Development and Localization of Virus-specific Antigens During the Multiplication of Herpes Simplex Virus in BHK 21 Cells

By L. J. N. ROSS, D. H. WATSON* AND P. WILDY

Department of Virology, University of Birmingham,
Birmingham 15, England

(Accepted 22 August 1967)

SUMMARY

Baby hamster kidney cells were infected with herpes simplex virus under one-step growth conditions. Virus-specific antigens were traced by 'direct' and 'indirect' immunofluorescence and their times of appearance estimated by agar-gel diffusion using an antiserum made by immunization of rabbits with herpes-virus-infected rabbit cells. Twelve distinct precipitin lines were detected by agar-gel diffusion and a succession of fluorescent patterns was distinguished. Quantitative estimations indicated progressive accumulation of antigen in various patterns within the nucleus, in the cytoplasm and at the cell membrane. These patterns were correlated with the cellular distortions seen with a conventional cytological technique.

INTRODUCTION

The formation of herpes-virus-specific antigens in cultured cells during virus multiplication has been studied in situ using immunofluorescence (Lebrun, 1956; Ross & Orlans, 1958; Roizman, 1961; Roizman, Spring & Roane, 1967; Shipkey et al. 1967). The patterns of immunofluorescence reported were all different, probably because of major differences in source of antibody, cell line, virus strain and staining technique. Recently, Roane & Roizman (1966) and Roizman et al. (1967) showed that it is possible to differentiate between virus-specific antigens present in the nucleus of infected cells and those in the cytoplasm by using antiserum produced in rabbits against herpes-virus-infected cells and human convalescent serum respectively.

A proper evaluation of the sequence of changes in cells as a result of infection can only be achieved under conditions of one-step growth and it is desirable to relate them to virus multiplication and to as many manifestations of infection as possible. In this paper we describe quantitative immunofluorescence studies made in BHK 21 cells infected with herpes simplex virus so as to give one-step growth. We have used antisemum prepared by immunization of rabbits with herpes-infected rabbit kidney cells and we have attempted to relate our observations to other events accompanying virus multiplication.

* M.R.C. Virus Research Group, University of Birmingham.


**METHODS**

*Tissue culture technique*

*Cells.* BHK 21 c13 cells (BHK) (Macpherson & Stoker, 1962) were grown in a modified Eagle’s medium (Vantsis & Wildy, 1962) containing 10% tryptose phosphate broth and 10% calf serum (ETC).

*Cell culture.* BHK cells were cultivated on a large scale as described by Watson *et al.* (1966) and Shedden & Wildy (1966). For cytological studies monolayers of cells were grown in miniature Petri dishes made by sealing brass curtain rings (15 mm. diam.) to coverslips with a mixture of 75% petroleum jelly and 25% paraffin wax. These rings had no cytotoxic effect when well covered with hydrocarbon mixture. Fifty thousand BHK cells in 0.5 ml. ETC were inoculated in each coverslip and after incubation at 37° in a humidified atmosphere of about 5% CO₂ and air for about 12 hr, monolayers consisting of randomly distributed well-spread cells were obtained.

*Virological techniques*

*Virus.* The HFEM strain of herpes simplex virus was used. It was produced in large amounts as described by Watson *et al.* (1966) in BHK cells and was harvested by disrupting the cells in distilled water in an ‘Electrosonic bath’ (Surgical Equipment Supplies Ltd., London, W. 3).

*Virus assay.* Infectivity titrations were performed by the plaque method described by Russell (1962).

*Particle counts* were made by the loop-drop method using phosphotungstate to provide negative contrast (Watson, Russell & Wildy, 1963).

*Serological techniques*

*Preparation of antisera.* Antisera to herpes-infected RK 13 cells were made by inoculation of young adult rabbits with eight 200 mg. doses of freeze-dried infected cell extract as described by Watson *et al.* (1966). Rabbits were bled 15 days after the last injection.

