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Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits

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Anti-nerve growth factor (anti-NGF) antibody has been shown to induce reactivation of latent herpes simplex virus type 1 (HSV-1) *in vitro*. We found that systemically administered anti-NGF induces ocular shedding of HSV-1 *in vivo* in rabbits harboring latent virus. Rabbits in which HSV-1 latency had been established were given intravenous injections of goat anti-NGF serum daily for 10 days beginning 42 days after primary viral infection. Tears were assayed for virus for 12 days beginning on the day of the first injection. All eight rabbits given high titer anti-NGF had infectious virus in their tears at least once during the 12-day period. Fifteen of 16 eyes were positive and the average duration of viral shedding for these eyes was 4.0 days. Latently infected rabbits receiving daily injections of nonimmune goat serum or saline for 10 consecutive days were controls. Only six of the 16 (38%) eyes from rabbits receiving nonimmune goat serum shed virus. Only one of 12 eyes from untreated rabbits shed virus. Sera from control rabbits had no detectable anti-NGF activity; titers in anti-NGF-treated rabbits ranged between 1 : 1000 and 1 : 10 000. NGF deprivation may act as a neuronal stressor and may share a common second messenger pathway with heat- or cold-stress induced reactivation of latent HSV-1.

Keywords: antibody; cornea; HSV latency and reactivation; nerve growth factor; rabbit; sensory and autonomic ganglia

Introduction

Infection with herpes simplex virus type 1 (HSV-1) is characterized by an epithelial or mucosal infection followed by entry of the virus into the nerve terminals innervating the site of infection. The virus proceeds via rapid retrograde axonal transport to the sensory ganglion where latent infection is established in neurons. Virus topically applied to the rabbit eye establishes HSV-1 latency in the trigeminal ganglion (TG). Once latent, reactivation (virus in tear film) can be induced by corneal nerve injury such as UV irradiation (Laycock *et al*, 1991), transection of corneal nerves, anterior superficial keratectomy (Beyer *et al*, 1990), and radial keratotomy (Haruta *et al*, 1987). Direct electrical (Green *et al*, 1981) and mechanical (Nesburn *et al*, 1976) stimulation of the TG also results in viral reactivation. In addition to these physical injuries, HSV-1 can also be induced to reactivate by both stress and stress-related pharmacologic agents such as heat stress (Sawtell and Thompson, 1992) and cold stress (Varnell *et al*, 1987, 1995), or by transcorneal iontophoresis of epinephrine (Hill *et al*, 1985, 1987a), timolol (Hill *et al*, 1987c), or 6-hydroxydopamine accompanied by topical dipivefrin hydrochloride (Hill *et al*, 1987b). Intravenous administration of cyclophosphamide and dexamethasone also induce HSV-1 reactivation (Haruta *et al*, 1989). Whether this is due to reduced immunosurveillance or to a toxic effect on trigeminal neurons remains to be determined.

About 70–80% of sensory neurons in the dorsal root or trigeminal ganglia (the HSV-1 target tissue) are dependent upon nerve growth factor (NGF) or survival and function (Johnson *et al*, 1980; Sikich, 1986). Numerous *in vitro* studies have shown that administration of NGF favors the establisment of latency over lytic infection and conversely that

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removal of NGF results in HSV reactivation. These studies were performed on primary neuron cultures as well as PC12 cell cultures, a noradrenergic rat phaeochromocytoma cell line (Block *et al*, 1994; Clements and Kennedy, 1989; Smith *et al*, 1992; Wilcox *et al*, 1990; Wilcox and Johnson, 1987, 1988). Taken together, these studies suggested that antibodies to NGF administered to latently infected animals might cause HSV reactivation. Here, we describe the effect of intravenous administration of anti-NGF sera on the induction of HSV-1 ocular shedding in latently infected rabbits.

