



Rescue of HSV-1 neurovirulence is associated with induction of brain interleukin-1 expression, prostaglandin synthesis and neuroendocrine responses

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Several HSV-1 neurovirulence genes have been mapped but the mechanisms by which they affect host-virus interactions are not known. We have previously mapped HSV-1 neurovirulence to the U_L53 gene region of the viral DNA by transfer of this gene from the neurovirulent R-19 strain to the non-neurovirulent R-15 strain in the generation of the p-71 recombinant, in which neurovirulence was rescued. In the present study we inoculated these strains into the paraventricular nucleus (PVN) of the hypothalamus of rats. We examined: (1) Clinical course of encephalitis. (2) Hypothalamic-pituitary-adrenocortical (HPA) axis function. (3) Brain cytokine gene mRNA expression and prostaglandin E₂ (PGE₂) production. (4) The relation of these parameters to viral replication and to cellular inflammation. In R-15 infected rats no signs of disease were observed. There was a temporary inflammatory reaction and IL-1 β transcription in the PVN area. The function of the HPA axis was similar to control rats. Only slight increase in brain PGE₂ production was found. In R-19 and p-71 infected rats, overt clinical signs of encephalitis and cellular inflammation in the PVN area were observed within 3 days post-infection (p.i.). All rats died between 4–7 days p.i. These strains induced IL-1 β transcription in the hypothalamus as well as in extra-hypothalamic brain regions in which no cellular inflammation was found. Basal serum ACTH and CS were markedly elevated and hypothalamic CRF-41 content was significantly reduced as compared to R-15 infected rats. Both strains markedly increased brain PGE₂ production. HSV-1 brain titers at 3 days p.i. were 100-fold lower than the inoculum titer although clinical signs of encephalitis were prominent. The results suggest that rescue of HSV-1 neurovirulence by the U_L53 gene region of the viral genome is associated with enhancement of viral-induced brain IL-1 β gene expression, increased brain PGE₂ synthesis and hypersecretion of HPA axis hormones. Viral-induced brain derived cytokines and prostaglandins may contribute to the clinical syndrome of acute herpetic encephalitis in particular at early stages of the disease when virus load in the brain is low and cellular infiltrates are not widespread.

Keywords: HSV-1; neurovirulence; U_L53 gene; interleukin 1; prostaglandins; adrenocortical axis

Introduction

The pathogenesis of herpes simplex virus type 1 (HSV-1) infection is dependent on viral as well as host factors. Several viral DNA sequences have been shown to control HSV-1 virulence and disease pattern (Field and Wildy, 1978; Thompson *et al*, 1983, 1985; Rosen *et al*, 1985; Becker *et al*, 1986; Field and Coen, 1986; Ben-Hur *et al*, 1987, 1988;

Day *et al*, 1988; Javier *et al*, 1988; Meignier *et al*, 1988; Chou *et al*, 1990; Izumi *et al*, 1990). We have previously mapped HSV-1 neurovirulence to the viral U_L53 gene region (Ben-Hur *et al*, 1987). This was done by co-transfection into cells of unit length viral DNA of the non-neurovirulent R-15 strain together with the U_L53 gene that was cloned from the closely related neurovirulent R-19 strain and selection of new neurovirulent recombinants (the p-71 strain) in the mouse brain (Figure 1). The U_L53 gene encodes a cell fusion protein that affects

plaque morphology and size *in vitro* (Ruyechan *et al*, 1979; Bond and Person, 1984; Pogue-Geile *et al*, 1984; Hutchinson *et al*, 1992). In the non-neurovirulent R-15 strain the U_L53 mRNA is expressed normally but it contains several point mutations (Moyal *et al*, 1992). Its protein product may be less active than in neurovirulent strains since R-15 has very low cell fusion activity, as seen by the tiny pinpoint plaques it produces in cell cultures. Indeed, a larger plaque size was rescued by the R-19 U_L53 gene together with a neurovirulent phenotype (Ben-Hur *et al*, 1987). There is no information on how the neurovirulence function mapped in the U_L53 gene region affects virus-host

