETC which was changed to maintenance medium 5 hr after plating.

Fixation. Coverslips were removed at intervals, fixed unwashed in acetone for 10 min. at room temperature, air dried for 30 min. and stained immediately.

Staining. The sandwich method was used throughout. Soluble (S) antigen was stained with six sera from young adults, convalescent from influenza A2 infection, with complement fixing titres of 128 to 256. They were used diluted 1/5 in saline buffered at pH 7.2. These sera were tested and did not cross react with the haemagglutinin of the MEL, WS or NWS strains of influenza A virus; the second layer of the stain was rabbit anti-human globulin conjugated with fluorescein isothiocyanate, FITC (Rinderknecht, 1960) or lissamine rhodamine B', (RB 200) (Chadwick, McEntegart & Nairn, 1958). Virus particle (V) antigen was stained with rabbit immune sera specific for WS or MEL, anti-haemagglutinin titres 3800 and 5200 respectively against 5 agglutinating doses of virus. These sera did not react in the complement fixation test with influenza A soluble antigen. The second layer was sheep or goat anti-rabbit globulin conjugated with either fluorochrome.

Specificity. The criteria of specificity were: (1) immunological staining of infected, but not of uninfected cells; (2) no staining after infection by the serologically unrelated myxoviruses, Newcastle disease virus and parainfluenza type 2; and (3) no staining by a 1/5 dilution of acute phase sera from the same patients.

Lack of cross reacting anti-haemagglutinin in human sera does not exclude the possibility of cross reacting staining of particle antigen in MEL- and NWS-infected cells. Anti-human globulin was therefore conjugated with RB 200 and anti-rabbit globulin with the different coloured fluorochrome, FITC. The rabbit conjugate would detect V antigen when cells were treated with specific rabbit antiserum. It could be shown that, at a time when conjugated anti-rabbit globulin first stained V antigen in the cytoplasm of infected cells, human sera, followed by anti-human globulin, stained the nucleus only. Thus S and V antigens could be distinguished from one another during the early stages of infection in the same cell. When the sera were conjugated with the reciprocal fluorochromes, FITC in place of RB 200, stained cells gave the same findings as before, but with the colours reversed (Fraser & Thormar, 1962, unpublished). The sequence of events shown in Pl. 1, figs. 1 to 4 thus represents synthesis and transport of soluble antigen only.

Nonspecific staining. Certain unadsorbed human and animal sera cause nonspecific staining of cells of other species when the sandwich technique of fluorescent antibody is applied. To outline nuclei and nucleoli of mouse L cells, we treated the cells with a human postvaccinial infant's serum which happened to stain nuclei slightly and nucleoli brightly. It neither stained nor interfered with the staining of influenza antigens, and scarcely stained BHK 21 cells.
Microscopy. A Reichert ‘Binolux’ microscope was used with UV-blue dark ground illumination. Photographs were taken on high speed Ektachrome film.

Ultraviolet irradiation. Virus samples were dialysed and irradiated in layers less than \( \frac{1}{2} \) mm. thick, 9 cm. from a Hanovia portable UV lamp.

Receptor destroying enzyme (RDE). Commercial samples were titrated by incubating a 1% suspension of human red blood cells in dilutions of RDE at 37° for 2 hr. Cells were then washed free of RDE, resuspended in normal saline and exposed to 5 agglutinating doses of MEL virus. The highest dilution showing nonagglutinated red cell suspension marked the end-point.

RESULTS

Comparison of complete with abortive infection

Continuous infection with the NWS strain and abortive infection by MEL and WSE viruses were confirmed by six serial passages on monolayers of about \( 10^{7} \) BHK21 cells. Initial inocula of \( 10^{8}, 10^{6}, \) and \( 10^{4} \) EID 50 of each virus were made, and washed out after one hour's adsorption when cultures were fed with 10 ml. ETC. This was followed by blind passage of the culture medium at intervals of 3 days' incubation at 35.5 and 37° each passage consisting of 1.0 ml. from the culture inoculated with \( 10^{8} \) EID 50, 1 ml. of a \( 10^{-4} \) dilution from the culture inoculated with \( 10^{6} \) EID 50 and 1 ml. of a \( 10^{-4} \) dilution from the culture inoculated with \( 10^{4} \) EID 50.