Results

The first indication that anti-NGF could induce HSV reactivation was obtained using serum with a relatively low antibody titer (1 : 20 000 to 1 : 30 000). Injections of this serum in six rabbits induced HSV-1 ocular shedding at least once over the 12-day test period (Experiment 1). Three of six rabbits receiving nonimmune goat serum, were positive for viral shedding. In both groups, virus was first detected in the tears on day 3. Rabbits receiving anti-NGF had eight of 12 eyes positive and the average duration of shedding was 2.0 days (Table 1). The rabbits receiving nonimmune goat serum had five of 12 eyes positive for virus and an average duration of shedding of 1.2 days. Chi-square analysis comparing positive eyes per total eyes revealed no significant difference (P=0.73) between the experimental (anti-NGF) group and the rabbits receiving nonimmune goat serum. There was a significant difference between the comparison of positive eye swabs per total swabs (P=0.0124, Table 1). The anti-NGF group was significantly different from the untreated group for eyes positive (P=0.0032) and total positive swabs (P=0.0007). The titers of goat anti-NGF antibody in the serum of rabbits at 5, 10 and 12 days after the first intravenous injection of anti-NGF goat serum ranged from 1 : 100 to 1 : 1000. Serum from rabbits that received nonimmune goat serum had no anti-NGF activity.

Anti-NGF with a titer 3 – 5 times higher than that used in Experiment 1 was used in another experiment using daily injections for 10 days. All eight rabbits that received the higher titer (1 : 80 000 to 1 : 100 000) antiserum, shed HSV-1 at least once over the 12-day test period (Experiment 2). All eyes but one (15/16) shed virus; the average duration of ocular shedding was 4.0 days (Table 2). Five of eight control rabbits and six of 16 eyes shed virus. Virus was first detected on day 3 in all groups. The average duration of shedding was 2.0 days in the control group. Chi-square analysis comparing positive eyes per total eyes or positive eye swabs per total swabs showed significantly greater HSV-1 shedding in the anti-NGF treatment group, compared to the control group. The titers of goat anti-NGF antibody in the serum of rabbits at 5, 10 and 12 days after the first intravenous injection of anti-NGF goat serum ranged from 1 : 1000 to 1 : 10 000. Serum from control rabbits that received nonimmune goat serum or phosphate buffered saline (PBS) had no anti-NGF activity.

Table 3 shows the percentage of latency associated transcript-positive (LAT^+) neurons in the trigeminal and superior cervical ganglia following intravenous injections with nonimmune goat sera, high and low titer anti-NGF sera, and PBS. The results reveal that there was no significant difference in the number of LAT^+ cells in any of the tissues examined. Analysis of the *in situ* hybridization results, as well as histological sections of the superior cervical and trigeminal ganglia, showed no morphological changes either in cell size or the frequency of cells undergoing atrophy (Figures 1 and 2).

Table 2 Anti-NGF-induced ocular reactivation[a]

Treatment	*Rabbits*	*No. positive (%)* *Eyes*	*Swabs*
Anti-NGF (high titer)			
3 rabbits	3/3 (100)	5/6 (83)	33/72 (46)
5 rabbits	5/5 (100)	10/10 (100)	27/120 (23)
Totals	**8/8 (100)**	**15/16 (93)[b]**	**60/192 (31)[c]**
Controls			
PBS, 3 rabbits	3/3 (100)	4/6 (67)	4/72 (5.6)
Goat serum, 5 rabbits	2/5 (40)	2/10 (20)	7/120 (5.8)
Totals	**5/8 (63)**	**6/16 (38)[b]**	**11/192 (5.7)[c]**

[a]Daily injections began on P.I. day 42 and were given for 10 consecutive days. [b]Chi-square two-tailed analysis P=0.049. [c]Chi-square two-tailed analysis P=0.0001

Table 1 Anti-NGF-induced ocular reactivation[a]

Treatment	*Rabbits*	*No. positive (%)* *Eyes*	*Swabs*
1 Anti-NGF (low titer)	6/6 (100)	8/12 (67)[b]	16/144 (11.0)[c]
2 Nonimmune goat serum	3/6 (50)	5/12 (42)[b]	6/144 (4.2)[c]
3 None	1/6 (17)	1/12 (8)[d,e]	2/144 (1.4)[f,g]

[a]Daily injections began on P.I day 42 and were given every other day for 10 days. [b]Chi-square two-tailed analysis P=0.7300, treatment 1*vs* 2 (eyes). [c]Chi-square two-tailed analysis P=0.0124, treatment 1 *vs* 2 (swabs). [d]Chi-square two-tailed analysis P=0.0593, treatment 2 *vs* 3 (eyes). [e]Chi-square two-tailed analysis P=0.0032, treatment 1 *vs* 3 (eyes). [f]Chi-square two-tailed analysis P=0.1515 treatment 2 *vs* 3 (swabs). [g]Chi-square two-tailed analysis P=0.0007, treatment 1 *vs* 3 (swabs)