Antiserum to rabbit γ-globulin was supplied by Dr A. S. Kelus. Pooled rabbit serum was fractionated on DEAE-cellulose and γ-globulin (IgG) was isolated. Sheep received three intramuscular injections of 10 to 30 mg. of γ-globulin in incomplete Freund’s adjuvant at monthly intervals. The sheep were bled 1 month after the last injection.

*Conjugation of antisera.* Antisera against herpes-infected RK 13 cells and against rabbit γ-globulin were conjugated to fluorescein isothiocyanate (Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.) as described by Marshall *et al.* (1958).

*Removal of non-specific staining components.* One ml. samples of conjugate were absorbed twice with an equal packed volume of guinea-pig liver powder (Coons & Kaplan, 1950) and 10⁸ BHK cells (moistened with PBS) for 10 min. at 4° with gentle stirring. After centrifugation at 4000 rev./min. in an MSE centrifuge for 10 min., the conjugate was carefully decanted and generally used without delay.

*Agar-gel immunodiffusion tests* were done in 1% Ionagar (Oxo Ltd, London) in 0·2 M-phosphate buffer pH 7 containing 0·85% sodium chloride, 0·1% sodium azide.
and 1% glycerol. Diffusion was permitted for 72 hr at room temperature. When precipitin lines were to be counted several dilutions of antigen samples were separately examined against dilutions of several antisera.

Cytological techniques

Fixation. For direct and indirect staining with conjugated antisera, cell monolayers were washed free of growth medium with buffered saline and fixed in acetone at $-20^\circ$ for 30 min. The cells were then washed in three changes of buffered saline.

For staining with gallocyanin + chrome alum, cells were fixed in methanol at room temperature for 10 min.

Staining for direct immunofluorescence. The fixed monolayers were drained and one drop of antiherpes conjugate was added. The coverslip was then kept in a moist chamber at $37^\circ$ for 30 min. Unreacted conjugate was then washed away in buffered saline for 30 min. with changes at 10 min. intervals.

Staining for indirect immunofluorescence. Monolayers were first treated for 30 min. at $37^\circ$ with one drop of unconjugated antiherpes serum diluted 1/2 in buffered saline. Excess serum was then washed away in buffered saline for 30 min. The monolayers were then treated at $37^\circ$ for 30 min. with one drop of anti-rabbit-globulin conjugate diluted 1/10 in buffered saline and then washed. Coverslips were mounted permanently in glycerol mountant (90% glycerol + 10% buffered saline).

Staining with gallocyanin + chrome alum. Cells fixed in methanol for 10 min. at room temperature were washed in distilled water and stained for 48 hr in Einarson's (1951) stain. They were washed in distilled water, dehydrated in acetone and mounted in D.P.X.

Cytological analysis. The preparations were examined with a Zeiss photomicroscope. For fluorescence microscopy, exciter filters BG 3/4, BG 12/4 and barrier filters 53, 50 and 44 were used and the condenser adjusted to bright field illumination. The total number of cells in each field was estimated after changing to phase contrast. Detailed analysis of the localization of virus specific antigens was made at a magnification of $\times 1000$. Each field contained from 8 to 16 cells and 30 fields were counted on each coverslip. Preliminary experiments showed that the percentage of fluorescent cells in each field did not vary significantly. (The coefficient of variation of the mean was 10 to 12%.) Estimations were done on duplicate coverslips in all the experiments.

RESULTS

Evaluation of techniques: necessary compromises

Ideally we should wish to find a technique allowing sensitive demonstration of antigen, fine discrimination of its situation and at the same time permitting fully quantitative experiments. Unfortunately the techniques we used are imperfect and the need for compromise was shown by preliminary experiments.