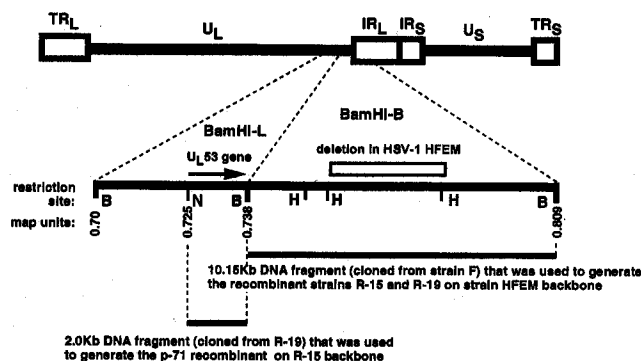


Figure 1 Physical map of the HSV-1 DNA with the positions of the DNA fragments BamHI-B, BamHI-L and its 2.0 Kb NruI-BamHI subfragment that contains the U_L53 gene. HSV-1 strain HFEM contains a 3.55 Kb deletion that resembles closely a HpaI fragment that is within the BamHI-B fragment. The R-15 and R-19 strains were generated by cotransfection of unit length HFEM DNA with the BamHI fragment B cloned from HSV-1 strain F (Rosen *et al*, 1985). In both recombinant strains, the BamHI-B region was restored to its full length as in strain F. However, R-15 was found to be completely avirulent when inoculated directly into the brain, while R-19 retained neurovirulence. The p-71 strain was generated by cotransfection of unit length R-15 DNA with the 2.0 Kb NruI-BamHI subfragment bordering the BamHI-B region (Ben-Hur *et al*, 1987). R-15 and R-19 are similar in size and restriction enzyme mapping of the BamHI-L region, but they differ in plaque size and in neurovirulence probably due to point mutations found in R-15 U_L53 gene. U_L, U_S, T_R_L, I_R_L, T_R_S and I_R_S indicate the unique long and short sequences and their terminal and internal repeat sequences in the HSV-1 genome. B: BamHI site, H: HpaI, N: NruI.

interactions and brain responses to infection *in vivo*. It may be expected that transfer of neurovirulence would be associated with viral replication to higher titers (Roizman and Kaplan, 1992). In addition, host brain responses to infection, which by themselves can affect clinical signs, may be altered by the viral neurovirulence function as well.

Activation of the HPA axis is one of the major responses to stressful stimuli, including infectious diseases. This axis consists of neurons containing corticotrophin releasing factor-41 (CRF-41) in the paraventricular nucleus (PVN) of the hypothalamus. These neurons send axons to the median eminence (ME), where CRF-41, the major secretagogue of ACTH, is released (Antoni, 1986; Whitnall, 1993). HPA axis responses to various stimuli are dependent on neural inputs mediated by central neurotransmitters, mainly by noradrenergic and serotonergic pathways (Plotsky *et al*, 1989; Fuller, 1992) and by the negative feedback exerted by glucocorticoids (Dallman *et al*, 1987; Weidenfeld and Feldman, 1993; Whitnall, 1993). Recently, we found (Ben-Hur *et al*, 1995) that corneal inoculation with HSV-1 led to an acute infection of the brainstem that caused significant changes in the function of the HPA axis in rats. Basal ACTH and corticosterone (CS) levels were markedly elevated and neural stressful stimulation failed to further increase the levels of these hormones. These effects were found without detectable viral replication in the hypothalamus.

Various stimuli such as ischemia, trauma and infection have been shown to induce cytokine gene expression and synthesis in the brain, and especially interleukin-1 (IL-1) (Waage *et al*, 1989; Ban *et al*, 1992; Yan *et al*, 1992). Cytokines are not only produced by activated immune cells that penetrate the injured brain, but are produced by brain cells as well (Liebermann *et al*, 1989; Giulian and Corpuz, 1993; Hopkins and Rothwell, 1995). There is emerging evidence that cytokines do not serve solely as messengers of the immune system (Schobitz *et al*, 1994; Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995). They also have important behavioral effects and cause pathological changes in the brain.

In the present study we examined several brain responses to HSV-1 intracerebral inoculation. We

Table 1 Clinical and virological follow-up after HSV-1 innoculation into the PVN

Virus strain	Clinical state (3 days p.i.)	Survival	Hyp	Isolation of virus from brain tissues*		F Cx
				Pons	Hipp	
R-15	asymptomatic	6/6 (100%)	2/4 (1 × 10 ²)	0/4	0/4	0/4
R-19	sick	0/6 (0%)	4/4 (1.5 × 10 ²)	1/4 (1 × 10 ²)	2/4 (1 × 10 ²)	2/4 (1 × 10 ²)
p-71	sick	0/6 (0%)	3/4 (1 × 10 ²)	4/4 (3 × 10 ²)	1/4 (2 × 10 ²)	2/4 (1 × 10 ²)

*number of positive tissues/rats (titer in mean pfu/tissue). Hyp: hypothalamus; Hipp: hippocampus; F Cx: frontal cortex

chose the hypothalamic paraventricular nucleus (PVN) as the site of inoculation because the PVN is part of the limbic system, and HSV-1 is neurotropic with a predilection to this system. In addition, this specific site of infection enabled us to study the direct effects of HSV-1 on secretory neurons responsible for the activation of pituitary-adrenocortical function.

We examined the following brain responses to infection:

- 1 Adrenocortical axis responses to HSV-1 infection were examined and under basal conditions and in response to stressful stimuli. The sensitivity of HPA axis responses to the negative feedback effect of exogenous GC was also investigated.
- 2 The effect of viral infection on cytokine gene expression was determined by hybridization of cytokine cDNA probes to mRNA isolated from various brain regions.
- 3 In addition, prostaglandin production in different brain regions was tested.

