DETECTION OF IMMEDIATE EARLY PROTEIN ICP27/IE63 AND THYMIDINE KINASE IN THE COURSE OF REACTIVATION OF LATENT HERPES SIMPLEX VIRUS 1 INFECTION

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Summary. – We followed the kinetics of reactivation of latent Herpes simplex virus 1 (HSV-1) infection established in rabbits by corneal route. The corresponding trigeminal ganglia (TG) were cultured and the culture medium was examined at daily intervals for release of infectious virus. Sections from the cultured TG fragments were stained with antisera against non-structural proteins such as the immediate early (IE) protein ICP27 and the early (E) proteins thymidine kinase (TK), the large subunit of ribonucleotide reductase (RR1), the *ori*-binding protein OBP and with a human serum obtained from volunteers immunized with an experimental subunit HSV-1 envelope (env) vaccine containing late structural proteins gB1, gC1, gD1 and gG1 (env antiserum). By indirect immunofluorescence (IF) test, ICP27 was detected in a few neurons from day 1 post explantation (p.e.), while TK was observed in neurons from day 2 p.e. Fluorescence with the human env antiserum was seen at day 3 p.e. The RR1 and OBP antisera stained productively infected Vero cells from 3 and 4 hrs post inoculation (p.i.), respectively. However, these sera showed no IF in cultured ganglion fragments at any interval examined. Our results showed the same cascade of HSV-1 IE and E protein expression during productive infection and reactivation *in vitro*.

Key words: HSV-1; ICP27; thymidine kinase; reactivation; latency; immunofluorescence; explantation technique

Introduction

During productive HSV-1 infection, the consecutive expression of α (IE), β (E) and γ (late, L) genes occurs. From the proteins synthesized only the latter are structural, virion components (Honess and Roizman, 1974). Transcription of IE genes, which contain special promoter sequences such as TAATGARAT, is initiated in the presence of a viral transcription factor α-TIF/VP16 as well as of some cellular cofactors (host cell factor (HCF), Oct-1) (Stern and Herr, 1991; O'Hare, 1993). Out of the five IE proteins (ICP4 of 175 K, ICP0 of 110 K, ICP22 of 68K, ICP27 of 63 K and ICP47 of 12 K), the first four are regulatory or transactivator proteins (Godowski and Knipe, 1986; Hayward, 1993), while the last interferes with antigen presentation by the MHC-I complex (Ahn *et al*., 1996). The most important transactivators acting at early stages p.i. are ICP4 and ICP0 (O'Hare and Hayward, 1985; DeLuca and Schaffer, 1985; Everett, 1986; Cai and Schaffer, 1992). Their absence results in a decreased synthesis of β proteins such as TK and RR1, which are essential for viral DNA synthesis especially in non-dividing neurons (Efstathiou *et al*., 1989; Tenser *et al*., 1991; Wilcox

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Abbreviations: CPE = cytopathic effect; DMEM = Dullbeco's Modified Eagle's Medium; E = early; env = envelope; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; HCF = host cell factor; HSV-1 = Herpes simplex virus 1; ICP = infected cell protein; IE = immediate early; IF = imunofluorescence; IPTG = isopropyl ß-thiogalactoside; LAT = latency-associated transcripts; L = late; MHC = major histocompatibility complex; OBP = *ori*-binding protein; p.e. = post explantation; p.i. = post inoculation; PCR = polymerase chain reaction; RR1 = ribonucleotide reductase (large subunit); SwAHu = swine anti-human; SwAMo = swine anti-mouse; TG = trigeminal ganglion; TK = thymidine kinase; UL = unique long; αTIF/VP16 = trans-inducing factor/virion protein 16

et al., 1992). ICP22 and ICP27 cooperate mainly at transactivation of structural γ proteins (Rice and Knipe, 1990; Jean *et al*., 2001). In addition, ICP27 is important for the selective transport of viral mRNAs (Phelan *et al*., 1996; Soliman *et al*., 1997) and stabilization of their 3'-ends (Brown *et al*., 1995).