Direct versus indirect fluorescent staining. Using monolayers incubated for various times after infection with herpes virus it was found that indirect staining produced a much more intense fluorescence than direct staining; it was particularly useful for revealing nuclear antigens. However, fine detail was frequently masked owing to intense fluorescence filling the whole cell. Direct staining on the other hand was less efficient in revealing early nuclear antigens, but was useful in demonstrating cyto-
plasmic granular antigens. Both methods of staining appeared specific as shown by the following tests:

(a) Direct staining was inhibited by pre-treatment with unconjugated anti-herpes serum but not with pre-immune serum.

(b) Uninfected cells did not stain by either the direct or indirect methods. Sometimes there was a faint fluorescence in the cytoplasm and nucleoli of uninfected cells. This was readily distinguishable from the fluorescence present in virus-infected preparations.

(c) Anti-rabbit γ-globulin conjugate failed to stain infected cells pretreated with pre-immune serum or anti-rabbit γ-globulin.

(d) No auto fluorescence was noted.

Infection in suspension versus infection of monolayers. Much work has now been done on the growth of herpes virus after infecting cells in suspension. Indeed the method is ideally suited to large-scale quantitative experiments so that the development of new virus can be followed by electron microscopy and antigens can be assessed by gel diffusion. Unfortunately, the method is not well suited for cytology because the infected cells do not spread out well on the glass. On the contrary, when cells are infected in monolayers they remain well spread out after infection but it is quite impossible to do particle counts or gel diffusion tests because the scale of operation is restricted.

For the above reasons both ‘suspension’ experiments and ‘monolayer’ experiments were done.

Experiment with cells infected in suspension

Five thousand million cells were suspended in ETC containing virus at an input multiplicity of 20 p.f.u./cell and stirred mechanically for 1 hr at 37°. The cells were then washed once in ETC and samples (1.8 x 10^8 cells) were dispensed in 20 oz. flat bottles containing 40 ml. ETC. All operations were done at 37°. Some of the cells were assayed for infectious centres under ETC containing 10% pooled human serum and 10^6 cells were dispensed per miniature Petri dish. At intervals the cells in the 20 oz. bottles were harvested, disrupted by standard ultrasonic treatment and the extracts were tested for infectious virus, virus particles and diffusible antigens. At the same time coverslip cultures were fixed and stained by the direct method (Table I). Lines which were obvious with the 5th and 6th hr samples were not seen with later extracts. However, this effect was not due to disappearance of these antigens later in the growth cycle but rather to excessive concentration; using dilutions of the later extracts, the missing lines were demonstrated.

Experiment with cells infected as monolayers

In parallel with the above experiment 5 x 10^4 cells from the same batch were inoculated into miniature Petri dishes and incubated overnight at 37°. Growth medium was removed and cells were overlaid with 0.1 ml. of virus inoculum at an added multiplicity of 100 p.f.u./cell. The cultures were incubated at 37° for 2 hr after which the inoculum was removed and the cells washed in ETC at 37° and incubated with 0.5 ml. ETC containing 10% pooled human serum. At intervals thereafter six coverslips were removed at each time; they were stained by the direct and indirect methods and also with gallocyanin. Uninfected cells did not fluoresce when stained either by the direct or indirect method. They looked normal in gallocyanin preparations. Infected
Development of herpes-virus antigens

cells showed signs of infection as early as 2 hr (Table 2). The sequence of changes observed is described below.

**Two hr.** The direct stain showed herpes-virus antigen present as weakly fluorescent fine dots at the cell membrane and in the cytoplasm. Indirect staining revealed diffuse weakly fluorescent material throughout the cytoplasm. No abnormality was observed in gallocyanin preparations.

