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THE INFLUENCE OF ANTIVIRAL SERUM ON CELLS INFECTED WITH HERPES SIMPLEX VIRUS TYPE 1

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Summary. — Homologous immune serum (IS) had an inhibitory effect on the accumulation of specific viral antigens in VERO cells infected with herpes simplex virus type 1. When infected cells were incubated in the presence of IS, lower amounts of viral, both intracellular and membrane, antigens were found by quantitative immunofluorescence and immune cytolysis as compared to same infected cells incubated in the absence of IS. The possible mechanisms of this effect are discussed.

Key words: herpes simplex virus; antibodies; quantitative immunofluorescence; immune cytolysis

Introduction

The molecular nature of the persistence of herpesviruses is explained by two hypotheses. According to the hypothesis of static latency, the viral DNA persists in neurons of the trigeminal, spinal and other sensory ganglia in a nonproductive form, which means that expression of the viral genetic information is repressed (Roizman, 1965). The hypothesis of dynamic latency implies continuous formation of viral progeny in a very limited number of ganglion cells. These two notions are not contradictory, as the silent genome may occasionally become expressed in single ganglion cells of a proportion of experimental animals with established latency (Rajčáni and Szántó, 1979). Baringer nad Swoveland (1974) indeed found persistent infection in 3 of 9 rabbits examined in the course of latency. Virions were seen in a total of 5 out of a great number of neurons in 2504 serial sections of the trigeminal ganglia.

Several authors admitted that homologous antibodies play a certain role in establishment and maintenance of latency. Stevens and Cook (1974) found that antibodies prevent reactivation of the latent virus in ganglion samples which were removed from donor mice with established latency and implanted into the peritoneal cavities of passively immunized recipients. Lehner (1975) suggested that antibodies react with the virus-coded antigens as well as with the virus-induced Fc receptors on the surface of infected cells

and thus may suppress the expression of viral genetic information and/or depress the synthesis of viral antigens. The effector molecule, anticipated to act inside such cells, is an hypothetical second messenger. Rajčáni *et al.* (1977) found a more than twofold decrease of the activation rate of latent HSV in ganglion fragments cultured in the presence of immune serum (IS) as compared to the same fragments cultured in the absence of IS. In addition, Bal *et al.* (1979) showed that pretreatment of guinea pig neural and ganglion tissues with homologous antiviral IgG 20 hr before and 48 hr after infection delayed the onset of virus replication in these cultures by 4-7 days respectively. According to Bal *et al.* (1979) it was difficult to distinguish whether the number of cells which became infected was reduced, or whether the expression of viral genetic information was halted by induction of an "antiviral state".

Golais and Sabó (1979) described an inhibitory effect of antiviral IgG on the activation of pseudorabies virus in latently infected VERO cells. They suggested that certain surface receptors, which bind the IgG, may participate in the control of viral gene expression. Nevertheless, the mechanism of action of antiviral IgG on infected cells remains obscure and needs further investigation. In the present experiments we aimed to follow the effect of homologous antibodies on highly susceptible VERO cells infected with herpes simplex virus type 1 (HSV 1).

Materials and Methods

Cell cultures. VERO cells were grown in Basal Eagle's medium (BEM) containing 5% inactivated calf serum (ICS). They were grown for 48 hr on coverslips in test tubes seeded with 1.5 ml of cell suspension (2×10^5 cell per ml).

Virus. The titre of the Kupka strain of HSV 1 was 2×10^8 plaque forming units (PFU) per ml. The virus was adsorbed for 30 min at multiplicity of 4 or 0.01 PFU/cell. Unadsorbed virus was removed by repeated washing with phosphate buffered saline (PBS). After adsorption, the cells were cultured in BEM containing 2% ICS (designated further on as normal serum, NS) or in BEM containing 5% rabbit IS (the final neutralizing titre of the medium was 12 in a virus neutralization test against 10^2 PFU of HSV 1).

Sera. (*1*) The IS added to BEM was obtained by immunization of rabbits with an antigen extract of HSV 1-infected cells treated with Nonidet-P40 as described by Kutinová *et al.* (1977). The serum was heated before use at 56° C for 30 min. (*2*) Antiviral IgG used for immunofluorescence (IF) staining and immune cytolysis was precipitated from hyperimmune serum to purified HSV 1 virions with $(NH_4)_2SO_4$ and purified by adsorption to DEAE-cellulose. (*3*) Control rabbit IgG was prepared in the same way. Both IgG samples were used at a concentration of 2 mg/ml. (*4*) The swine anti-rabbit conjugate (SwAR; SEVAC, Prague) was fractionated on DEAE-cellulose; the fraction eluted with 0.15 mol/l NaCl in 0.01 mol/l phosphate buffer was used for IF staining at a concentration of 2 mg/ml. (*5*) Commercial guinea pig complement (Bioveta, Ivanovice na Hané, Czechoslovakia) was diluted 1 : 10 in PBS, pH 6.9.

