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REPRODUCTION OF HERPES SIMPLEX VIRUS IN HeLa CELLS STUDIED BY IMMUNOFLUORESCENCE AND CYTOCHEMICAL METHODS

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Summary. — Using the direst fluorescent antibody (FA), method, we observed in HeLa cells infected with herpes simplex (HS) virus several fluorescent bodies of different localization (large and small fluorescent granules in the nucleus, and granular and diffuse fluorescence in the cytoplasm or on the nuclear membrane). We established a certain quantitative relationship between the amount of infective virus in cells and the amount of specific antigen in the acute infection. The specific viral antigen could be demonstrated by the direct FA method if the titre of infective virus in cells reached at least $10^{2.5}$—$10^{3.0}$ $TCID_{50}$/0.1 ml.

Cytochemical studies of HS virus-infected cells revealed markedly increased activities of acid phosphatase (ACP) and non-specific esterase (NE) as compared to uninfected cells. The activities of alkaline phosphatase (ALP), adenosine triphosphatase (ATPase) and adenosine monophosphatase (AMPase) were generally low and their increase could be detected only in later stages of infection when the cells already displayed marked morphological changes. A high activity of succinic dehydrogenase (SD) was found mainly in giant polynucleate cells in later stages of infection, what illustrates a certain metabolic activity in these degenerating cells.

Introduction

Studies on the reproduction of HS virus in various tissue cultures and detection of specific viral antigens by the FA method contributed a number of new findings concerning the antigenic structure of HS virus and the different localization of the antigens in the cell in the course of infection (Roane and Roizman, 1966; Roizman *et al.*, 1967; Shipkey *et al.*, 1957; Céder and Váczi, 1968; Ross *et al.*, 1968).

In the present work we studied the reproduction of HS virus in HeLa cells at various multiplicities of infection and the localization of specific viral antigens in cells by the FA method. We also followed the relationship between the amounts of infective intracellular virus and the specific viral antigen as detected by conjugates prepared from two different imune sera. A further subject of this study was the cytochemical assay of activities of some cellular enzymes, considered as the expression of a certain metabolic response of cells to HS virus infection.

Materials and Methods

Virus. A virus strain, designated HSZP, isolated at our Institute from herpetic vesicles on lips of a patient, was used. The strain uderwent 59 passages in chorioallantoic membranes of embryonated eggs, 132 passages in cell cultures from rabbit lungs, line ZP 1/58, and 130 passages in HeLa cell cultures. The infective titre of virus amounted to $10^{6.0}$ $TCID_{50}$ per 0.1 ml.

Tissue cultures. The line of HeLa cells used has been cultivated in our Institute since 1955 in a medium containing 40% of human serum in Hanks' solution, 0.1% of yeast extract and antibiotics. The present experiments were performed with HeLa cells passaged in medium 199 with 10% of heated horse serum. For the immunofluorescent and cytochemical studies, we used coverslip cultures seeded with 100 000 cells per 1 ml. Two days old cultures were infected with 0.1 ml of HS virus per 1 ml of medium.

Virus titration was carried out in tube cultures of HeLa cells according to the cytopathic effect (Szántó, 1963). For the titration of intracellular and extracellular virus, we used cells and medium, respectively, remaining after withdrawal of coverslip cultures from the tubes.

FA method. Two conjugates were employed for detection of specific herpetic antigen. One was prepared from a hyperimmune rabbit serum with a virus neutralization titre of 1 : 256, and the other from a human immune serum with a titre of 1 : 128. Both conjugates were prepared as described before (Leššo *et al.*, 1963). Coverslips with grown cell cultures withdrawn at various time intervals post infection (p.i.) were fixed with acetone for 10 minutes and stained with the respective conjugate for 30 minutes. Uninfected HeLa cell cultures and preparations stained with a heterologous conjugate (against Newcastle disease virus) served as controls.

Cytochemical methods. The activities of ALP, ACP, NE, membrane ATPase, AMPase and SD were assayed by the methods described previously (Leššo and Mayer, 1968).