It is well known that HSV-1 establishes latency in regional sensory ganglion cells. The only transcripts detected during latency in relative abundance are the latency-associated ones (LAT). They do not have the features of mRNA (Rock *et al*., 1987; Spivack and Fraser, 1987; Ho, 1992). Thus, the key problem in explaining the molecular events at initiation of HSV-1 reactivation is the absence of viral transactivator α-TIF/VP16. With the exception of Tal-Singer *et al*. (1997), who have found that TK is the first protein synthesized at reactivation, all investigators have stressed that the order of transcription of IE and E genes in the course of HSV-1 reactivation is similar to that during productive infection (Margolis *et al*., 1992; Minagawa *et al*., 1994; Devi-Rao *et al*., 1994; Halford *et al*., 1996; Režuchová *et al*., 2003). When mutants deleted in both ICP0 genes were used to establish latency, the number of LAT as well as the reactivation rate decreased (Cai *et al*., 1993; Wilcox *et al*., 1997; Halford and Schaffer, 2001).

In our previous paper (Režuchová *et al*., 2003) we have shown that a minimal amount of ICP4 mRNA may be found during latency in trigeminal ganglion (TG) extracts, while the ICP0 mRNA was a reliable marker indicating the onset of reactivation in TG explants.

Here we describe the kinetics of appearance of IE and E HSV-1 proteins detectable by indirect IF test using antisera prepared against purified recombinant proteins (Košovský *et al*., 2001; Ďurmanová *et al*., 2001). Out of the four antisera used to detect the IE and E polypeptides by IF test, only two (anti-ICP27 and anti-TK) gave positive results; while the attempts to visualize the expression of RR1 and OBP during reactivation in TG sections remained unsuccessful.

Materials and Methods

Virus and cells. HSV-1 strain Kupka is an isolate from a case of human keratitis kindly provided by Dr. J. Benda, Central Military Hospital, Prague. This virus strain establishes latency in up to 90% of animals but extremely rarely causes lethal encephalitis (Rajčáni *et al*., 1977). HSV-1 strain 17 was a kind gift from Dr. M. Preston, the Institute of Virology, University of Glasgow, Glasgow, UK. The viruses were propagated in Vero cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of calf serum, 20 U/ml penicillin, 20 µg/ml streptomycin and 40 µg/ml gentamycine at 37°C in 5% CO_2 atmosphere.

Animals. Albino rabbits (domestic breed Dobrá Voda) weighing 2.5–3.0 kg were infected into right scarified cornea with 2.5 x 10^5 PFU in 50 µl. Virus replication in the cornea was followed for 14 days by swabbing the conjunctival sac at 3–4 day intervals p.i. Specimens from infected rabbits were examined for HSV-1 in Vero cells. At days 63 to 262 p.i. the animals were killed in deep anesthesia (Vetanarcol, Richter Pharma), their both trigeminal (Gasserian) ganglia (TG) were removed, minced under sterile conditions and cultured in the RPMI medium supplemented with 10% of fetal calf serum (FCS) and antibiotics (as described above) for up to 7 days. At daily intervals samples of the medium were taken for virus titration. In addition, the corresponding ganglion fragments were washed in phosphate-buffered saline (PBS), pooled and frozen in liquid propan-butan for IF test. For late time intervals in culture, namely the days 3 and 4, the medium was changed for fresh one.

Preparation of antisera. The IE protein ICP27/IE63 and the E proteins TK, RR1 and OBP/UL9 were prepared as fusion proteins using the Pin Point Xa-1 expression system (Promega) and purified as described previously (Košovský *et al*., 2001; Ďurmanová *et al*., 2001). Each immune serum for IF test was prepared by immunization of 10 adult Balb/c mice with the corresponding purified fusion protein (10–15 µg/dose) mixed with the Al-Span oil adjuvant (Sevapharma) according to the manufacturers instructions. The antigens were administered at days 0, 21 and 35 by intraperitoneal route. At day 45 (10 days after the last antigen dose) the animals were bled from the orbital sinus. As a positive control for staining structural (env) virion proteins, the human env antiserum was used. This serum was obtained from human volunteers immunized with an experimental HSV-1 subunit vaccine containing the glycoproteins B1, C1, D1 and G1 (Weiss *et al*., 1994; Rajčáni *et al*., 1995) and who were seronegative before immunization.

