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Effects of Interferon-γ Gene Therapy in the Murine Central Nervous System and Concentrations in Cerebrospinal Fluid after Intrathecal or Intracerebral Administration

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Abstract: Systemically administered murine interferon-γ (IFN-γ) has a modest therapeutic effect in a murine model of cryptococcal meningo-encephalitis, and markedly potentiates conventional chemotherapy. Viral vector-mediated gene transfer is a new potential strategy for delivery of therapy to the central nervous system (CNS). To investigate whether levels of IFN-γ higher than those with systemic administration can be achieved in the CNS with intra-CNS therapy, we studied IFN-γ levels in CSF after gene therapy in normal mice with a non-replicating adenovirus vector containing the murine IFN-γ gene, comparing two routes of vector administration. Two doses, $10^{7.3}$ or $10^{8.3}$ virus/mouse given in 50 μL were compared. Mean (n = 5/group) IFN-γ levels in the CSF (by ELISA) after diluent, $10^{7.3}$ or $10^{8.3}$ viruses given intrathecally (IT) were 0, 13510, >30000 pg ml^{-1} on day 5, and 320, 5190, 20740 pg ml^{-1} on day 10, respectively. In contrast, after intracerebral (IC) injection, levels were 0, 700, 835 pg ml^{-1} on day 5, and 10, 160 and 2160 pg ml^{-1} on day 10. Serum IFN-γ levels were lower: 17, 168, 1139 pg ml^{-1} on day 5 and 65, 159, 305 pg ml^{-1} on day 10 after IT, but were 166, 700, 4375 and 141, 46, 138 pg ml^{-1} on days 5 and 10, respectively after IC administration of virus. CSF leukocytosis was noted on day 5, only after virus; 1250 and 1375 WBC mm^{-3} after $10^{7.3}$ and $10^{8.3}$ virus given IT, and 0 and 417 after IC administration of virus; all had cleared by day 10. Mice given $10^{8.3}$ virus became hypoactive by day 3, with some having ruffled fur and ataxia, especially after IT; after $10^{8.3}$ virus given IC signs were less severe. Few given $10^{7.3}$ virus exhibited clinical signs. By day 10, animals in IC group had recovered and those in the IT group had improved. Histopathology showed meningitis with mononuclear infiltration only after virus by either route. This was more consistent with $10^{8.3}$ virus, and was more prevalent on day 10. Thus, we show very high IFN-γ concentrations in the CSF, which were greater after IT administration of vector than after IC administration (where presumably parenchymal levels are higher), using gene therapy. This was complicated however, by transient meningitis with the high virus dose. The route that might give therapeutic benefit against meningoencephalitis with a pathogen needs additional study.

Key words: Gene therapy, interferon-γ, central nervous system

INTRODUCTION

Over the last several years there have been rapid advances in the area of replacement of genes in situ as a therapeutic method for the treatment of various diseases. Several viral vectors, such as retroviral, adenoviral and lentiviral constructs have been used[1,2]. In addition, other mechanisms of delivery of the desired gene, e.g., with liposomal carriers, have been used[1]. This exciting new approach to therapy also raises the potential of site-specific immunomodulatory therapy using cytokines for the treatment of refractory infectious or inflammatory diseases.

Gene therapy to the central nervous system (CNS) is of particular interest. Decay of transgene expression, primarily due to an induced adaptive immune response to the adenoviral proteins, has been a problem with adenoviral vectors in other tissues, yet in

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immunodeficient animals persistence of transgene expression has been noted[3]. The lack of classical antigen presenting dendritic cells and diminished lymphatic clearance in the parenchyma of the brain might be considered in some ways similar to the tissues of an immunodeficient animal, and thus an adaptive response to the adenoviral antigens cannot be mounted in this compartment. Induction of an adaptive immune response to adenoviral antigens and subsequent CNS inflammation has been shown to occur via lymphatic drainage of the meninges and choroid plexus, when the vector is injected into the ventricular space or spinal fluid[4]. However, the priming of an adaptive immune response to adenoviral antigens may be avoided if the virus is carefully injected directly in the brain parenchyma, eliciting instead only a transient nonspecific inflammatory response[4]. It is hoped this would result in stable transgene expression and not induce an autoimmunity to brain tissue resulting in neurological damage[4]. This suggests that gene transfer to immune-privileged sites, such as the CNS, may have greater potential than transfer to nonimmune-privileged tissues.

Several interleukins have been administered using gene-therapy techniques[1,5-12]. In particular, administration of the murine interferon-γ (IFN-γ) in a nonreplicating adenovirus vector, Ad5E1-IFNγ via a pulmonary route to rats has proven to enhance the resistance of the rats to pulmonary challenge with *Pseudomonas*[13].

In previous studies we demonstrated that exogenous administration of recombinant murine interleukin-12 or IFN-γ alone or in combination with conventional antifungal therapy was moderately efficacious in the treatment of meningeal cryptococcal infection in mice[14-16]. The attractiveness of immunomodulation through a gene-therapy approach would be the potential for achieving sustained levels of IFN-γ through the expression of the gene in tissues that had taken up the vector. This would also reduce the number of treatments with purified cytokine (e.g., IFN-γ) needed and have pharmacokinetic advantages over exogenously administered compound with respect to rapid systemic clearance and CNS penetration. Thus, immunomodulation of cells that can kill pathogens in the CNS, such as *Cryptococcus neoformans*, might enhance the efficacy of these cells, particularly if the IFN-γ is made by cells in the CNS, thereby obviating the need for delivery across the blood-brain-barrier. Supportive of this concept are the results of Lampson[17] showing that IFN-γ activates microglia, which are putative antigen presenting cells in the brain; microglia may also act as effector cells during infection. Though gene therapy to the CNS has been explored for the treatment of congenital, degenerative, oncologic and other diseases, it has been rarely studied with respect to CNS infection[18].