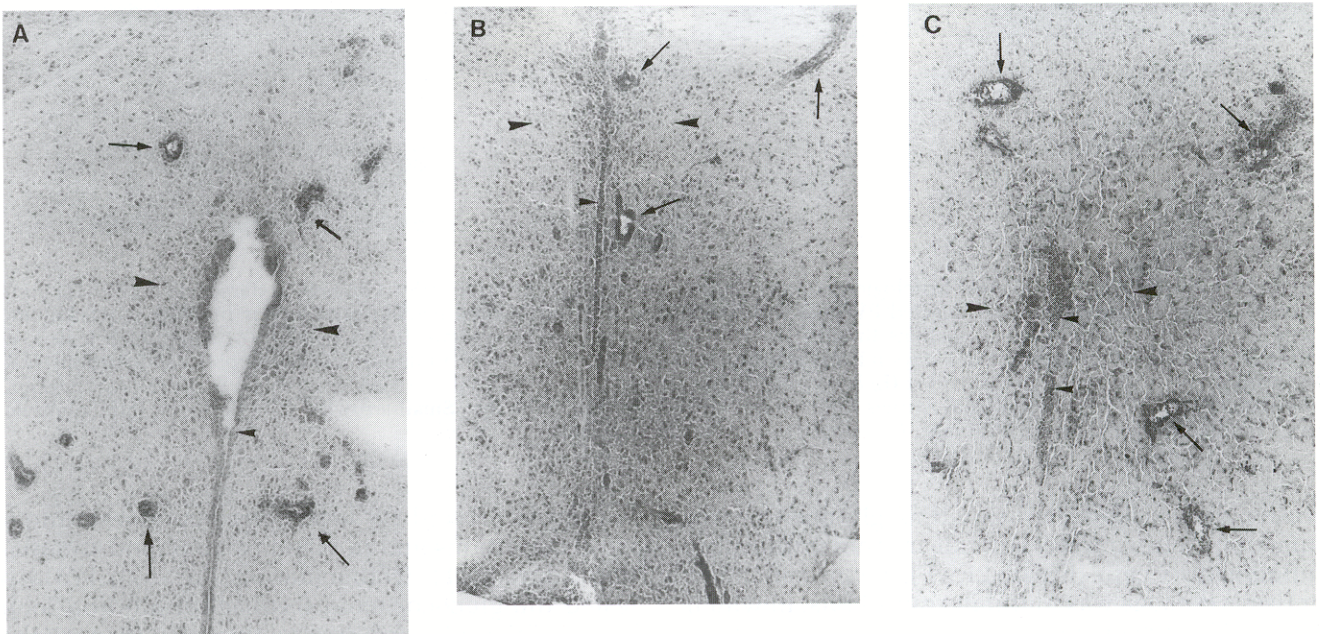
We compared the effects of the three closely related recombinant HSV-1 strains R-15, R-19 and p-71 (Figure 1) to determine the association of their clinical neurovirulent properties with the various tested brain responses and with the degree of viral replication in the brain.

Results

Rats were injected stereotactically with 5×10^4 pfu/rat of various HSV-1 strains into the PVN and then followed clinically for at least a week (Table 1).

This dose caused a reproducible disease in the time span of 1 week. All rats that were infected with the apathogenic strain R-15 survived the infection without any clinical signs of disease. All rats that were infected with the pathogenic strains R-19 and p-71 developed signs of encephalitis and died by the end of the first week postinfection (p.i.). By the third day p.i., all R-19 and p-71 infected animals showed severe irritability and cachexia. The rats lost 30% of their pre-infection body weight. Later, they developed continuous seizure like activity and died. The infectious virus was isolated from the rat brains at 3 days p.i. (Table 1). Strain R-15 was found in the hypothalamus of some rats in low titers (10^2 pfu/tissue), but not in other brain regions. The pathogenic R-19 and p-71 were not restricted to the injection site, and spread to other brain regions, such as the frontal cortex, pons and hippocampus. Their brain titers were $4-7 \times 10^2$ pfu/brain, which is a little higher than R-15 titer, but still very low and a hundred-fold lower than the inoculum titer.

At 3 days p.i., all HSV-1 strains (including the apathogenic strain R-15) induced a significant perivascular inflammatory response in the hypothalamus and particularly in the PVN area, as shown by hematoxylin-eosin stained sections (Figure 2A–C). However, within 7 days, p.i., the hypothalamic inflammation which was seen in R-15 infected brains decreased and normal cytoarchitecture was restored in the PVN (Figure 2D). More edema and necrosis at 3 days p.i. was seen in R-19 and p-71 infected brains as compared to R-15. However, brain areas distant from the inoculation site (frontal



