Detection of Herpes Simplex Virus Induced Polypeptides in Rabbit Trigeminal Ganglia

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The objective of this study was to follow herpes simplex virus type 1 (HSV-1) genome expression in rabbit trigeminal ganglia during primary, latent, and artificially reactivated infections using monospecific antiserum to representatives of the sequentially produced alpha, beta, and gamma group polypeptides. Rabbits with or without electrode implants were inoculated with $10^5$ plaque forming units of McKrae strain HSV-1 following scarification and monitored by daily preocular tear film culture. Animals were killed during primary, latent, and artificially reactivated infections. Representative trigeminal ganglion sections from each animal were reacted with either preimmune rabbit serum, anti-HSV-1, anti-ICP-4, anti-ICP-8, or anti-ICP-5 sera. During primary infection, staining was evident in 30-40% of the ganglion cell nuclei with all antisera. During latency, staining with anti-ICP-4 was detected in 14 out of 18 ganglia tested; 10-15% of the ganglion cell nuclei per section exhibited positive staining. A +/− staining pattern with anti-ICP-8 was obtained in 5 out of 14 of the ganglia tested; 1-3% of the nuclei per section exhibited this staining pattern. Staining with anti-HSV-1 and anti-ICP-5 was not detected. During induced reactivation, staining of ganglion cell nuclei with all antisera was observed, but the number of cells staining per section was decreased compared to that observed during primary disease. Invest Ophthalmol Vis Sci 25:1436–1440, 1984

Contemporary theory concerning the natural history of herpes virus infection is summarized by the neuronal hypothesis. This theory assumes that as the result of primary herpetic infection, virus invades superficial nerve endings and is subsequently transported intraaxonally to corresponding ganglia where a latent infection is established. Upon activation (either spontaneously or by various induction techniques), virus or virus-specified products are transported centrifugally in axons to target organs, where they are released with or without the production of characteristic herpetic lesions.

Although the state and activity of the HSV-1 genome during periods of silent infection have not been defined, the bulk of experimental evidence favors conservation of the virus in a nonreplicating form. Several investigators have detected virus-specific DNA in explant cultures from ganglia of latently infected mice, and others have reported the detection of HSV-specific ribonucleic acid in human sensory ganglia, suggesting that some genome transcription must be occurring within the ganglia between episodes of active peripheral herpetic infection. Two additional pieces of experimental evidence further support the concept of viral genome conservation during latent infection. When latent ganglion infection was compared with acute and reactivating infection via nucleic acid hybridization, a dramatic increase in both viral DNA genome equivalents and messenger RNA was observed during acute and reactivating herpetic infection. When monospecific hyperimmune antiserum to an immediate early polypeptide of molecular weight 175,000 designated VP175 or infected cell polypeptide-4 (ICP-4) and hyperimmune antiserum to HSV-1 were used to document persistence of an alpha group antigen in rabbit trigeminal ganglia during latency (22–200 days postinfection), 90% of the ganglia examined exhibited positive staining with ICP-4. In contrast, all samples examined during primary infection (days 3–12 postinfection) exhibited positive staining when reacted with either anti-HSV-1 or anti-ICP-4 serum.

The current study is a natural extension of this work. Trigeminal ganglia from rabbits during primary, latent, and reactivating HSV-1 infections were sectioned serially and processed for indirect immunofluorescent staining using hyperimmune antiserum to HSV-1; monospecific hyperimmune antisera to immediate early and early nonstructural polypeptides (ICP-4, ICP-8, respectively); and a structural HSV-1

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induced polypeptide (ICP-5). Immunofluorescent staining results during latent and reactivating infection were compared with those results obtained during acute ganglionic infection.

**Materials and Methods**

**Inoculation**

Twenty-one New Zealand white rabbits (NZW) were inoculated with $10^6$ plaque forming units of the McKrae strain of herpes simplex virus type 1 (HSV-1) after corneal scarification. Following ocular instillation of the virus, the lids were closed and massaged for 15 sec. Animals were maintained at the Neurosensory Center, Baylor College of Medicine in accordance with the ARVO Resolution on the Use of Animals in Research.