Microscopy and photomicrography. In fluorescence microscopy, the "Grosse Luminiszenzeinrichtung" with the HBO 200 mercury lamp and in light microscopy a "Lumipan" (both C. Zeiss, Jena) were used. Fluorescent micrographs were taken on a 24 × 36 mm RF 3 film (U.S.S.R.) with exposures of 20—60 seconds. Other photographs were taken on 9 × 12 cm ORWO NO 20 plates.

Results

Detection of viral antigen by the FA method

Viral antigen was followed in HeLa cells infected at either high (10 $TCID_{50}$/cell) or low (0.1 $TCID_{50}$/cell) input multiplicity at intervals of 5, 8, 12, 16, 24, 32 and 48 hours p.i.

At high multiplicity of infection, the specific viral antigen was observed 5 hours p.i. in cell nuclei as grains and larger granules (Figs 1 and 2). The specific antigen in this phase of infection was localized not only in the cell nucleus, but also on the nuclear membrane and in the paranuclear area of the cytoplasm as a granular and diffuse fluorescence. A similar picture was observed at 8 hours p.i. (Figs 3 and 4). At 12—16 hours p.i. most of the viral antigen was localized in the vicinity of the nuclear membrane and the paranuclear area of cytoplasm as a diffuse fluorescence (Fig. 5). The fluorescense of the antigen showing this localization was very intensive. At this stage of the infection there proceeded a marked accumulation of the antigen, so that approx. 70% of the cells contained viral antigen. Also at this stage, a great number of polynucleate cells, so-called polycaryocytes the cytoplasm of which contained a large amount of antigen showing diffuse fluorescence, could be seen. At 24 hours p.i. the cells showed marked morphological changes (Fig. 6).

At the low multiplicity of infection the specific antigen was first demonstrated at 16 hours p.i. The localization and development of the antigen in the

course of infection was similar to that found at the high multiplicity of infection. A substantial increase in the amount of the antigen occurred from 24—32 hours p.i.

These observations were made using the conjugate prepared from hyperimmune rabbit serum. By use of the conjugate prepared from immune human serum we detected the specific antigen only in the cytoplasm of infected cells.

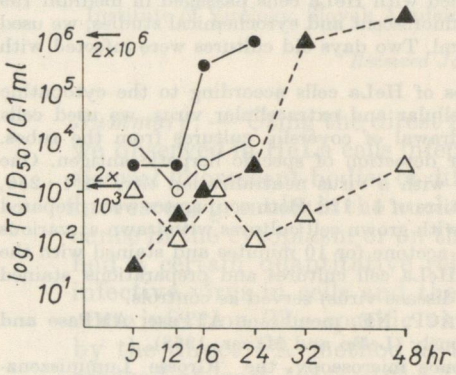

Fig. 7.
Multiplication of HS virus in HeLa cell cultures
● and ▲ — titres of intracellular virus at high and low multiplicity of infection
○ and △ — titres of extracellular virus at high and low multiplicity of infection
The arrows indicate size of inoculum in $TCID_{50}$.

Detection of virus by the titration method

In parallel with the FA assay we followed the infective HS virus in HeLa cells by the titration method. The results of titration of both intracellular (ICV) and extracellular (ECV) virus after infection with both high and low multiplicity are illustrated in Fig. 7. With the high multiplicity of infection, the titre of ICV at 5 hours p.i. was $10^{3.0}$ $TCID_{50}$/0.1 ml and it markedly increased in the period from 12—16 hours p.i. With the low multiplicity of infection, the titre of ICV at 16 hours p.i. was $10^{3.0}$ $TCID_{50}$/0.1 ml and its substantial increase occurred from 24—32 hours p.i. Values of ECV titres were generally lower in both cases. A more detailed comparison of the assay of infective virus and specific antigen (direct FA method) is presented in Table 1. It can be concluded that the specific viral antigen cannot be demonstrated in HS virus-infected HeLa cells unless the titre of ICV is equal or higher than $10^{2.5}$—$10^{3.0}$ $TCID_{50}$/0.1 ml.