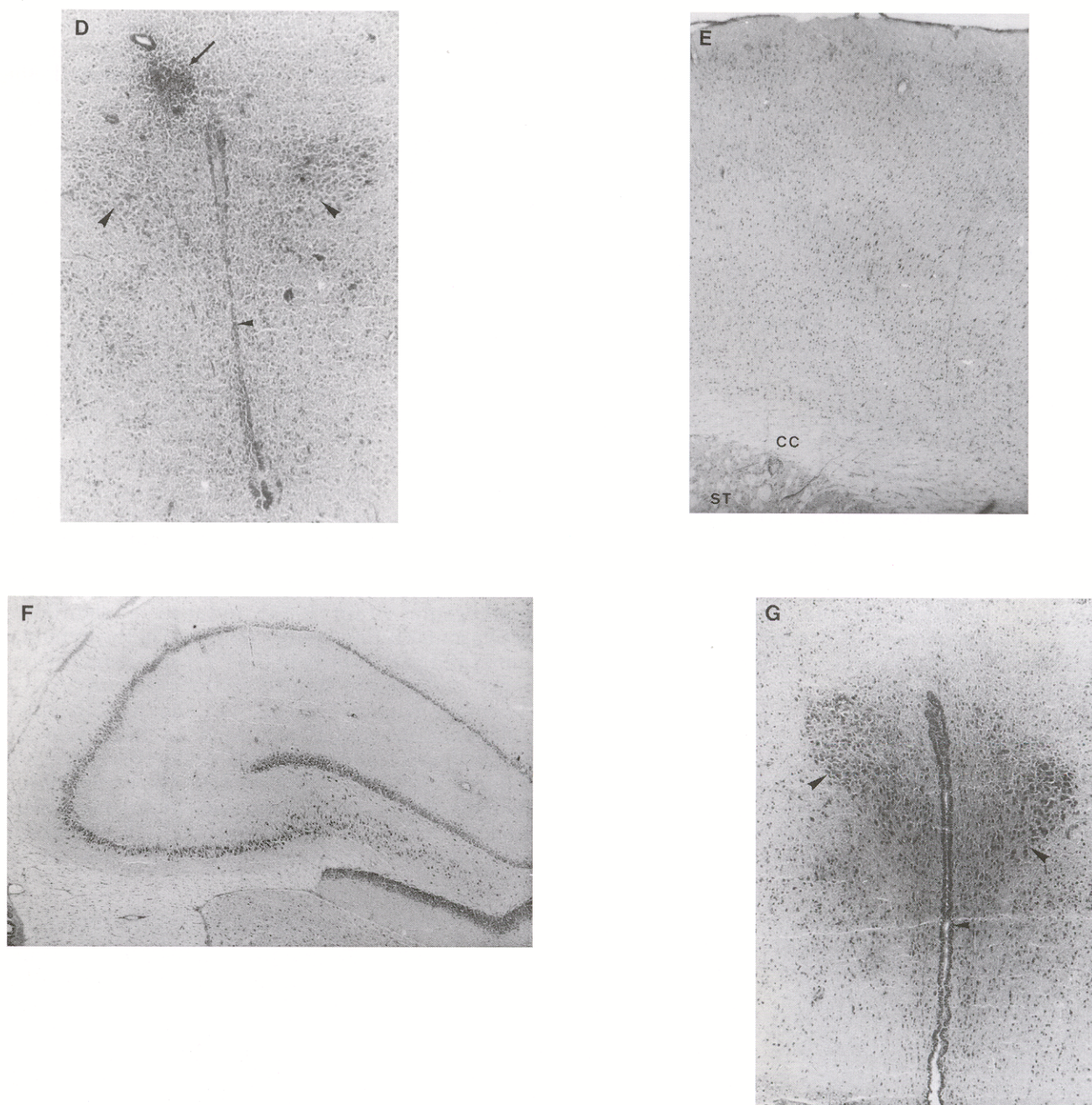


Figure 2 Hematoxylin-eosin stained histological sections of brains infected with various HSV-1 strains by stereotaxic inoculation into the PVN of the hypothalamus. All infected PVNs looked distorted at 3 days p.i., with marked perivascular infiltration, edema, and some necrosis. This was found in sections through the PVN in rats infected with strain R-15 (A), R-19 (B) and p-71 (C). In R-15 infected rats the PVN re-organized at 7 days p.i. and showed just residual inflammatory infiltrates (D). Extrahypothalamic regions of R-19 infected rats, such as the frontal cortex (E) and dorsal hippocampus (F) were normal at 3 days p.i. Vehicle (Vero cells suspension) inoculated PVNs (G) were normal too. Arrows indicate perivascular cellular infiltrates; Small arrowheads indicate the third ventricle; Large arrowheads indicate the PVN area; CC: corpus callosum; ST: striatum.

cortex and hippocampus), although positive for infectious virus, were still clear from infiltrating inflammatory cells (Figure 2E–F). No inflammation or tissue damage was found in control rats, in which Vero cells suspension was injected into the PVN (Figure 2G).

Serum ACTH and CS levels were measured under basal conditions and in response to photic stimulation 3 days post PVN inoculation (Figure 3). We also tested the glucocorticoid feedback inhibition loop by administration of dexamethasone prior to the stress. In control rats, photic stimulation induced a