**Ocular Culture**

Beginning day 3 postinoculation, all eyes were monitored daily through day 21 and every third day thereafter until killing for the presence of infectious virus shedding by quantitative ocular culture. Sterile cotton tipped applicators (Schering Medical Scientific) were passed over the upper and lower conjunctival cul-de-sac, lightly rolled over the cornea, and retained in the nasal fornix for 5–10 sec for maximum tear film adsorption. Swabs were eluted with agitation for 15 sec in 0.5 ml of Hanks’ balanced salt solution (HBSS, Gibco) containing 1.25 μg/ml fungizone, 200 μg/ml gentamicin, 200 IU/ml penicillin, and sterile distilled water, adjusted to a final pH of 7.4 with NaHCO₃. Fifty-microliter aliquots of the HBSS/tear film mixture were adsorbed onto confluent 16-mm Vero cell monolayers for 5 min at 37°C in high humidity and 5% CO₂. All cultures were monitored daily by inverted light microscopy for the development of cytopathology consistent with HSV-1 infection. HSV-1 titers were determined by quantitative culture using multiple regression analysis.

**Antiserum Preparation—ICP-4, ICP-8, and ICP-5**

Monospecific antiserum was prepared to each of the purified polypeptides as previously described.

**Indirect Immunofluorescence Test**

The indirect immunofluorescence test described by Porter et al was employed with minor modifications. Serial sections (4–6 μm) were fixed in acetone at room temperature for 5 min and air dried. Before staining the sections were rehydrated in phosphate buffered saline for 5 min at room temperature. Sections were treated for 30 min at room temperature with either antiserum or preimmune rabbit serum diluted 1:2 or 1:8 in phosphate buffered saline. Sections then were washed three times in phosphate buffered saline and treated for 30 min with fluorescein conjugated goat anti-rabbit gamma globulin (FITC-GAR, Hyland Laboratories). Sections were washed three times in phosphate buffered saline, air dried, and mounted in 50% glycerol in phosphate buffered saline. A minimum of 12 sections (containing an average of 150 neurons per section) from each ganglion were stained and observed. To confirm initial results, several experiments were carried out in double-blind fashion.

**Electrode Implantation**

Monopolar stainless steel electrodes were implanted using stereotaxic coordinates as previously described.

**Experimental Design**

Eight rabbits were killed during primary infection days 2–9 postinoculation (positive bilateral tear film cultures). Seven animals were killed during latent infection (negative bilateral tear film cultures) on days 41–119 postinfection. The remaining 6 rabbits were killed following induction by direct electrical ganglionic stimulation consisting of a series of pulses of 1 msec duration at a frequency of 10 Hz with voltages ranging from 0.5 mV to 4 mV. Two trigeminal ganglia (TG) were harvested at 12 hr postinduction, 4 TG at 24 hr postinduction, and 6 TG at 48 hr postinduction. Virus was not recovered from precocurant tear film cultures in the rabbits killed at 12 and 24 hr poststimulation. Virus was recovered from precocious tear film in two of the three rabbits killed at 48 hr postinduction. Five noninoculated rabbits were killed and used as negative controls. Ganglia were processed for indirect immunofluorescent staining.

**Results**

No immunofluorescent staining was evident in ganglion samples from the noninfected rabbits or from samples reacted with preimmune rabbit serum during primary, latent, and reactivating HSV-1 infections (Fig. 1A). Immunofluorescent staining results with anti-HSV-1 and monospecific hyperimmune antiserum to ICP-4, ICP-8, and ICP-5 are summarized below.