Experimental procedures. Cultures infected with 4 PFU of HSV 1 per cell were replenished with BEM containing either IS or NS incubated for 4, 8, 12 and 16 hr; cells infected at a multiplicity of 0.01 PFU were incubated for 48 hr. Infected cells incubated in the presence of either serum as well as uninfected cells and infected cells removed immediately after adsorption (time 0) were treated as follows: *1*) After washing with PBS, the cells were overlayed with the immune or control IgG and SwAR conjugate [detection of membrane viral antigen (VA) by quantitative immunofluorescence (QIF)]; *2*) after washing with PBS, the cells were treated with immune and nonimmune IgG (for 30 min at 37° C) and complement (30 min at 37° C) and stained with 0.025% trypan blue for evaluation of immune cytolysis as described by Karmysheva *et al.*

Table 1. Results of QIF_i measurements of VA on the surface of VERO cells infected with HSV 1 (multiplicity 4 PFU per cell)

Hr p.i.	Homologous antibodies in medium absent (NS)	present (IS)	P
4	3.8 ± 1.3*	4.1 + 1.1	> 0.05
8	14.3 ± 5.8	9.6 ± 3.5	< 0.003
12	60.4 ± 29.0	23.1 ± 10.0	< 0.0001
16	52.2 ± 15.3	36.3 ± 19.1	< 0.01

* Mean values and their standard deviations as measured with a × 40 objective (over an area of 8 μm^2) in 35 cells (unfixed cells stained with immune IgG and SwAR conjugate).

(1974); (*3*) the cells were drained, fixed in acetone and stained with the immune or control IgG and SwAR conjugate (QIF measurements of the total intracellular VA); and (*4*) the ce ls were fixed in Bouin's solution and stained with haematoxylin and erythrosin for the estimation of the percentage of mitoses and the number of inclusion bodies.

The coverslips stained according to the procedures 2 and 4 were viewed in an Amplival (Zeiss, Jena) microscope. The intensity of IF in cells stained according to procedures 1 and 3 was measured in a Fluoval microscope equipped with a microphotometer (Zeiss Photometrie, Jena). The relative intensity of IF was measured as described by Rajčáni *et al.* (1979) using either a × 40 objective (evaluation of membrane VA) or a × 16 objective. The data were evaluated statistically by the method of Fischer.

Results

Changes in VERO cells infected with HSV1 at a multiplicity of 4 PFU per cell

In infected cells incubated in the absence of IS, first cytopathic changes were seen by 8 hr p. i. About 5% of cells contained intracellular inclusions and some were rounded. Mitosis was seen in 1.4% of cells. By 12 hr p. i., about 49% of cells contained inclusion bodies. The number of cells containing viral antigen increased from 25% at 4 hr to 39.5% at 8 hr p. i. By 12 hr p. i., practically all cells contained the VA. Later on, rounding of cells and their detachment from the glass was enhanced. The development of the cytopathic effect (CPE) was slightly delayed in infected cells incubated in the presence of IS. VA was found in 14% of cells by 4 hr, in 20% of cells at 8 hr

Table 2. Results of QIF measurements of total VA in fixed VERO cells infected with HSV 1 (multiplicity 4 PFU per cell)

Hr p.i.	Homologous antibodies in medium absent (NS)	present (IS)	P
4	5.5 ± 2.5*	5.3 ± 2.2	> 0.05
8	20.9 ± 6.8	23.2 ± 8.6	0.42
12	62.3 ± 12.4**	43.6 ± 20.5***	< 0.0001

* Mean values of QIF and their standard deviations as measured with a × 16 objective (over an area of 40 μm^2) in 35 cells (fixed cells stained with immune IgG and conjugate SwAR).

** Cells stained with nonimmune IgG 11.4 ± 5.8.

*** Cells stained with nonimmune IgG 7.3 ± 3.9 (similar cells stained only with the SwAR conjugate are shown in Fig. 3).

(about 3% of cells developed inclusion bodies) and in 24% of cells at 12 hr p. i. (inclusion bodies seen in 4.1% of cells). The number of mitoses was 1.5%.