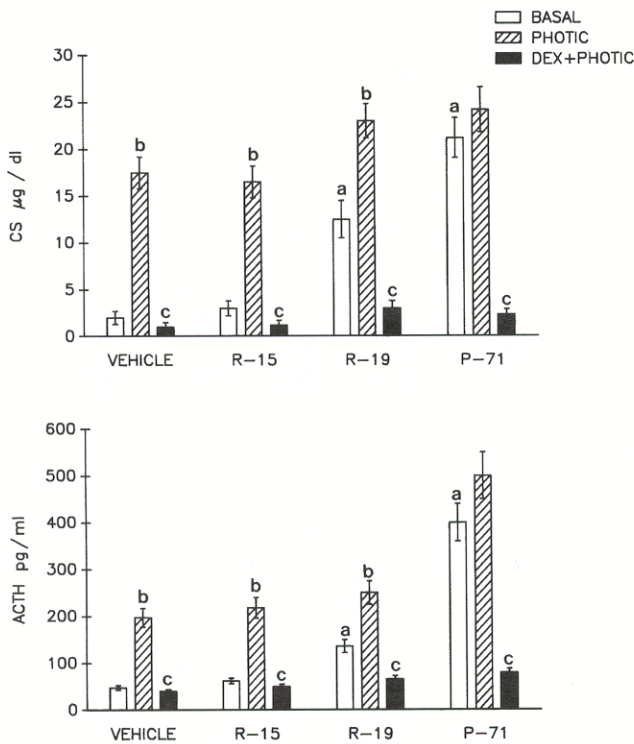


Figure 3 Serum levels of ACTH and CS 3 days post PVN inoculation with various HSV-1 strains or vehicle. Hormones were measured under basal conditions and in response to photic stimulation with and without dexamethasone (dex) pretreatment. The values represent the mean \pm s.e.m. of 6–8 animals. (a) $P < 0.05$ as compared to basal levels in vehicle or R-15 inoculated rats. (b) $P < 0.05$ as compared to the respective basal levels. (c) $P < 0.05$ as compared to the respective hormone levels following photic stimulation.

Table 2 CRF-41 content in the median eminence (ME) of rats inoculated with HSV-1 recombinant strains into the PVN

HSV-1 strain	CRF-41 pg/ME
control	3850 \pm 120
R-15	3900 \pm 150
R-19	1500 \pm 45*
p-71	470 \pm 25*

* $P < 0.05$ as compared to control or R-15 inoculated rats

significant rise in serum ACTH and CS. This effect was completely blocked by pretreatment with dexamethasone. Basal ACTH and CS were significantly elevated in rats that were infected in the PVN with the pathogenic R-19 strain. Exposure to photic stress induced further rise in the levels of these hormones and dexamethasone completely suppressed them to basal levels similar to control rats. Basal ACTH and CS levels were even higher in rats infected with HSV-1 p-71 than in R-19 infected animals. These hormone levels could not be further

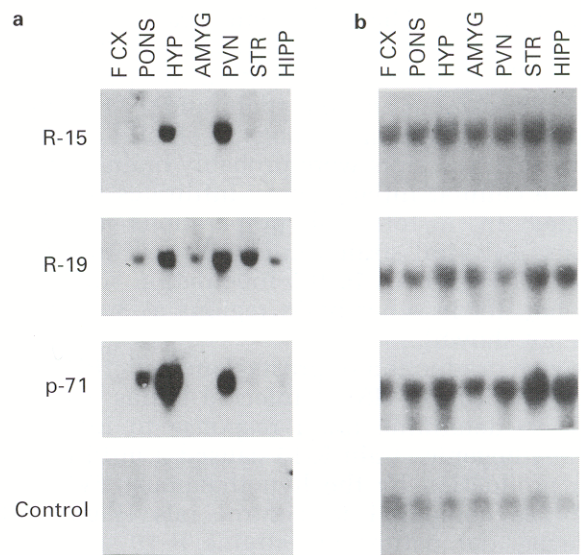


Figure 4 Autoradiogram of Northern blot hybridization analysis of total RNA (20 μ g/lane) isolated from various brain regions of groups of rats infected with various HSV-1 strains or vehicle (five rats per group) with IL-1 β cDNA probe. Filters were also hybridized with β -Actin cDNA probe to assure that comparable amounts of RNA were loaded (data not shown). FCX: frontal cortex, HYP: hypothalamus, AMYG: amygdala, STR: striatum, HIPP: dorsal hippocampus.

elevated by photic stress and dexamethasone completely blocked their levels. In contrast to the pathogenic strains, the HSV-1 R-15 strain caused only a slight elevation in basal serum ACTH and CS levels. Also, the responses to photic stimulation and dexamethasone were similar to control rats (Figure 3). In addition, it was of interest to examine whether the neuroendocrine effects of HSV-1 are specific to the PVN. Therefore, the virulent strain R-19 was inoculated into the frontal cortex. These rats were still clinically asymptomatic at 3 days p.i. and HPA axis responses at this time were similar to control rats (data not shown). However, these rats developed lethal encephalitis within the next week.

Table 2 shows the ME CRF-41 content measured 4 days after inoculation of HSV-1 strains into the PVN. It may be seen that while the pathogenic R-19 and p-71 strains caused a marked depletion in CRF-41, the apathogenic strain R-15 did not affect CRF-41 content when compared to control rats. CRF-41 depletion was greater with strain p-71 than with strain R-19 infection. This finding correlates with the higher basal ACTH and CS serum levels in p-71 infected than in R-19 infected rats.

Cytokine gene mRNA transcript expression in various brain regions at 3 days after PVN inoculation was examined to correlate them with the clinical effects of HSV-1 encephalitis (Figure 4). IL-1 β and TNF α mRNA transcription was not detected in any tested brain area of control rats. R-15 induced IL-1 β transcription only in the hypota-

lamus and PVN. The pathogenic strains induced a stronger response in these regions. In addition, R-19 induced IL-1 β gene expression in the pons, striatum, hippocampus and amygdala, and p-71 induced IL-1 β transcription in the pons. It should be noted that these transcripts were probably brain derived, because cellular inflammatory infiltrates were not detected in histological preparations of these regions. All viral strains induced weak transcription of TNF α mRNA in the inoculation site of the PVN but not in other brain regions at 3 days p.i. (data not shown).