Table 1. A comparison of the detection of HS virus in HeLa cells by the FA and titration methods

Input multiplicity of infection ($TCID_{50}$ per cell)	Hours after inoculation							
	8		16		24		48	
	FA	Titre*	FA	Titre	FA	Titre	FA	Titre
10	+	3.50	+	4.5	+	5.25	+	6
1	+	3.25	+	3.5	+	4.25	+	5.25
0.1	0	2.25	+	2.75	+	4.0	+	5.25
0.01	0	1.25	0	1.75	+	3.25	+	4.5
0.001	Not done		0	1.0	0	1.0	±	2.5
0.0001	Not done		0	1.0	0	1.0	0	1.75

* log $TCID_{50}$/0.1 ml values.
+ and 0: presence and absence of viral antigen, respectively; ±: weak fluorescence in a few cells.

Enzymic activities in HS virus-infected cells

Activities of the enzymes studied were assayed in experiments at both high and low multiplicity of infection. Since there were no differences in enzyme activities due to different multiplicities of infection, we present only the results obtained with the high multiplicity (Table 2). It can be seen that

Table 2. Cytochemical assay of enzymic activities in HS virus-infected HeLa cells

Hours p.i.	ALP		ACP		NE		ATPase		AMPase		SD	
	I	C	I	C	I	C	I	C	I	C	I	C
5	+	+	++	+	+	+	+	+	+	+	+	+
8	+	+	++	+	++	+	+	+	+	+	+	+
12	+	+	++	+	++	+	+	+	+	+	++	+
16	++	+	++++	+	+++	+	++	+	++	+	++	+
24	++	+	+++	+	+++	+	++	+	++	+	+++	+

The titre of inoculum was 10^6 $TCID_{50}$/0.1 ml. I and C: infected and uninfected (control) cells, respectively.

\+ Weak reaction present in 5—10% of cells.
++ Medium intensity reaction present in 20—40% of cells.
+++ Strong reaction present in 40—60% of cells.
++++ Very strong reaction present in the majority of cells.

an increase in the activity of ACP and NE took place soon after infection of the cells with HS virus and that the activity of these lysosomal enzymes was generally high as compared to the uninfected cells. The activity of ACP was located near the nuclear membrane in the paranuclear area of the cytoplasm (Fig. 8). The reaction for ACP became strongly diffuse and spread throughout the whole cytoplasm in the later stage of infection (16—24 hours p.i.) (Fig. 9). At this interval, some polynucleate cells showed even a decrease of ACP activity which can be ascribed to a release of the enzyme from damaged cells into the medium.

The activities of ALP, ATPase and AMPase in infected cells did not differ from those in uninfected controls in the early stage of infection. An increase in their activities appeared only in the last stage of infection (16—24 hours p.i.) and it was confined to round, degenerating cells (Fig. 10).

The activity of SD in infected cells at 12—24 hours p.i. was higher than in control cells. A high enzymic activity was observed mainly in giant polycaryocytes as a strong diffuse reaction (Figs 11 and 12). The reaction for SD was localized in granules and rods in the cytoplasm close to the nuclear membrane; it is known that the activity of SD is bound to mitochondria.

Discussion

Reports on the antigenic structure of HS virus have often been contradictory, apparently due to the use of diverse experimental techniques, cell substrates and both the quality and origin of the antisera employed. While

Lebrun (1956) and Ross and Orlans (1958), using the indirect FA technique and human immune serum, detected the specific viral antigen in the nuclei of infected HEp-2 and HeLa cells, respectively, Roizman *et al.* (1967), as well as ourselves, did not confirm these observations by the use of a conjugate prepared from human gamma-globulin. Roizman *et al.* (1967) assume that the antibodies present in human gamma-globulin fraction possess a reduced immunogenic reactivity, what, however, does nor necessarily represent their general property.

The appearance, function and localization of various fluorescent bodies were studied by Roizman *et al.* (1967) in HEp-2 cells, by Géder and Váczi (1968) and Géder *et al.* (1968) in BSC_1 and HEp-2 cells, and by Ross *et al.* (1968) in BHK 21 cells. These authors, in addition to data similar to ours, reported a diffuse fluorescence in cell nuclei shortly after the infection. On the other hand, Ross *et al.* (1968), similarly to us, were unable to detect this type of fluorescence by the direct FA technique. It seems likely that the latter method is not sensitive enough for demonstration of this type of fluorescence.