Table 1. Complete and abortive infections on serial passage of influenza A strains in BHK21 cells

<table>
<thead>
<tr>
<th>Strain ...</th>
<th>NWS</th>
<th>WSE</th>
<th>MEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passage no. ...</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>CPE</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>HAD %</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HA</td>
<td>128</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>EID 50 log_{10} Pre</td>
<td>3-5</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>EID 50 log_{10} Post</td>
<td>7-5</td>
<td>..</td>
<td>..</td>
</tr>
</tbody>
</table>

CPE = Cytopathic effect on monolayer.
++ Complete destruction.
HAD % Percentage of cells showing haemadsorption.
HA = Titre of haemagglutinin.
EID_{pre} Pre-incubation infectious titre = residual virus.
EID_{post} Post-incubation infectious titre.
0 Not present.
.. Not done.

Table 1 is a composite table showing the results of several characteristic experiments. MEL and WSE produced haemagglutinin at the first passage, but produced no cytopathic effects after the first passage, no haemadsorption after the second passage and at the sixth passage the cultures were free of virus. At the second passage, the number of haemadsorbing cells, 0-1% of about \( 10^{7} \) cells, was roughly equivalent to the number of EID 50 remaining from the first inoculum. The product of the first MEL passage, MEL-C13 virus (see methods) therefore was supposed to be non-infectious, except for
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this fraction. NWS caused rounding of cells and destruction of the monolayer, and haemadsorption by all cells could be demonstrated; the cultures yielded infectious virus at all passages except in the undiluted series which failed to produce haemadsorption or infectious virus at the fourth passage. This is supported by Milliken’s observations (personal communication).

Fluorescent staining

The time when soluble antigen first became stainable, and the intracellular sites at which it was seen in infected cells, were compared in the three types of infection, NWS, WSE and MEL. Groups of monolayers were infected simultaneously with each of the three strains and coverslips taken from each group at the same points of time during the infectious cycle.

Coverslips were removed half hourly from 1 to 7½ hr after infection and at then 9, 12, 15, 24 and 36 hr, by which time most cells were rounded and coming off the glass. Infection in suspension with 5 EID 50 per cell and on the monolayer with 30 EID 50 per cell gave similar results. No difference could be detected by immunofluorescent staining between the three varieties of infection. There was, in particular, no delay in the apparent movement of S antigen from nucleus to cytoplasm and certainly no retention of antigen in the nucleus of any cell. This was made particularly easy to follow because of the complete ‘emptying’ of the nucleus at one stage in the sequence of events which was as follows.

Synthesis. There was some asynchrony of response to infection, but S antigen could first be seen in the nucleus at 2½ hr and was present in all nuclei at 4 hr (Pl. 1, fig. 1). V antigen appeared in the cytoplasm of different cells from 3½ hr after infection and all cells were stained at 5½ hr, though specific fluorescence was weak in a proportion of cells. V antigen was not found in nuclei at any time.

Transport. Staining of S antigen began to appear in the perinuclear area at 4½ to 5½ hr and by 7½ hr all nuclei were apparently unstained, though sharply outlined by specifically stained material (Pl. 1, fig. 2).

Release. From 5½ hr onwards S antigen extended progressively to the margin of the cell and by 9 to 12 hr cells which had not rounded up showed long, branching, faintly stained filaments (Pl. 1, fig. 4), reminiscent of the projections first described by Hoyle (1954) using dark ground microscopy. Many specifically stained granules lay free on the surface surrounding the cell (Pl. 1, fig. 4). Thus soluble antigen followed the same sequence of synthesis and transportation in the abortive, WSE and MEL, as in the complete, NWS, infection by influenza A virus.

Accessory findings. After the initial emptying of the nucleus secondary specific staining appeared in the following circumstances. The nuclei remained unstained from 7½ until 9 to 12 hr after infection when, in response to some convalescent sera, many cells began to show intranuclear rings, single, occasionally double, or twisted like a figure of eight. The rings became progressively brighter over the next 12 hr (Pl. 1, fig. 3). The centre of the intranuclear rings remained unstained, but specific staining in the cytoplasm of the cell became more intense as though the cell continued to synthesize soluble antigen. This stage may be seen also in Pl. 1, fig. 4.
**Comparison of infected BHK21 cells with infected L cells**

It was necessary to see whether the soluble antigen of influenza A virus was retained in the nucleus of L cells in the same way as fowl plague virus. In our line of L cells no retention of influenza A antigen was found. Specific staining was much less intense and could not be detected until 6 to 7½ hr after infection with MEL or NWS. The nuclear appearances were different from those in BHK21 cells. The structure of the nuclei was emphasized by producing nonspecific fluorescent staining. Using a human serum (see methods) which induced nonspecific staining of nuclei faintly, and of nucleoli markedly, we were able to trace the changes seen in Pl. 1, figs. 5 and 6.