Table 3 LAT expressing in rabbit ganglia[a]

Treatment	Number of positives (%) Ganglia TG	SCG
Anti-NGF (low titer)	178/1170 (15.2)	39/495 (7.8)
Anti-NGF (high titer)	140/1200 (11.7)	34/512 (6.7)
Nonimmune goat sera	93/978 (9.5)	22/457 (4.8)
PBS	62/1054 (5.9)	26/471 (5.5)

[a]Cumulative averages from three ganglia in each group except the PBS treatment group that had only two ganglia

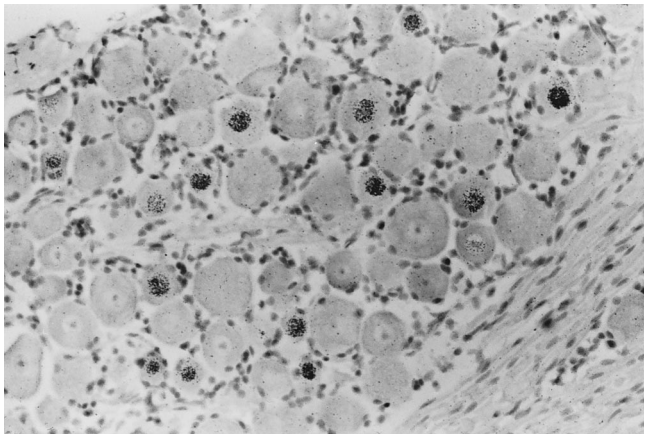

Figure 1 LAT-expressing cells in trigeminal ganglia.

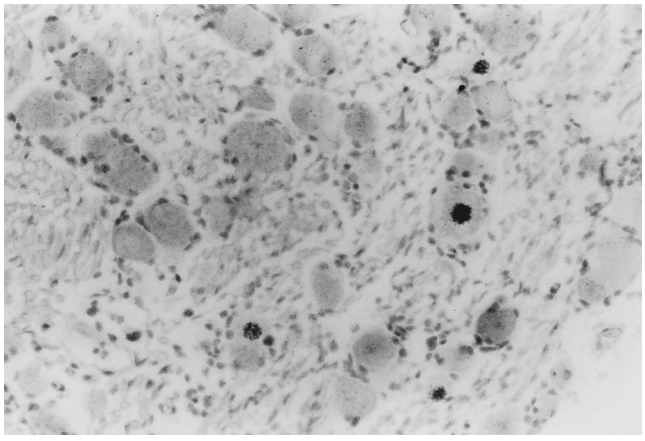

Figure 2 LAT-expressing cells in superior cervical ganglia.

Both the TG and SCG from selected groups were analysed by quantitative PCR to determine if any treatment alters the HSV genome copy number per neuron (Table 4). No statistical differences were detected between any of the treatment groups regarding the copy number of HSV DNA per latently infected neural cell ($P > 0.2$).

Table 4 Quantitation of HSV-DNA in rabbit ganglia[a]

Treatment	Ganglia TG	SCG
Anti-NGF (low titer)	20.3	12.5
Anti-NGF (high titer)	22.2	12.9
Nonimmune goat sera	16.1	11.8
PBS	19.3	13.7
Untreated	18.7	14.2

[a]Cumulative averages from three ganglia in each group except the PBS treatment group that had only two ganglia and untreated group which had 10 ganglia. Rabbits were sacrificed on post-inoculation days ranging from 52–58. This was 1 to 2 days after final treatment. Values expressed as mean genome copy number per 100 cells

Discussion

Wilcox *et al* (1987, 1988, 1989) showed that the addition of anti-NGF antibody to cultures of latently infected neurons induced HSV-1 reactivation. Our study shows a similar result following intravenous injection of latently infected rabbits with anti-NGF serum. Laycock *et al* (1994) reported that intraperitoneal administration of anti-NGF antibodies induced viral shedding in latently infected mice. While our report shows that anti-NGF given to latently infected rabbits can cause reactivation, Laycock *et al* (1994) reported only a slight (10%) increase in frequency of induction. They reported that administration of murine NGF to mice undergoing UVB-induction did not block HSV reactivation. They further reported that treatment of mice with rabbit anti-NGF resulted in atrophy of neurons in the superior cervical ganglia, but that this atrophy did not result in the death of neurons. However, we detected no change in the morphology of the superior cervical or trigeminal ganglia and no change in the number of LAT^+ neurons. While there are species differences between the anti-NGF antibodies used in the Laycock study and our study (rabbit anti-murine NGF *vs* goat anti-immune NGF, respectively), there are probably significant differences regarding the epitope specificity and the potential for cross-reactivity of any anti-NGF antibody with members of the structurally-related neurotrophins (brain derived neurotrophic factor and neurotrophins 3, 4/5, and 6). Alternatively, these results could simply reflect differences in viral reactivation frequencies between latently infected mice and rabbits (Hill *et al*, 1996c).