Immune cytolysis was more active in infected cells kept in the absence of IS as compared to cells kept in the presence of IS. By 8-16 hr p. i., lysis occurred in 55—75% of the former but only in 3.2—5.5% of the latter (Fig. 1). Immune cytolysis was not observed in infected cells treated with the control IgG. Nonimmune IgG added to infected cells cultured in the absence of IS did not sufficiently activate the complement system as caused only slight cytolysis. This confirmed the assumption that an optimal concentration of VA as well as an appropriate antibody concentration are necessary for activation of the complement system.

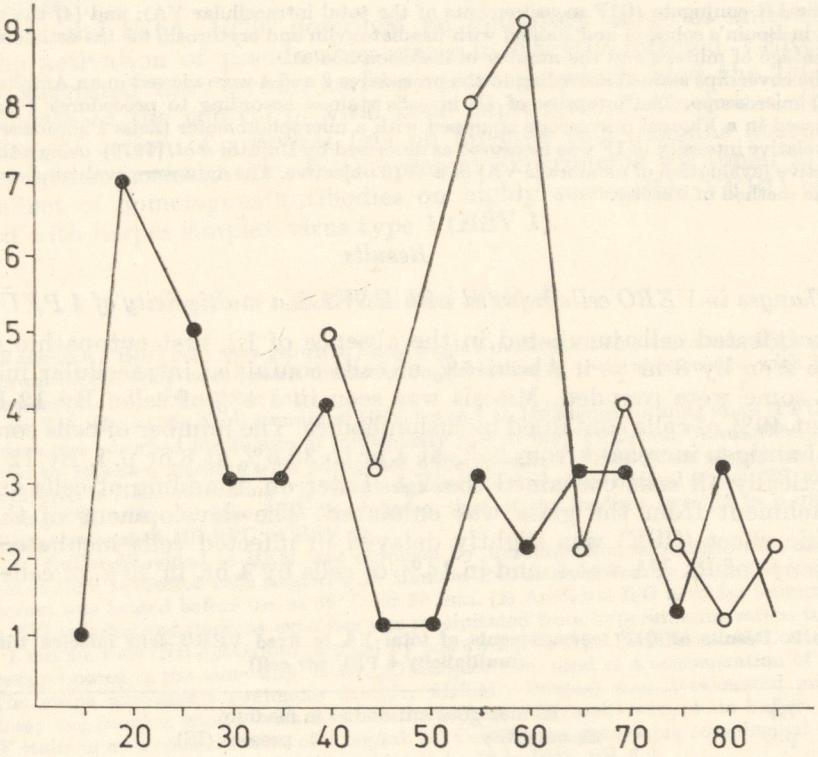

Fig. 2.

Distribution of the relative intensity of IF of VA in VERO cells by 12 hr p. i. with 4 PFU of HSV 1 per cell as measured by QIF

Abscissa: relative intensity of IF; ordinate: number of cells revealing the given relative intensity of IF.

○ Cells incubated in the absence of IS

● Cells incubated in the presence of IS

Table 3. Detection of surface VA in VERO cells by 48 hr p. i. at low multiplicity (0.01 PFU per cell) as measured by QIF

Method	Homologous antibodies in medium absent (NS)	present (IS)	P
Immune cytolysis[1])	90.2%	1.5%	
QIF[2])	62.5 ± 22.7	15.4 ± 7.3	< 0.0001

1) Per cent of cells revealing VA.
2) Mean values and standard deviations of the surface VA in unfixed cells treated with immune IgG and conjugate SwAR (objective × 40, measured in 35 cells).

The results of QIF measurements in unfixed cells stained with immune IgG, reflecting the accumulation of membrane VA in infected cells cultured in the presence of either IS or NS, are shown in Table 1. The binding of immune IgG and conjugate to the surface of unfixed cells by 4 hr p. i. was similar regardless whether the cells were kept in the presence or absence of IS. At further intervals, the binding of immune IgG and conjugate was higher in cells kept in the absence of IS. Fixed cells cultured in the presence of IS showed a lower total binding of immune IgG and conjugate at 12 hr p. i. (Table 2, Fig. 2). At this interval, the amount of total VA antigen was the highest in fixed cells.