IF test. Vero cell monolayers grown on coverslips were infected with the HSV-1 strain 17 at a multiplicity of 10 PFU/cell. At hourly intervals from 2 to 12 hrs p.i., the infected as well as control cells were washed with PBS, air-dried and fixed in cold acetone for 10 mins. Then the cells were stained with each mouse antiserum and with pre-immunization (control) sera diluted 1:20–1:40 and with an anti-mouse conjugate (SwA-Mo/FITC, Sevapharma) diluted 1:40 for 40 mins. Eight to ten µm thick semi serial cryostat sections of cultured rabbit TG fragments were also fixed in cold acetone for 10 mins and stained in parallel with each antiserum (a mouse ICP27 antiserum diluted 1:20, a mouse TK antiserum diluted 1:20, a mouse RR1 antiserum diluted 1:20, a mouse OBP antiserum diluted 1:20, a control mouse serum diluted 1:20, and a human HSV-1 antiserum diluted 1:40). The sera were adsorbed to uninfected Vero cells and to uninfected rabbit brain suspensions to avoid non-specific staining and then they were applied in a humid chamber at 37°C for 40 mins. After serum treatment, the slides were washed in PBS, incubated with corresponding conjugates (SwA-Mo/FITC or SwA-Hu/FITC) at room temperature for 40 mins, washed 3 times with PBS, counterstained with 0.01% thiasin red (Fluka) and then mounted in a Tris-glycerol solution (5 mmol/l Tris-HCl pH 8.0 in 90% glycerol). The slides were observed under a Nikon E400 fluorescence microscope.