Table 1. Development of herpes antigens during virus growth

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>% fluorescent cells</th>
<th>No precipitin lines</th>
<th>Virus particles/cell</th>
<th>Infectivity (p.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1½</td>
<td>0</td>
<td>0</td>
<td>160</td>
<td>0.92</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>150</td>
<td>0.47</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>3</td>
<td>560</td>
<td>Not done</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>5</td>
<td>1000</td>
<td>0.28</td>
</tr>
<tr>
<td>5</td>
<td>52</td>
<td>6</td>
<td>1400</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td>58</td>
<td>8</td>
<td>1350</td>
<td>0.34</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>10</td>
<td>5400</td>
<td>1.6</td>
</tr>
<tr>
<td>11</td>
<td>69</td>
<td>12</td>
<td>6420</td>
<td>7</td>
</tr>
<tr>
<td>14</td>
<td>70</td>
<td>12</td>
<td>8400</td>
<td>8.2</td>
</tr>
<tr>
<td>24</td>
<td>64</td>
<td>12</td>
<td>11700</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Table 2. Development and location of herpes antigens during virus growth

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>% nuclei phase I*</th>
<th>% nuclei phase II</th>
<th>% nuclei phase III (% +ve)</th>
<th>Cytoplasm</th>
<th>% cells abnormal</th>
<th>Predominant appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1†</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No abnormality</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>Nucleolar</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>5</td>
<td>0</td>
<td>86</td>
<td>84</td>
<td>fragmentation</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>15</td>
<td>0</td>
<td>87</td>
<td>92</td>
<td>Margination</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>46</td>
<td>10</td>
<td>83</td>
<td>97</td>
<td>Chromatin</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>50</td>
<td>38</td>
<td>98</td>
<td>98</td>
<td>Hyaline nuclei</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>33</td>
<td>50</td>
<td>98</td>
<td>100</td>
<td>Normal</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>20</td>
<td>71</td>
<td>95</td>
<td>100</td>
<td>Normal</td>
</tr>
</tbody>
</table>

* For explanation of phases I, II and III see text and plate.
† Time since adding inoculum; adsorption period 2 hr.

**Three hr.** Indirect staining showed bright fluorescent patches in the nucleus of the majority of the cells referred to as phase I (Pl. 1, fig. 1 a). The nuclear membrane was fluorescent along the whole periphery and the cytoplasm was filled with diffuse fluorescent material. Some areas of brighter fluorescence were sometimes seen near the nuclear membrane. In most cells direct staining showed faint fluorescent patches in the nuclei and minute powdery granules at or just outside the nuclear membrane which was fluorescent in part or along its entire periphery. Nucleoli of most cells were fragmented as shown by gallocyanin staining.

**Four to 6 hr.** Indirect staining showed a great increase in area and in intensity of the fluorescent nuclear patches. In most of the cells, referred to as phase II (Pl. 1, fig. 1 b), the nuclei were filled with an intense fluorescence except for the marginated chromatin
and some dark areas which looked rarified on phase contrast. The cytoplasm was brighter than at 3 hr and it was possible to see in some cells areas of strongly fluorescent material localized just outside the nuclear membrane. Direct staining also revealed a relative gain in area and in intensity of the nuclear fluorescence in most cells. In the cytoplasm, brightly fluorescent granules, larger in size than earlier, were observed (Pl. 1, fig. 2); some being in close association with the nuclear membrane and others situated away from it. Some of these granules appeared to be hollow spheres. A thin band of fluorescent material was observed in parts of the cell periphery. In gallocyanin preparations chromatin was marginated and granules appeared in the nucleus of nearly all cells.

Nine hours. Indirect staining showed very bright fluorescence throughout most of the cells, referred to as phase III (Pl. 1, fig. 3). It was sometimes possible to detect one or two granules embedded in a fainter fluorescent matrix of phase II. Many cells were round and the cytoplasm appeared constricted. It was completely filled with diffuse intense fluorescence and there was a distinct layer of fluorescent material at the cell membrane. Direct staining (Pl. 1, fig. 4) revealed in about 50% of the cells nuclear fluorescent granules which appeared to be similar in shape to the cytoplasmic granules referred to already (Pl. 1, fig. 2). The plane of focus of these granules was within the nucleus. At this stage, the cytoplasmic granules were rather indistinct and the cytoplasm appeared to be filled with intense fluorescent material in most of the cells. It was hard to decide whether this change in the cytoplasm was due to rounding of the cells or whether there was actual fusion of the granules. Most of the cells were round and the nucleoplasm looked hyaline in gallocyanin preparations. From 9 hr onwards there was an increase in the proportion of cells in phase III but rounding of the cells seriously interfered with cytological examination.