Infected cells previously kept in the presence of IS and treated with the anti-rabbit conjugate only revealed fine granular fluorescence indicating that specific IgG molecules became attached to the surface of infected cells in the course of culturing (Fig. 3). By contrast, infected cells cultured in the absence of IS showed an extremely faint fluorescence when stained with the SwAR conjugate.

The binding of the control IgG and conjugate to the surface of infected cells also deserves attention. The IF was more intensive in unfixed cells incubated in the absence of IS (relative intensity 27.1 ± 18.8 at 12 hr p. i.) as compared to cells incubated in the presence of IS (relative intensity 2.5 ± 1 at 12 hr p. i.). IF staining of fixed cells by nonimmune IgG at 4-8 hr revealed a very slight background fluorescence. Nonspecific binding of the control IgG to infected cells was evident at 12 hr p. i. and again it was higher in cells cultured in the absence of IS (11.4 ± 5.8) as compared to cells cultured in the presence of IS (7.2 ± 3.9). As mentioned above, infected cells cultured in the presence of IS revealed binding of antibodies in the course of culturing. Such binding of antibodies may interfere with the attachment of the nonimmune IgG molecules to the virus-induced Fc-receptors. The present findings do not allow to distinguish whether less Fc receptors were formed or whether they were less accessible for steric reasons.

Changes in VERO cells infected with HSV 1 at a low multiplicity of infection (0.01 PFU per cell)

At a low virus input, the CPE developed more slowly. By 48 hr p. i. all cells incubated in the absence of IS showed rounding and desquamation

from the glass surface. In cells kept in te presence of IS the CPE was less extensive and about 25% of the cells showed rounding. The results of QIF measurements and of the evaluation of immune cytolysis are given in Table 3. Only 1.5% of infected cells showed lysis when kept in the presence of IS as compared to about 90.2% of cells kept in the absence of IS. The accumulation of the surface antigen as detected by immune IgG was 4 times lower in the former cells as compared to the latter.

Discussion

The present data showed that HSV 1-infected cells cultured in the presence of IS showed low accumulation of intracellular and surface membrane antigens and the development of a CPE was inhibited. This effect was possibly related to the interaction of homologous antibodies with the virus-coded surface antigens. The difference in accumulation of specific VA was more clear-cut in cells infected at a low multiplicity. It was fourfold when measured by QIF and up to 60-fold when estimated by immune cytolysis. The latter approach indicates only a great accumulation of antigen.

As already mentioned, antibodies inhibit activation of the nonproductive HSV 1 in neurons of ganglia implanted into the peritoneal cavities of recipient mice, if the recipients are passively immunized with specific IgG (Stevens and Cook, 1974). The frequency of the latent HSV activation is lower in ganglion fragments cultured in the presence of IS (Rajčáni *et al.*, 1977). The data obtained with acutely infected cells broaden our knowledge concerning the effect of homologous antibodies on the course of virus replication. Inhibition of the CPE may favour the maintenance of the morphological integrity of infected cells. Antibodies cannot only mask the virus-coded surface antigens — inhibiting the access of immunocompetent cells — but they may indirectly influence the course of their synthesis.

Scupham *et al.* (1979) studied the influence of antibodies and other membrane-active substances on the appearance of E1 and E2 glycoproteins in Sindbis virus-infected cells. They found an impaired processing of precursor polypeptides and explained this by changes in the fluidity of the cytoplasmic membranes. The results of Karmysheva *et al.* (1979, 1980) confirmed the early inhibition of the accumulation of VA and the development of a CPE in cells infected with Sindbis virus incubated in the presence of IS. A feed-back effect of specific antibody on the budding of the ribonucleoprotein of vesicular stomatitis virus was demonstrated in neurons infected in vitro (Dubois-Dalcq *et al.*, 1980). Modulation of virus maturation occurred upon interaction of antibody molecules with the virus-coded antigens (G protein?) on the surface of infected neurons. The existence of intracellular regulatory mechanisms mediated by secondary messengers after action of effector substances on surface receptors is a widely accepted phenomenon well documented by interferon, lectins or hormones.

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Explanation of Micrographs (Plate XIII):

Fig. 1. Early stage of immune cytolysis (swelling) in VERO cells 16 hr p. i. with HSV 1, incubated in the absence of IS. Viral staining with trypan blue, × 600.

Fig. 3. Binding of antibodies to infected cells incubated in the presence of IS 12 hr p. i. is indicated by granular fluorescence scattered over the whole cell surface. Fixed cells stained with the conjugate to rabbit immunoglobulins. × 540.