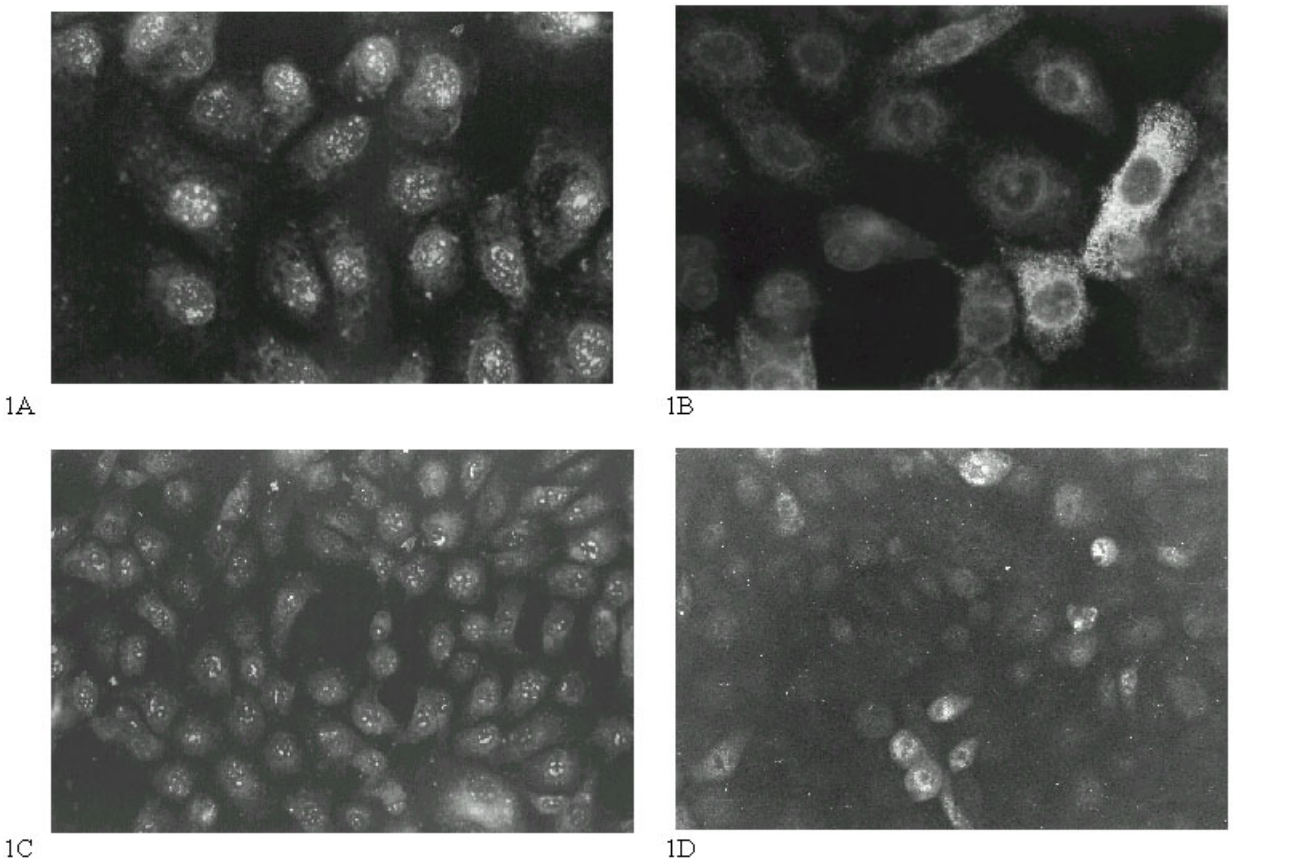

Fig. 1

Detection of IE protein ICP27 and E proteins RR1, OBP and TK during productive infection of Vero cells with HSV-1

A. Fine granular fluorescence of ICP27 in the nuclei at 3 hrs p.i. Magnification 400x.
B. Fine granular fluorescence of RR1 in the cytoplasm at 3 hrs p.i. Magnification 400x.
C. Granular fluorescence of OBP in the nuclei at 4 hrs p.i. Magnification 200x.
D. Granular fluorescence of TK in the nuclei at 6 hrs p.i. Magnification 200x.

Results

Detection of IE and E proteins in the course of productive infection

In acute productive infection, accumulation of fine granular fluorescence of the IE protein ICP27 in the nuclei of infected cells was observed from 3 hrs p.i. (Fig. 1A), while at the same time interval, the E protein RR1 appeared within the cytoplasm (Fig. 1B). Accumulation of the E protein OBP could be observed in the nuclei from 4 hrs p.i. (Fig. 1C). Finally, TK could be find in the nuclei from 6 hrs p.i. (Fig. 1D, Table 1). Summing up, all the four antisera detected the corresponding IE and E antigens during acute productive infection of Vero cells (Figs 1A-D) provided a high enough virus dose (>1 PFU/cell) had been inoculated. No fluorescence was seen either in non-infected or infected cells stained with the pre-immune sera.

Detection of IE and E proteins in the course of reactivation

The appearance of virus-specific non-structural as well as structural HSV-1 proteins was followed in explanted TG fragments from rabbits, which had been infected into the right scarified cornea 2–5 months ago. When the ganglion fragments were kept in culture for several days, their satellite cells and neurons underwent proliferation and apoptosis, respectively. The TG fragments from 21 rabbits were cultured for up to 7 days. During this time period, the right (homolateral) TGs released virus in 17 cases (81%), while the left (contralateral) TGs in 10 cases (47%) only. The number of homolateral ganglia reactivating the virus increased from nearly 5% at day 3 to nearly 30% at day 7 (Table 2). Using the human env antiserum a few neurons and several satellite cells revealed fluorescence by day 3 p.e. (Fig. 2A). The number of virus-producing cells increased

Table 1. Detection of selected IE and E HSV-1 proteins in productively infected Vero cells by indirect IF test

Protein	Result of IF test – time p.i. (hrs)							
	2	3	4	5	6	8	10	12
ICP27	–	+	+	+	+	+	+	+
TK	–	–	–	–	+	+	+	+
RR1	–	+	+	+	+	+	+	+
OBP	–	–	+	+	+	+	+	+

(+), (–) = positive, negative. For other abbreviations see their list at the front page.

Table 2. Release of infectious virus into medium of TG explant cultures in the course of reactivation

Tissue	Virus release[a] (day p.e.)					Total	
	3	4	5	6	7	Positivity	Negativity
RTG	1/21 (4.8%)	1/21 (4.8%)	4/21 (19.0%)	5/21 (24.0%)	6/21 (29.0%)	17/21 (81.0%)	4/21 (19.0%)
LTG	2/21 (9.5%)	2/21 (9.5%)	1/21 (4.8%)	2/21 (9.5%)	3/21 (14%)	10/21 (47.0%)	11/21 (52.0%)

RTG = right TG, LTG = left TG.
[a]Positive/total rabbits.