Ex vivo production of prostaglandin E₂ (PGE₂) by various regions of HSV-1 infected brains was also tested to determine brain response to infection. The apathogenic strain R-15 caused a mild rise in PGE₂ production by the hypothalamus, PVN and cortex as compared to control rats (Figure 5). However, the pathogenic strains R-19 and p-71 induced marked increase (ranging from 2- to 10-fold rise) in PGE₂ production by the various brain regions tested. The increase in PGE₂ production was most pronounced in the frontal cortex and hippocampus although these regions still lacked inflammatory infiltrates.

Discussion

In the present study we examined several brain responses during experimental HSV-1 encephalitis including the activation of the HPA axis, induction of cytokine gene expression and prostaglandin E₂ synthesis. The use of closely related recombinant viruses enabled us to examine whether the degree of HSV-1 replication in the brain and the induction of neuroendocrine and inflammatory brain responses to infection are dependent on the viral neurovirulence function mapped in the UL53 gene region.

The apathogenic HSV-1 R-15 strain could only be isolated from the hypothalamus, and not from other brain regions. This strain caused an inflammatory response around the site of injection, induced IL-1 β gene expression in this region but did not cause fever or clinical signs of encephalitis. Also, R-15 induced only very slight elevation in basal ACTH and CS levels and in PGE₂ synthesis. In contrast, the pathogenic R-19 and p-71 strains caused lethal encephalitis. At 3 days p.i., the invading inflammatory response was still restricted to the site of inoculation, even though viruses spread to other brain regions. These strains induced marked increase in basal ACTH and CS levels and depleted hypothalamic CRF. In addition, these pathogenic strains induced IL-1 β gene expression and PGE₂ synthesis in extrahypothalamic brain areas. Therefore, rescue of neurovirulence by the UL53 gene region is associated with enhanced viral spread in the brain and mildly increased virus titer as well as

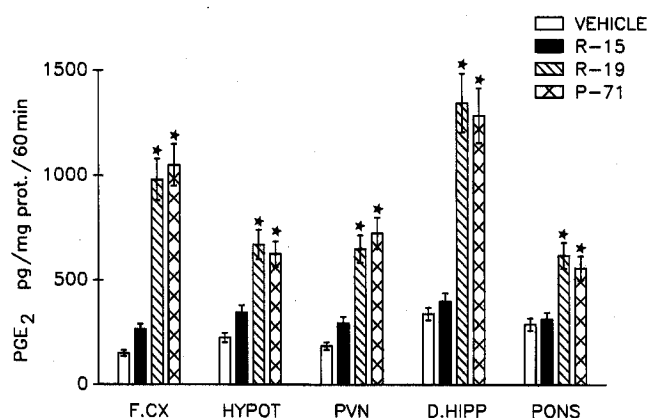


Figure 5 *Ex vivo* production of PGE₂ by slices from different brain regions of rats, 3 days post PVN-inoculation with various HSV-1 strains. Values represent the mean \pm s.e.m. of 8–12 tissue slices taken from four rats per each experimental group. * $P < 0.05$ as compared to the respective brain region in vehicle inoculated rats. F.CX - frontal cortex; Hypot - hypothalamus; PVN - paraventricular nucleus; D.HIPP - dorsal hippocampus.

with enhancement of several brain responses to infection, namely the induction of brain IL-1 gene expression, PGE₂ synthesis and HPA axis hormones. The mildly elevated virus titer and its spread in the brain may be responsible for the neurovirulent properties of the R-19 and p-71 strains. It remains to be shown whether activation of cytokines genes, prostaglandins and neuroendocrine responses are due to the increase in virus replication or whether the UL53 gene product is directly involved in neurotoxicity and in activation of these inflammatory mediators. It should also be pointed out that HSV-1 R-19 and p-71 titers in the brain were consistently very low. These titers were $4-7 \times 10^2$ pfu/brain, which is 100-fold lower than the inoculum titer and within the same order as for the apathogenic R-15 titer in the brain at this stage. The findings of low neurovirulent virus load in the brain and absence of widespread cellular infiltrates suggest that clinical disease may result not only from destruction of brain cells by lytic viral replication, but also from additional mediators that are produced due to virus - host brain interaction. The notion that severity of HSV-1 encephalitis may be relatively independent of viral replication has been suggested (Javier *et al*, 1988) in another experimental system in which an HSV-1 neurovirulence function was mapped in a different region of the viral genome and was found to be dissociated from high titer viral replication.