Other studies have demonstrated a relationship between NGF and HSV. Aloe (1987) reported that newborn rats given NGF were resistant to HSV for a limited time; this resistance was particularly evident in sympathetic neurons. Dicou *et al* (1991) reported increased titers of anti-NGF in sera of HSV-infected patients with active herpetic disease compared to latently infected patients who had no

signs of disease. These authors suggested that the anti-NGF antibodies reduced the available concentrations of NGF and thus elicited viral reactivation. Dicou *et al* (1991), using an unspecified strain of virus, found no difference in anti-NGF titers between HSV-infected and uninfected rabbits. Also, rabbits were infected by intravenous injection as opposed to a more relevant route such as epidermal, nasal, or ocular application. Furthermore, 100% of human subjects had recurrent herpetic disease and 60% were experiencing clinical symptoms when their sera were titered for anti-NGF antibody; however, the study did not reveal whether the rabbit groups were currently undergoing or had ever experienced either primary or reactivated viral shedding or disease when their sera were analysed (Dicou *et al*, 1991). Thus, whether endogenous, circulating anti-NGF antibodies play a role in the rabbit model of HSV reactivation is still an open equation.

NGF is required for survival and differentiation of sympathetic and sensory neurons and is involved in various metabolic and physiologic processes in embryonic and neonatal neurons. The survival and function of mature, differentiated neurons in adult animals are much less acutely dependent on NGF compared to immature neurons. However, our study and those of Wilcox and Johnson (1987, 1988) suggest that NGF has a continuing influence on the physiological homeostasis of adult differentiated neurons and that anti-NGF treatment or NGF deprivation activates second messenger pathways that result in viral reactivation. In an *in vitro* neuronal cell culture study by Smith *et al* (1992), the protein kinase inhibitor 2-aminopurine was found to inhibit reactivation induced by treatment with anti-NGF antibodies or a cyclic AMP (cAMP) analog, but not reactivation induced by phorbol myristate acetate. This would suggest the existence of at least two pathways of HSV reactivation, and that NGF deprivation could act as a neuronal stressor.

In conclusion, the results of this study suggest that the correlation between HSV induction and NGF deprivation seen *in vitro* is valid in an *in vivo* model. NGF deprivation could indeed fall into the same category as stress-related HSV induction since they appear to share dependence on cAMP-mediated second messenger pathways.

Materials and methods

Viral strain
HSV-1 strain McKrae was propagated in primary rabbit kidney (PRK) cells and titered by plaque assay on green monkey kidney cell (CV-1) monolayers.

Rabbits and viral inoculation
The unscarified corneas of New Zealand White rabbits (3–4 kg) were inoculated with 25 μl of a suspension of McKrae strain HSV-1 ($1-2\times10^5$ plaque-forming units). Primary corneal infection was verified by slit lamp biomicroscopic examination (SLE) on days 5–7 after infection. The use of rabbits in these experiments conformed to the ARVO Resolution on the Use of Animals in Research.

Tear-film swabs
Tears were collected by rotation of sterile Dacron-tipped swabs in the upper *cul-de-sac*, across the corneal epithelium, and into the lower *cul-de-sac*, where the swab was allowed to absorb tears. The swabs were placed in tubes containing PRK monolayers and incubated for 18–24 h at 37°C. Subsequently, the swabs were removed, and Eagle's minimum essential medium with 2% fetal bovine serum was added. The tubes were monitored daily for 11–14 days for the appearance of cytopathic effect indicative of HSV-1 infection.