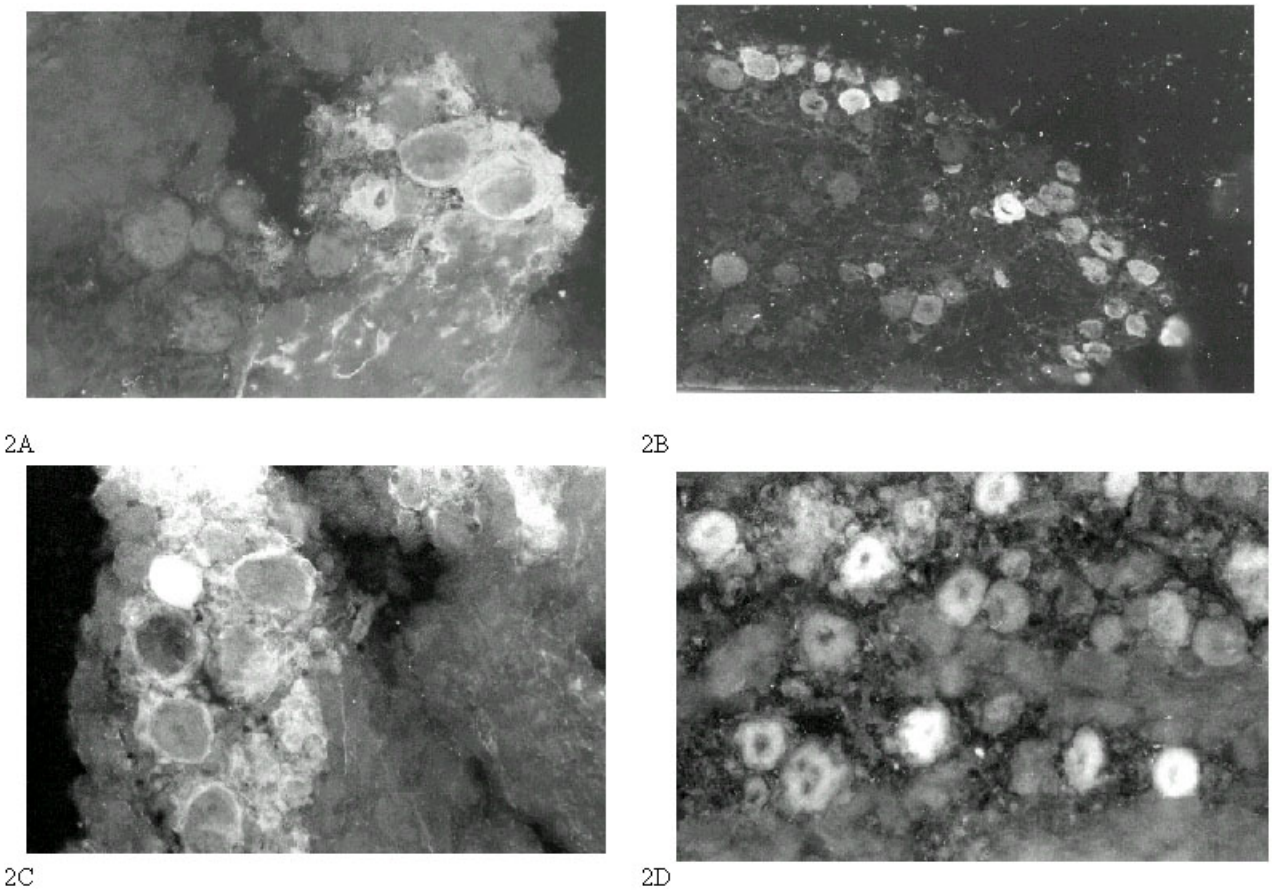

Fig. 2

Detection of IE protein ICP27, E protein TK and L envelope proteins during reactivation of latency in TG explants

A. Fluorescence of env proteins in some neurons and non-neural satellite cells at 3 days p.e. Magnification 400x.
B. Fluorescence of ICP27 in a neuron at day 1 p.e. Magnification 200x.
C. Fluorescence of ICP27 in a few neurons and non-neural satellite cells at day 5 p.e. Magnification 400x.
D. Fluorescence of TK in a few neurons at day 2 p.e. Magnification 400x.

later so that the TG fragments examined at latest time intervals in culture showed overwhelming positivity in predominantly non-neural cells.