Roizman *et al.* (1967) moreover postulated that antigens responsible for the diffuse nuclear and cytoplasmic fluorescence are synthesized shortly after the infection and require arginine-treated cells for their formation. Antigens corresponding to nuclear and cytoplasmic granules appear a bit later and do not require arginine. This early nuclear and perinuclear fluorescence was also observed by Géder *et al.* (1968) in HEp-2 cells and it was found resistant to the inhibitory effect of cytosine arabinoside; the latter fact speaks for the lack of requirement of DNA synthesis.

All these data support the view that several antigenic components are formed in HS virus-infected cells. The question as to whether the site of accumulation of fluorescent bodies coincides with that of synthesis of the specific antigen remains unanswered, however. Recent morphological studies on HS virus (Morgan *et al.*, 1968; Nii *et al.*, 1968; Darlington and Moss, 1968 and the results of Hochberg and Becker (1968) showed that the so-called "naked" viral particles occurring in cell nuclei are also infective; this fact would support the hypothesis that the antigens as a specific product of HS virus can be synthesized both in the nucleus and the cytoplasm of cells. In the present work we found that there exists a certain quantitative relationship between the amount of infective virus and the amount of specific antigen as detected by the direct FA method in cells undergoing acute herpetic infection. A raise of infective ICV led to a parallel increase of the amount of the specific antigen which could be demonstrated only under the condition that a certain minimal amount of infective virus was present in the cell.

In following enzymic activities in HS virus-infected cells we found a high activity of two lysosomal enzymes, namely ACP and NE. We assume that this was due to the herpetic infection, since the enzymic activities steadily increased with virus multiplication in the cells for the whole period of infection. A similarly high activity of ACP was reported by Chardonnet *et al.* (1962) in adenovirus 5-infected human kidney cell cultures. There might be just plain speculations abouth the possible role of these lysosomal enzymes in virus reproduction; nevertheless, their importance in the process of infection

appears probable. They may participate at the stripping off of virus and the release of viral DNA in the cytoplasm, or they may play a role in the onset of viral cytopathic effect on cells (Allison and Malucci, 1965).

The activities of ALP, membrane ATPase and AMPase were low and they increased only in the later stages of infection. We assume that the increased activities are caused by autolytic processes in cells morphologically injured by virus infection. Epstein and Holt (1963) estimated electron optically a high ATPase activity on cell membrane and outer viral envelope, what was observed in our experiments only in giant polynucleate rounded cells undergoing degeneration.

The activity od SD was enhanced in infected cells 12—24 hours p.i. as compared to uninfected cells. It is rather difficult to find a plausible explanation of the finding that a high activity of SD, an oxidative enzyme, was present in giant polycaryocytes which are considered as an expression of certain degeneration of cells. The increased activity of SD in cells infected with HS and adenoviruses was reported also by other authors (Gilbert, 1963; Nosik, 1964).

The present results about the enzymic activities of HS virus-infected cells offered evidence of profound changes in the metabolism of infected cells. The significance of these metabolic changes remains, however, so far unknown.

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Explanation of Photomicrographs:

HeLa cells infected with HS virus.

Fig. 1. Strong specific fluorescence in the form of large granules and small grains in the cell nucleus at 5 hours p.i. ×400.
Fig. 2. Specific granular fluorescence in the cell nucleus and a diffuse fluorescence on the nuclear membrane, 5 hours p.i. ×900.
Fig. 3. Diffuse and granular fluorescence of specific antigen on the nuclear membrane and in the cytoplasm, 8—12 hours p.i. ×400.
Fig. 4. Diffuse fluorescence of specific antigen near the nuclear membrane, 8—12 hours p.i. ×900.
Fig. 5. Strong specific fluorescence of antigen near the nuclear membrane and in the cytoplasm, 12—16 hours p.i. ×400.
Fig. 6. Presence of giant polycaryocytes showing a strong diffuse fluorescence in the cytoplasm, 24 hours p.i. ×250.
Fig. 8. Reaction for ACP, 8—12 hours p.i. Gomory's method. ×300.
Fig. 9. Reaction for ACP, 16—24 hours p.i. The azocopulation method. ×300.
Fig. 10. Reaction for ALP, 16—24 hours p.i. The azocopulation method. ×400.
Fig. 11. Reaction for SD, 16 hours p.i. ×300.
Fig. 12. Reaction for SD, 24 hours p.i. ×300.