Viral induced IL-1 β gene expression and PGE₂ synthesis occurred in the hypothalamus as well as in extrahypothalamic brain regions, which are distant from the site of HSV-1 inoculation. The enhanced hypothalamic IL-1 β gene expression and PGE₂ synthesis may be due to the presence of local

cellular mononuclear infiltrates as shown in Figure 1A–C. On the other hand, the induction of these inflammatory mediators in extrahypothalamic brain regions that were still lacking cellular mononuclear infiltrates suggests that they are derived from brain cells. While R-19 induced IL-1 β transcription in the pons, striatum, hippocampus and amygdala, the effect of pathogenic p-71 strain was detected only in the pons. This differential effect may be due to the enhanced CS responses in p-71 infected rats as compared to R-19. It is possible that the markedly elevated CS levels induced by p-71 infection inhibited IL-1 β expression in the hippocampus, striatum and amygdala. This is supported by previous studies showing that glucocorticoids suppress IL-1 expression in mononuclear cells and in the brain (Knudsen *et al*, 1987; Lew *et al*, 1988; Schobitz *et al*, 1994).

It is well established that IL-1 and prostaglandins cause fever and major behavioral modifications, termed collectively as sickness behavior. This includes somnolence, cachexia and decreased locomotor and exploratory activity. In addition, injection of IL-1 into the hippocampus can elicit epileptogenic EEG activity (Kapas *et al*, 1994). It has been also suggested that brain derived IL-1 participates in neurodegeneration *in vivo*, and this effect can be blocked by the IL-1 receptor antagonist (Rothwell and Relton, 1993). Therefore, it is possible that clinical signs of acute HSV-1 encephalitis may be due, at least in part, to viral induced hyperproduction of IL-1 and prostaglandins in the brain. The very slight TNF α gene expression, found only in the PVN, suggests that this cytokine does not play an important role in the early stages of HSV-1 encephalitis.

The present study demonstrates that rescue of neurovirulence was also associated with activation of the HPA axis, as manifested by depletion of CRF-41 from the ME and hypersecretion of ACTH and CS. Our results indicate that CRF-41 is directly involved in mediating the ACTH response to HSV-1 infection because depletion of CRF-41 from the ME apparently reflects its release into the hypothalamic pituitary portal system. It appears that the effects of the p-71 recombinant on the HPA axis were more pronounced compared to those of the neurovirulent R-19 parental strain. This is evident from the finding that p-71 caused a marked depletion (8 fold *versus* 2.5 fold caused by R-19) of CRF-41 from the ME and subsequently higher secretion of ACTH and CS under basal conditions. Although basal ACTH and CS were higher by 3- and 6-fold respectively in R-19 infected rats compared to controls, these rats could respond to a neurogenic stressful stimulus by a further rise in the serum level of these hormones. This suggests that PVN neurons, which are responsible for the activation of the HPA axis are still functioning at this stage

after HSV-1 inoculation into the PVN. The lack of ACTH and CS responses to photic stimulation in p-71 infected animals is probably due to the already maximal basal levels of these hormones, so that a further stress-induced rise could not be achieved. Our results also show that pretreatment with dexamethasone completely abolished HSV-1 induced HPA axis activation, indicating that the glucocorticoid negative feedback mechanism is still intact on the first 3 days of HSV-1 encephalitis. It should be noted that the avirulent R-15 strain did not activate the HPA axis, although its titer in the hypothalamus and the degree of the local cellular inflammation were similar to those of the virulent R-19 and p-71 strains. This further indicates that activation of the HPA axis was not due to the inflammatory response or lytic viral replication but was dependent on the viral neurovirulence function.

In conclusion, this study shows that genetic transfer of neurovirulence was associated with enhancement of viral-induced brain IL-1 β gene expression, prostaglandin synthesis and activation of the HPA axis. Although neurovirulent strains could spread in the brain and replicate a little better than the non-neurovirulent strain, their titers were consistently very low. It is suggested that brain derived cytokines and prostaglandins may contribute to the clinical syndrome of HSV-1 encephalitis, particularly during early stages of the disease, when virus load is low and cellular inflammation did not yet evolve.

Materials and methods

Viruses

Herpes simplex virus type 1 recombinant strains R-15, R-19 and p-71 were propagated on monkey kidney cells (Vero) to a titer of 10⁷ plaque forming units (pfu)/ml. R-15 and R-19 are recombinant viruses that were generated by co-transfection of unit length HSV-1 strain HFEM DNA with cloned BamHI fragment B (0.738–0.809 map units [mu] of the viral genome) from HSV-1 strain F in order to replace the deletion in the HFEM genome (0.762–0.789 mu) (Rosen *et al*, 1985). p-71 is a recombinant virus that was generated (Ben-Hur *et al*, 1987) by co-transfection of unit length R-15 DNA together with a 2.0 Kb NruI-BamHI fragment (derived from the right hand side of BamHI fragment L, 0.725–0.738 mu, and containing the U_L53 gene) cloned from strain R-19. This recombinant was isolated from an infected mouse brain. The molecular construction of HSV-1 recombinant strains R-15, R-19 and p-71 is shown in Figure 1).

Animals

The experiments were carried out on male rats of the Hebrew University strain, weighing approximately 200 g when infected. They were housed in

the animal room of our laboratory in groups of 5/cage, under artificial illumination between 6:00 and 18:00 h. Purina chow and water were available *ad libitum*. Ambient temperature was 22–23°C. Animals were transferred to individual cages 24 h prior to neuroendocrine experiments. All neuroendocrine experiments were performed between 7:00 and 11:00.