In parallel sections of the homolateral (right) TG explants we detected fluorescence only with the ICP27 and TK antisera, but not with the RR1 and OBP antisera (Table 3).

Table 3. Detection of selected IE and E HSV-1 proteins in cultured TG fragments by indirect IF test

Protein	Results of IF test (day p.e.)							
	0	1	2	3	4	5	6	7
ICP27	–	+	+	+	+	+	–	–
TK	–	–	+	+	+	+	+	+
RR1	–	–	–	–	–	–	–	–
OBP	–	–	–	–	–	–	–	–
Env proteins[a]	–	–	–	+	+	+	+	+

[a]Env proteins detected with the serum from human volunteers immunized with HSV-1 glycoproteins B1, C1, D1 and G1.
(+), (–) = positive, negative. For other abbreviations see their list at the front page.

Staining with the ICP27 antiserum was seen from days 1 to 5 p.e. At the earliest time interval (day 1), the ICP27/IE63 antigen was expressed in single neurons only (Fig. 2B); later on, this IE protein was found not only in neurons but also in satellite cells (Fig. 2C). These findings indicated that neurons rather then satellite cells are the initiation site of HSV-1 reactivation, i.e. the most probable site of latency. The E protein TK was detected in neurons at day 2 p.e. (Fig. 2D). Here again, satellites in addition to neurons were found positive at later time intervals in culture (data not shown).

Similarly as in productively infected Vero cells, the onset of TK fluorescence was delayed as compared with that of ICP27/IE63 (Table 3).

Discussion

Neurons have traditionally been regarded as the primary site of HSV-1 latency since the presence of viral DNA has been experimentally proved within these regional sensory ganglion cells (Cook, 1974; Docherty and Chopan, 1974). Our demonstration of IE and E proteins first in neurons and then in non-neural cells brings further support for the above assumption. Satellites and other non-neural cells become probably infected from the reactivated virus, which can spread not only back to peripheral tissues along axons, but also within the ganglion itself. It has been claimed that the reactivation starts in neurons, in which the latency had been established, i.e. in the absence of virus replication and under conditions, which favor LAT expression (Margolis *et al*., 1992; Preston, 2000). Only about 3% of total neurons are permissive for HSV-1 infection (Sawtell, 1997). Satellite cells are probably more permissive for HSV-1 replication than neurons. Therefore, they may be less suitable for establishing latency. In addition, the IE HSV-1 promoters may not be activated in non-neural cells (Loiacono *et al*., 2002), which argues against a dominant role of satellites in harboring latent HSV-1 and/or in initiation of reactivation. However, Taus and Mitchell (2001) have described activation of ICP4 synthesis in Schwann cells of ganglia with established latency. The presence of ICP4 mRNA has been repeatedly described in non-cultured ganglia during latency, i.e. in the absence of any clear-cut reactivation stimuli (Kramer and Coen, 1995; Kramer *et al*., 1998; Režuchová *et al*., 2003), while the presence of TK mRNA under such conditions has been found rarely (Kramer and Coen, 1995). As ICP4 interacts with the LAT promoter (Batchelor *et al*., 1994) it may play a dual regulatory role, either balancing latency maintenance and/or promoting reactivation. We showed that in rabbit ganglion explants the expression of latent HSV-1 starts with ICP0 and ICP27 transcription, which can be detected along with the already present ICP4 transcripts at 4 hrs p.e. (Režuchová *et al*., 2003). Other authors have found that HSV-1 mutants deleted in both ICP0 genes to establish latency produced decreased amounts of LAT transcribed in neurons as well as the frequency of reactivation (Cai *et al*., 1993; Wilcox *et al*., 1997; Halford and Schaffer, 2001).

We demonstrated the appearance of the IE ICP27 by 24 hrs and the E protein TK by 48 hrs in TG culture. Both findings are in accord with our assumption of keeping the order of IE and E proteins expression during reactivation as described at productive virus infection. Unfortunately, we were unable to detect the RR1 and OBP proteins with our sera, which were able to visualize these proteins in productively infected Vero cells. This fact can be explained by insufficient concentration of the proteins in question in neurons during reactivation. Localization of the IE protein ICP4 in ganglion sections before or during reactivation is the matter of recent investigation.

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