**Primary Infection (Table 1)**

Ganglion samples harvested during primary infection reacted with all antisera. Staining with anti-HSV-1 serum was diffuse with cytoplasmic as well as nuclear staining observed (Fig. 1B). Twenty to 25%
Fig. 1. A, Section reacted with preimmune rabbit serum, diluted 1:2. Lack of specific nuclear staining is illustrated in positive control consisting of rabbit trigeminal ganglion cell bodies during primary infection 4 days after ocular inoculation with HSV-1; positive tear film culture. Artifactual staining is noted around cut edges of cells (×860). B, Section reacted with anti-HSV-1, diluted 1:8. Diffuse, nongranular nuclear and cytoplasmic staining of TG cell bodies (arrows) during primary infection 3 days after ocular inoculation with HSV-1; positive tear film culture (×320). C, Section reacted with anti-ICP-4, diluted 1:4. Solid nuclear fluorescence in TG cell bodies during primary infection, day 4 postinoculation; positive tear film cultures (×400). D, Section reacted with anti-ICP-8, diluted 1:4. Fine, “dust-like” staining confined to the nucleus of TG cell bodies during primary infection; 3 days postinoculation with HSV-1; positive tear film culture (×400). E, Section reacted with anti-ICP-5, diluted 1:4. Fine “dust-like” staining similar to the reaction with anti-ICP-8 is present over nuclei of TG cells during primary infection, 6 days postinoculation; positive tear film culture. Arrows illustrate fine cytoplasmic staining observed in some of the neurons (×460). F, Section reacted with anti-ICP-4, diluted 1:4. Solid nuclear staining in TG cell bodies during latent infection, 64 days postinoculation; negative bilateral tear film cultures (×380). G, Section reacted with anti-ICP-4, diluted 1:4. Coarse granules overlying solid background fluorescence in nuclei of TG cell bodies 24 hr following electrical induction; negative tear film culture (×720). H, Section reacted with anti-ICP-8, diluted 1:4. Fine nuclear fluorescence of varying intensity in cord of TG cell bodies 48 hr following electrical induction; positive tear film culture (×380). I, Section reacted with anti-ICP-5, diluted 1:4. Fine nuclear staining 48 hr following electrical induction; positive tear film culture (×420).

of ganglion cells per section exhibited staining. Staining with anti-ICP-4 serum was restricted to the nucleus and was granular to solid in pattern (Fig. 1C). Although the staining pattern often appeared solid, a distinct coarse granularity superimposed on a solid background was appreciated upon focusing in more than one plane. Twenty-five to 40% of the neurons per TG section exhibited staining. Staining with anti-ICP-8 serum resulted in immunofluorescence in 5–10% of the neurons per section (Fig. 1D). The
Table 1. Reactivity of trigeminal ganglia obtained from rabbits sacrificed during primary, latent, or artificially reactivated HSV-1 infections with antisera to ICP-4, ICP-8, ICP-5, or HSV-1

<table>
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* Animals killed during primary infection (days 3-9) had bilateral positive tear film cultures.
† Numbers in parentheses indicate the number of ganglia tested per antiserum group.
‡ The total number of ganglia tested in the anti-ICP-4 column is greater than two ganglia per animal because duplicate lots of anti-ICP-4 were used with same animals.
§ ND = not done.
‖ Animals killed during latent infection (days 41-119 postinfection) had bilateral negative tear film cultures.
|| Animals were killed at 12, 24, or 48 hr following electrical induction. All tear film cultures were negative at 12 and 24 hr postinduction. Two of the animals killed at 48 hr had positive tear film cultures.

staining was limited to the nucleus and appeared to be of a fine “dust-like” consistency.

Nuclear fluorescence was exhibited in 3-8% of neurons per section when sections were reacted with anti-ICP-5 serum. (Fig. 1E). The staining pattern was similar to that obtained with anti-ICP-8 serum. Cytoplasmic staining with anti-ICP-5 serum was present in 2-3% of the neurons per section.