There was no perinucleolar space in uninfected cells, but 4½ hr after infection a perinucleolar halo developed that was quite unstained by antisera to S antigen (Pl. 1, fig. 5). About 2 hr later the first S antigen became stainable as a bright ring round the dark nucleolar halo (Pl. 1, fig. 6). This was succeeded by stippled and also diffuse staining of the remainder of the nucleus; S antigen was present in the cytoplasm at 12 hr, not at 9 hr. Rabbit anti-V sera did not stain intranuclear structures.

**Effect of multiplicity in BKH21 cells**

The ratio of plaque forming units added per cell represented a ratio of physical particles per cell which was at least ten times greater, and it was therefore possible that both abortive infection and the fluorescence results might have been characteristic of a von Magnus effect only (von Magnus, 1951). Moffat et al. (1960), Hillis et al. (1960) and Watson (1961) reported alterations in the site and amount of influenza antigens when multiplicities of infection were altered. Accordingly, virus was diluted to give a ratio of one EID 50 per 10^4° cells. At this proportion, examination of several coverslips showed five positive cells 4 hr after infection, all showing nuclear staining of soluble antigen and three positive cells at 7½ hr, all showing cytoplasmic localization of soluble antigen. Intermediate virus/cell ratios, 1:100 and 1:500 also showed the same sequence as before. The best preparation of virus had a particle/infectivity ratio of about 8:1 (Milliken, personal communication). It was therefore clear that the sequence of staining reactions was not dependent on multiplicity of infection.

**Properties of abortive MEL virus**

The noninfectious MEL-C13 virus was investigated by comparing with MEL virus its content of soluble antigen, properties of adsorption and interfering power, and ability to enter eclipse or to interact genetically with NWS virus.

**Content of soluble antigen**

The extrusion of soluble antigen, revealed by fluorescent staining, suggested that it was likely to be incorporated in the virus particle. MEL-C13 grown overnight on BHK21 cells infected at a multiplicity of about 10, and concentrated to 1920 agglutinating doses per ml. by the method of Drescher, Hennessy & Davenport (1962) was treated with ether (Hoyle, 1952) to release soluble antigen. We found that it gave the same end point, 1/256, in a complement fixation test with human convalescent serum as did soluble antigen made from infectious MEL virus. Similar results were given by
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the same viruses twice adsorbed and eluted from human red cells, but MEL-C13 was very sensitive to ether after this treatment and the yield of antigen was 25 to 50% less.

Penetration and disposal of C13-MEL haemagglutinin

Table 2 shows the recovery of haemagglutinin from 10⁶ BHK21 cells exposed on the monolayer to 128 agglutinating doses of MEL or MEL-C13 virus, then washed and disrupted by ultrasonic treatment at intervals during incubation. Equal adsorption and loss of haemagglutinin occurred with both preparations. Undisrupted cells did not release detectable amounts of haemagglutinin. The intracellular location of adsorbed virus antigen was confirmed by fluorescent tracing after the method of Boand, Kempf & Hanson (1957) for phagocytes and Fraser & Gharpure (1962) for polyoma infected BHK 21 cells. At the time of infection all cells showed specifically stained cytoplasmic

Table 2. The recovery of virus haemagglutinin from undisrupted BHK21 cells at intervals after infection with 128 agglutinating doses of MEL or C13-MEL virus

<table>
<thead>
<tr>
<th>Virus recovered (agglutinating doses)</th>
<th>Hours after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL</td>
<td>½  1  1½  2  2½  24</td>
</tr>
<tr>
<td>C13-MEL</td>
<td>48  6  6  2  2  512*</td>
</tr>
</tbody>
</table>

* New virus haemagglutinin.

Table 3. The interfering effect of 480 agglutinating doses of MEL virus irradiated with UV for varying lengths of time. Challenged with NWS 10⁵ EID 50

<table>
<thead>
<tr>
<th>Time irradiated (in sec.)</th>
<th>Control without interfering agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>+ +</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>45</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>64</td>
</tr>
</tbody>
</table>

Yield of challenge Virus* Expt. 1: + + Expt. 2: + +

* = Challenge not possible because of cytopathic effect of partially inactivated virus. * = Haemagglutinin titre.