DISCUSSION

Our results showed a progressive accumulation of virus specific antigens during growth. This was evident in the suspension experiment in which, as time went by, increasing numbers of lines were formed in gel-diffusion tests and the intensity of fluorescence steadily increased. With these crude techniques we were unable to tell whether early virus proteins were superseded later by other proteins like they are during the replicative cycle of bacteriophage T₂ (Cohen, 1965). But in the monolayer experiment we were able to distinguish characteristic patterns of immunofluorescence in infected cells and to show that these succeeded each other in a particular order. Moreover, these patterns were related to the morphological changes in the cells judged by phase-contrast microscopy and stained preparations.

We should, of course, wish to relate our findings with previous work in which we studied the formation of various virus specific enzymes (Keir et al. 1966; Klemperer et al. 1967), the formation of virus components (Russell et al. 1964) and the progressive morphological changes in infected cells (Love & Wildy, 1963; Wildy et al. 1961; Watson, Wildy & Russell, 1964), but we are not yet able to do this usefully partly because our herpes antiserum contains multiple antibodies. We are now trying to prepare monospecific sera and when that has been achieved many useful comparisons will be possible. For the present we can only reflect that our intranuclear and cytoplasmic fluorescent patches and granules may well correspond with the various
Development of herpes-virus antigens

bodies seen by Love & Wildy (1963) and Watson et al. (1964) because they appear in similar situations at similar times.

So far, immunofluorescence studies on herpes-infected cells have been difficult to reconcile with each other. The most likely reason for this is that antisera of very different quality have been used. Our results most resemble those of Roizman's group (Roane & Roizman, 1966; Roizman et al. 1967). This group showed a serological difference between nuclear granules and the nuclear 'amorphous mass' as well as between cytoplasmic granules and 'diffuse' antigens. They also showed that the amorphous nuclear and diffuse cytoplasmic fluorescence appear early while the granules appear later and require arginine. Early intranuclear fluorescence was also noted by Géder et al. (1967), who also showed that it was not suppressed by cytosine arabinoside, suggesting that it was concerned with an early function not requiring DNA synthesis. We believe that the fluorescence we observed on the cell membrane was due to the antigens demonstrated by Roane & Roizman (1964) and Watkins (1965). We cannot yet say whether the sites of fluorescence represent sites of antigen synthesis or simply sites of accumulation. There is evidence of protein synthesis in the cytoplasm of herpes-infected cells (Sydiskis & Roizman, 1966). More information on this point may be available when monospecific antisera have been produced.

The authors would like to thank Dr W. I. H. Shedden for his advice in the early stages of the work. Valuable technical assistance was provided by Christine Bridgewater and Heather Martin. The work was partly supported by a grant from Glaxo Laboratories Ltd.

REFERENCES


I22 L. J. N. ROSS, D. H. WATSON AND P. WILDY


*(Received 26 July 1967)*

**EXPLANATION OF PLATE**

Fig. 1. Indirect stain 3 hr after infection. (a) Cell in phase I showing fluorescent nuclear patches. Note fluorescence at nuclear membrane and diffuse cytoplasmic fluorescence. (b) Cell in phase II showing fluorescence of practically the whole nucleus. Also note areas of bright cytoplasmic fluorescence.

Fig. 2. Direct stain 6 hr after infection showing fluorescent cytoplasmic granules. Note faint fluorescence at cell membrane.

Fig. 3. Indirect stain 9 hr after infection showing round intensely fluorescent cells (phase III). Note fluorescence at the cell membrane.

Fig. 4. Direct stain 9 hr after infection showing fluorescent nuclear granules.