Latent Infection (Table 1)

During latency, staining with anti-ICP-4 serum was observed in 14 out of 18 of the ganglia examined (Fig. 1F). Granular staining superimposed on a solid background was confined to the nucleus. One to 3% of the neurons per TG section in 5 out of 14 of the ganglia examined exhibited a +/- staining pattern when reacted with anti-ICP-8 serum. No fluorescence was evident when anti-ICP-5 serum or anti-HSV-1 serum were reacted with the latently infected ganglion sections.

Artificially Reactivated Infection (Table 1)

Trigeminal ganglion sections from rabbits killed at 12 and 24 hr postinduction exhibited positive staining with anti-ICP-4 and anti-ICP-8 sera (Fig. 1G, H). The staining pattern was similar to that observed during latent infection. Cords or groups of neuron cell bodies reacted positively with anti-ICP-4 serum in contrast to positive staining in isolated cells observed during latency. TG sections from animals killed 48 hr postinduction showed positive or +/- staining with all monospecific antisera tested (anti-ICP-4, anti-ICP-8, and anti-ICP-5). Approximately 20-30% of the neurons per ganglion section reacted with anti-ICP-4 serum. Anti-ICP-8 serum reacted with approximately 8-10% of the neurons per section. Anti-ICP-5 serum reacted with 5-6% of the neurons (Fig. 11). The staining patterns were predominantly nuclear with occasional light-to-moderate cytoplasmic staining.

Discussion

In this study, a functional definition of active and latent disease was used to identify primary, latent, and artificially reactivated infection. During primary and reactivating infection, infectious virus could be recovered from both preocular tear film and cell-free TG homogenates. Following clinical resolution of the primary stage of infection, a silent or latent infection ensued during which infectious virus could not be recovered from either preocular tear film or cell-free homogenates of TG. During this stage, the potential for production of infectious virus was retained as demonstrated by the recovery of infectious virus from whole cell and organ culture explants of the TG.17

It has been determined that HSV-1 viral polypeptides are sequentially synthesized in three interlinked cascade regulated groups designated alpha, beta, and gamma.18 The alpha and beta groups contain largely...
nonstructural polypeptides and are synthesized at highest rates 3–4 and 4–7 hr postinfection, respectively. The gamma group contains major structural polypeptides and are synthesized later. Monospecific antisera representative of the alpha, beta, and gamma group HSV-1 polypeptides were used in order to indirectly assess viral genome expression in TG neurons during primary, latent, and reactivating disease.

Use of these antisera revealed that a polypeptide representing each of the groups was represented antigenically within TG neurons during primary infection. During latency, staining was obtained with anti-ICP-4 serum and to a minor extent, with anti-ICP-8 serum (fewer cells, weaker staining). The number of cells staining with anti-ICP-4 decreased to approximately 10% per section compared with 25–40% per section during primary infection as previously reported.12 The significance of this staining with anti-ICP-8 is presently under study. Whether this decrease represents cell drop out (secondary to cell death), aborted infection, or decrease in polypeptide concentration to undetectable levels is currently unknown. During early induced reactivation (12 and 24 hr postinduction), staining was evident only with anti-ICP-4 and anti-ICP-8 sera. However, the number of ganglion cells staining as well as the intensity of staining was increased compared with that observed during latency. By 48 hr postinduction, staining was observed when sections were reacted with all antisera. Whether induced reactivation produces the same pattern of response as normal spontaneous reactivation is not known.

The data presented in this report supports the concept of HSV-1 genome conservation within the neuron in the static state with only partial expression during periods of latent infection. The detection of positive nuclear fluorescence by monospecific antisera to ICP-5 following induced reactivation probably represents de novo gamma viral polypeptide synthesis, as it is not present during latency. Increases both in intensity of staining and in number of cells staining with ICP-4 in reactivating infection suggest some component of de novo synthesis of ICP-4, even though the antigen is found within TG neuron nuclei during all stages of infection.

Key words: HSV, latency, trigeminal ganglia, polypeptides, immunofluorescence

References