Spontaneous viral shedding
Ocular swabs were performed beginning on post-inoculation (P.I.) day 20. Culturing of the eye was stopped when infectious virus was detected. Slit-lamp examination was performed on P.I. day 19 before initiating swabbing to determine that no active lesion was present. Both eyes of all rabbits in all groups shed virus once during post-inoculation days 20–40, confirming the establishment of latency. The majority of positive viral cultures occurred between P.I. days 20–30. No eyes were shedding when the intravenous injections began.

Antibodies to mouse nerve growth factor
NGF was purified from mouse submandibular salivary glands (Bocchini *et al*, 1969), emulsified in Freund's adjuvant, and injected intradermally into a goat. Injections consisting of 1.0 mg NGF in adjuvant were given at 3-week intervals. Anti-NGF titers in goat serum were determined at 3-week intervals. Titers are expressed as the reciprocal of the highest dilution that prevented neurite outgrowth from chick embryo dorsal root ganglia *in vitro* (Fenton, 1970). Anti-NGF titers in rabbit sera were also determined using the same neurite outgrowth assay.

In situ *hybridization*
Excised ganglia taken on selected days after infection were fixed in 4% paraformaldehyde for 18 to 24 h at 4°C and processed by procedures described by Stroop *et al* (1984). Ganglia were taken 1–2 days after the experiments ended. The P.I. days of sacrifice ranged from 54 to 58. Tissues were paraffin embedded, and 7 μm-thick sections were cut. PCR was used to synthesize a [^{3}H]DNA probe for use in the *in situ* hybridization by incorporating tritiated dNTPs into the reaction mixtures (Hill *et al*, 1996a). The primer pair sequence for the LAT probe

and details of the methods have been reported (Hill *et al*, 1996a). The probe solution (35 μl per section) was applied, and slides were heated to 94°C for 5 min and then incubated overnight at 44°C. Excess probe was removed by washing, and autoradiography was performed with Kodak NTB-2 nuclear emulsion (Biotech International, Inc., New Haven, CT). After 10 days of incubation at 4°C, the slides were developed in Dektol (Kodak Corp., Rochester, NY), and the sections were stained with Harris hematoxylin and eosin (Harleco Eosin Y, 1% Alcoholic Solution; EM Corp., Chestnut Hill, MA).

Calculation of the percentage of LAT$^+$ neurons
Masked observers counted the number of LAT$^+$ neurons and the total number of neurons. The ophthalamic region was the primary region counted; however, all regions appeared to contain approximately the same proportion of positive neurons. Neurons were distinguished by the size of their cell body (Konigsmark, 1970). Only LAT$^+$ neurons with a visible nucleus were counted. The number of neurons counted ranged from 400 to 1200 per ganglia.

Quantitation of HSV DNA by PCR
DNA was extracted from homogenized ganglia by a standard procedure (Hill *et al*, 1996a). The procedures of Coen (1990, 1994) as modified by Hill *et al* (1996a,b) were employed for the quantitation of rabbit alpha actin (124 bp) and HSV-1 ribonucleotide reductase (243 bp). These sequences were coamplified from each DNA sample to quantitate the HSV DNA copy number per neuronal cells. The primer pair sequences and the details of the PCR have been given previously (Hill *et al*, 1996a,b,d). The PCR products were analysed by dot blotting and hybridization with ^{32}P-labeled probes. Quantitation was performed densitometrically with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Experimental design
Experiment 1 Rabbits harboring latent HSV-1 were randomly assigned to either the experimental (anti-NGF) group or the control group. Six rabbits received intravenous injections of 10 ml of pooled immune (anti-NGF) goat serum with titers between 1 : 20 000 and 1 : 30 000 every other day for 10 days beginning on day 42 after HSV infection. Each injection was given slowly for 20 – 25 min. The control group (n=6) received nonimmune goat serum by the same route on the same schedule. Eye swabs were collected from all eyes every day for 12 consecutive days. Blood was taken on days 5, 10 and 12.

Experiment 2 Rabbits harboring latent HSV-1 were randomly assigned to either an experimental (anti-NGF) group or a control group. The experimental groups (n=3 and n=5, done separately) received intravenous injections of 10 ml of pooled immune (anti-NGF) goat serum with titers between 1 : 80 000 and 1 : 100 000 daily for 10 consecutive days beginning on day 42 after HSV infection. The control groups received either nonimmune goat serum (n=5) or phosphate buffered saline (PBS) (n=3) by the same route on the same schedule. Eye swabs were collected from all eyes every day for 12 days. Blood was taken on days 5, 10 and 12.

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