Table 4. A comparison of the interfering effect of UV-irradiated MEL virus and C13-MEL virus

<table>
<thead>
<tr>
<th>Interfering agent</th>
<th>Nil</th>
<th>MEL-C13 100 AD</th>
<th>MEL-C13 1,000 AD</th>
<th>MEL-irradiated 100 AD</th>
<th>MEL-irradiated 480 AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1 BHK 21 cells</td>
<td>64*</td>
<td>96</td>
<td>96</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>Expt. 2 BHK 21 cells</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>. .</td>
<td>0</td>
</tr>
<tr>
<td>Expt. 3 Chick embryo</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>64</td>
<td>0</td>
</tr>
</tbody>
</table>

* Yield of challenge virus in agglutinating doses (AD). . . = not done.
granules; 2 hr after infection all cells were unstained except for a few containing single flecks of specific staining, usually near the cell nucleus. Adsorption, penetration and disposal of C13-grown MEL virus would seem to follow a normal course.

Interference. Infectious MEL virus, after inactivation by UV irradiation interferes effectively with the growth of super-infecting virus. Under the conditions stated in methods, the exposure required to produce optimum interference was 30 sec. (Table 3). The interfering effect of dialysed MEL-C13 was compared with that of UV-irradiated, dialysed MEL in monolayers of BHK21 cells and in the allantoic cavity of the chick embryo using challenge doses of 100 EID 50 of NWS or of influenza B (Lee). Because of the presence of residual virus, MEL-C13 was UV irradiated before use in eggs, but not before use in BHK21 cells in which successive cycles of MEL infection do not occur. It will be seen from Table 4 that MEL-C13 is not an interfering agent in conditions where UV-irradiated MEL interferes effectively.

Phenotypic mixing

Since MEL-C13 could penetrate the BHK21 cells and become undetectable tests were made to see whether, during double infection of the cells with active virus, reactivation or rescue might occur sufficient to produce phenotypic mixing as revealed by the release of doubly neutralizable haemagglutinin (Hirst & Gottlieb, 1953; Fraser, 1953).

Table 5 shows that no phenotypic mixing occurred even when the amount of C13-MEL used was 200 times greater than the corresponding effective amount of MEL.

These results, together with lack of interference and non-infectiveness, suggest that the defects in abortive MEL virus lie in functions which follow elimination of ingested haemagglutinin. Presumably, the virus nucleic acid is absent or fails to function for unknown reasons.

### Table 5. Double neutralization tests on the 1st-cycle yield of mixed infection in BHK21 cells with NWS, MEL and MEL-C13 viruses

<table>
<thead>
<tr>
<th>Diluent</th>
<th>NWS only</th>
<th>MEL only</th>
<th>NWS and MEL</th>
<th>NWS and MEL-C13</th>
<th>100 AD</th>
<th>1000 AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>32*</td>
<td>22</td>
<td>24</td>
<td>24</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Anti-NWS serum</td>
<td>0</td>
<td>14</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Anti-MEL serum</td>
<td>24</td>
<td>0</td>
<td>1</td>
<td>24</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Type of yield</td>
<td>NWS</td>
<td>MEL</td>
<td>Double</td>
<td>NWS</td>
<td>NWS</td>
<td>NWS</td>
</tr>
</tbody>
</table>

* Titre in agglutinating doses (AD).

DISCUSSION

The synthesis of soluble antigen of influenza A virus in the nucleus of BHK 21 cells and its migration from the nucleus are evidently unlike the retention of soluble antigen of fowl plague virus in the abortive infection of L cells reported by Franklin & Breitenfeld (1959). Since there was no apparent difference in this respect between NWS infection, which is complete, and MEL which is abortive, retention of soluble
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The antigen of a myxovirus in the nucleus of infected cells is not the sole cause for the production of non-infectious virus. It may be that the accumulation of fowl plague antigen in the nucleus of L cells and of chick embryo cells (Rott & Scholtissek, 1963) is the result of the defect which causes abortive infection and not its prime cause.