Inoculation of viruses

Animals were anesthetized with sodium-pentobarbital (35 mg/Kg) and were stereotactically bilaterally inoculated into the PVN with various HSV-1 strains (2×10^4 pfu to each side in a volume of 2 μ l). Stereotaxic coordinates with bregma as zero reference point (Pellegrino *et al*, 1967) were: anterior 0.0, lateral 0.3 (bilateral), height 7.5. Control animals were injected with uninfected Vero cells suspension.

Dissection of brain areas

Following decapitation, brains were rapidly removed and dissected on ice under sterile conditions for the various experiments. The borders of the brain regions taken, with bregma as zero reference point according to Pellegrino *et al* (1967) were (in mm): frontal cortex, anterior 6 to anterior 4; hypothalamus, anterior 0 to posterior 3.5; PVN, anterior 0.5 to posterior 0.5; striatum, anterior 2 to anterior 0.5; dorsal hippocampus, posterior 1.5 to posterior 3; amygdala, anterior 0.5 to posterior 2; pons, posterior 4 to posterior 7.

Isolation and titration of HSV-1 from brain

Following decapitation, various brain tissues were dissected out and frozen at –40°C until virus determination. Viral isolation and titration was done as previously described (Ben-Hur *et al*, 1983; Gordon *et al*, 1983) by dounce homogenization of tissues in phosphate-buffered-saline and seeding them on BSC-1 cell cultures. Presence of virus was determined by the appearance of a typical cytopathic effect (CPE). Positive tissues were then titrated by infecting BSC-1 monolayers with serial dilutions of the respective homogenate, covering the cell cultures with 1% agar, and counting gentian violet staining plaques that appeared after 3 days under the agar.

Determination of the HPA axis function

6–8 animals per experimental group were sacrificed either under basal condition or following photic stressful stimulation. 3.5 hours prior to stress exposure the rats were injected intraperitoneally with either vehicle or dexamethasone (5 μ g/100 g dissolved in 0.5 ml saline containing 1% ethanol). The animals were then exposed in a dark room to a photo-stimulator emitting flashes at the rate of 4/sec for 4 min. The animals were sacrificed 10 min after the onset of the stimulus. Upon sacrifice trunk

blood was collected in chilled tubes and sera stored at –40°C until assayed for ACTH and CS. For CRF-41 determination, brains from animals under basal conditions were removed and immediately placed on ice. The ME was uniformly excised under a binocular microscope and was placed in 0.5 ml ice cold 0.1 M HCl. The tissues were stored at –80°C until assayed for immunoreactive CRF-41 content. The content of immunoreactive CRF-41 in the ME was measured in tissue extracts by RIA using a specific anti-CRF-41 antiserum obtained from the Pasteur Institute (Paris) as previously described (Weidenfeld *et al*, 1989). Serum ACTH concentration was determined by RIA using materials from a kit obtained from CIS (France). CS was determined by RIA as previously described (Weidenfeld *et al*, 1989).

PGE₂ determination

The *ex-vivo* production of PGE₂ was determined in brain tissues obtained from HSV-1 or vehicle inoculated rats as previously described (Weidenfeld *et al*, 1992). The tissue slices (10–15 mg each) were placed in 1 ml of ice-cold oxygenated Krebs-Ringer buffer, pH 7.4. The supernatants were decanted and replaced by 1 ml of fresh buffer. After further oxygenation, the tubes were tightly closed and incubated at 37°C in a shaking water bath for 60 min. The supernatants were removed and stored at –70°C until assayed for PGE₂ content. The tissues were homogenized in 1 ml water and protein was determined. PGE₂ was determined by RIA as previously described (Weidenfeld *et al*, 1992).

Data analysis

All data (ACTH, CS, CRF-41, PGE₂) are presented as the mean \pm s.e.m. Statistical analysis of the data was performed using 2-way analysis of variance (ANOVA) followed by student-Neuman-Keuls test. Differences were considered significant at $P < 0.05$.

Cytokine genes mRNA detection

On day 3 p.i. the rats brains were removed and dissected under sterile conditions and immediately frozen in liquid nitrogen. Each brain region was pooled from five rats. Total cellular RNA was isolated using the guanidium-isothiocyanate/cesium chloride method (Chirgwin *et al*, 1979). 20 μ g of RNA per sample were electrophoresed in 1.5% agarose gels and transferred to nylon filters (Biotrans) either by the Northern blot method (Thomas, 1980) or with a vacuum blotter (Biorad, 90 min blot, 5 psi). Hybridization was performed with nick translated DNA probes (BRL kit) at 42°C for 16 h. The filters were washed by stepwise lowering of salt concentration from $2 \times$ SSC to $0.5 \times$ SSC and raising the temperature from room temperature to 60°C and were then

exposed to X-ray film. Size of RNA transcripts was determined according to RNA markers (BRL RNA ladder). The amounts of RNA blotted in each lane were compared by hybridization to a DNA probe of the constitutive gene β -Actin. Murine IL-1 β , TNF- α and β -Actin cDNAs (inserted in pBR322 vectors) were a gift from Prof. Yechiel Becker, Department of Molecular Virology, The Hebrew University Medical School. The inserts were cut out and isolated after electrophoresis in low melting agarose.

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