We also know that the production of noninfectious material by MEL virus in adult mouse brain can be counteracted by double infection with NWS and MEL virus. Infectious MEL virus then appears in the progeny (Fraser, 1959). The same has been found true of double infection of BHK21 cells (Milliken, personal communication). If the defect of MEL, WSE and other egg-adapted influenza strains in mammalian cells can be restored by NWS and its variants, there should be an opportunity, by analogy with defective mutants of phage (Benzer, 1961) of identifying the defect by a combination of chemical and genetic studies (Epstein et al. 1963; Cooper, 1965).

Migration of soluble antigen from nucleus to cytoplasm and its extrusion from the cell were easy to trace and it has been shown that this staining pattern did not represent a false cross reaction between the V antigen and unrecognized particle antibody in the human convalescent sera.

The perinucleolar ring seen in influenza infected L cells, which was specifically stained by all convalescent influenza sera tested, was not seen in infected BHK21 cells, nor was a perinucleolar zone apparent in them at any time. The formation of perinucleolar haloes has been reported in polyoma-virus-infected nuclei (Bereczky, Dmochowski & Grey, 1961) and Fraser & Crawford (1965) noted that early synthesis of polyoma antigen is perinucleolar. It is interesting that nucleolar activity should be associated with the synthesis of RNA virus (influenza) as well as a DNA virus (polyoma).

The appearance of secondary immunological staining within the BHK21 nucleus 9 hr after infection was not due to typical antibody to S antigen, as judged by the failure of some sera to stain intranuclear rings, nor were the rings stained by specific V antibody. The nature of the ring-like structure is unknown, but Crawford & Gharpure (personal communication) have noted intranuclear bodies in electron micrographs of influenza-infected BHK21 cells which may well represent the unstained area within the brightly stained fluorescent ring of Pl. 1, fig. 3. It is also possible that they are similar to the intranuclear structures seen in influenza B infection by Berkaloff & Colobert (1963).

Complement fixation tests showed soluble antigen present in the abortive MEL haemagglutinin; soluble antigen migrated from the nucleus, through the cytoplasm to the outside of the cell; adsorption and disposal of the abortive virus followed the same course as complete egg-grown virus; but infectivity was lost, interference was absent and phenotypic mixing failed to take place. Genetic interaction was not tested completely because of the difficulty of excluding the formation of recombinants by the residual infectious inoculum in MEL-C13 virus.

We have shown that our findings are independent of multiplicity of infection. Since incomplete virus of the von Magnus type results from multiple infection and retains some degree of infectivity, interfering power (von Magnus, 1954) and ability to interact genetically with infectious virus (Burnet, Lind & Stevens, 1955), there is a clear difference between abortive infection and the formation of incomplete virus. We do not know whether the difference represents absence of RNA from, or the presence of noninfectious RNA in, the abortive haemagglutinin.
The experiments were carried out with the technical co-operation of Miss Joyce Anderson; the prints were produced by Mr Alan McIlroy. I am specially indebted to Dr C. Ross and the courtesy of Professor Norman Grist of the Regional Virus Laboratory, Ruchill Hospital, for numerous convalescent human sera of known specificity and titre.

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MOFFAT, M. A. J., HOLTERTMAN, O. A. & HILLIS, W. D. (1960). The development of soluble (S) and viral (V) antigens of influenza A virus in tissue culture as studied by the fluorescent antibody technique. 2. Studies employing a high multiplicity of infection in beef embryo kidney cells. Acta path. microbiol. scand. 50, 409.


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EXPLANATION OF PLATE

The staining of influenza-infected BHK21 cells and L cells with convalescent human sera and fluorescein conjugate of anti-human globulin at intervals after infection. The sequence of staining reactions in BHK21 cells is identical for MEL, WSE and NWS viruses. Bar = 10 μ.

**BHK21 cells**

Fig. 1. Nuclear staining 3½ hours after infection.
Fig. 2. ‘Empty’ nuclei, ‘positive’ cytoplasms 7½ hr after infection.
Fig. 3. Intranuclear rings 12 hr after infection.
Fig. 4. Filaments and extruded granules 15 hr after infection. The staining of nonfilamentous rounded cells is cytoplasmic. A nuclear ring is seen faintly through the cytoplasm of the central cell.

**L cells**

Fig. 5. Unstained perinucleolar halo 4½ hr after infection. All staining nonspecific.
Fig. 6. Specifically stained ring surrounding perinucleolar halo 6½ hr after infection.