Adenovirus vectors containing the murine IFN-γ gene have been administered via a pulmonary route[1,13,19]. Although murine IFN-γ has been injected directly into the CNS[20], few data are available on the direct administration of an adenovirus vector containing the murine IFN-γ gene into the CNS nor the resultant cytokine levels in CSF. Two studies have reported the direct inoculation of an adenoviral vector carrying the murine IFN-γ gene into brain parenchyma and have shown transfection of a variety of cell types in the brain[21,22]. Furthermore, these authors reported repression of tumor growth in the brain by the vector proposed to be through the antiangiogenic activity of the IFN-γ[21, 22]. Interestingly, they also reported the presence of increased concentrations of IFN-γ in the serum of the transfected mice[22].

In preparation for a possible future study of IFN-γ therapy of CNS infection, we needed to determine the consequences of direct injection of the Ad5E1-IFNγ vector into the CSF by different routes, namely intrathecally or intracerebrally, with respect to safety and IFN-γ production. Since previous studies using the Ad5E1-IFNγ vector have shown that IFN-γ is produced in the lungs for approximately 7 days, with peak concentrations occurring about three days after instillation of the viral vector[13], we assessed IFN-γ concentrations over a 10-day period. The present study was carried out to compare two different routes of vector administration to the CNS and to measure the resultant concentrations of IFN-γ present in the CSF and the serum.

MATERIALS AND METHODS

Five-week-old male BALB/c mice (ave. wgt. 17.6 g) were used in this study under an approved Institutional Animal Care and Use Protocol from the California Institute for Medical Research. On day 0, mice were given an intrathecal (IT) or intracerebral (IC) injection of different concentrations of Ad5E1-IFNγ virus[13] (adenovirus with murine interferon-γ [IFN-γ] gene), kindly provided by Dr. Jack Gauldie, McMaster University, Hamilton, Ontario. This virus contains the CMV promoter followed by a SV40 virus poly A. Control animals received phosphate-buffered saline (PBS). For IC injection, mice were anesthetized using methoxyflurane fumes and given virus or PBS. The injection was done at a point 4 to 5 mm posterior to the eyes on the midline. Mice recovered rapidly from anesthesia and no deaths occurred. Mice given the viral doses by IT injection were anesthetized using a mixture of ketamine and xylazine. Once the animal was in a surgical plane of anesthesia, the fur on the dorsal

surface over the lumbar region was shaved and a small incision made. The viral suspension in 50 μL of PBS was injected into the lumbar space and the incision closed with surgical clips. Animals were kept warm and monitored for recovery from anesthesia. Three groups of 10 mice each were injected IC and 3 groups injected IT. Mice received $2x10^7$ or $2x10^8$ virus particles of IFN vector or PBS for each route of injection. Mice were observed daily for clinical signs of illness or deleterious effects.

On days 5 and 10 post-injection, five predesignated mice from each group were bled under anesthesia and then euthanatized using CO_2 anoxia. CSF samples were obtained by puncture of the cisternal space using a microcapillary pipette. The brain of each mouse was aseptically removed and placed in 10% buffered formalin for histological study. Tissues were embedded in paraffin, sectioned and stained by Luxol fast blue- hematoxylin and eosin for histopathological assessment of cellular influxes and possible demyelination.

Each CSF sample (ca. 10 μL or more) was split for determination of total WBC count, as well as determination of IFN-γ concentration. For total leukocyte (WBC) counts, 5 μL of CSF was diluted with 20 μL of sterile saline and WBC enumerated by hemacytometer counting. Cells were removed from CSF (ca. 5 μL) by centrifugation and CSF stored at -80°C until assayed for IFN-γ determination. On the day of assay, 5 μL of CSF was diluted with 45 μL of diluent provided with the ELISA kit. The concentration of IFN in each CSF was then determined by ELISA assay using a kit specific for murine IFN-γ (Endogen, Woburn, Mass.). Serum concentrations of IFN-γ were determined following the manufacturer's instructions for dilution of the serum.

Statistical analyses were done using GraphPad Prism ver. 3.01 (GraphPad Software, San Diego, Calif.) by a nonparametric Kruskal-Wallis test followed by a Dunn's post-test for multiple comparisons. P value of < 0.05 was considered significant.

RESULTS

Clinical parameters: After injection of PBS or adenoviral vector, body weight, WBC in the CSF and clinical appearance were followed through the course of the 10 days of the experiment. Figure 1 shows the change in body weight of the animals in the various groups. In comparison with day 0, mice given PBS IT had gained weight ($p<0.05$) by day 10 and those given $2x10^8$ virus IT had lost weight ($p<0.05$) by day 5, other comparisons were not significant. In contrast, no significant changes in body weight were found for the groups given PBS or virus via the IC route Fig. 1.

Similar to effects on body weight, the CSF leukocytosis observed in mice given virus was more severe in those injected by the IT route (Fig. 2). On day 5, those given either viral inocula via the IT route had similar numbers of WBC in the CSF, which subsided by day 10 and were greater than the WBC in the CSF of mice given the virus IC (Fig. 2). However, no statistical differences were noted due to the small numbers of samples available for counting (Fig. 2).

Histology: Histological assessment of the brain tissues of mice showed that only animals given virus showed any pathological response (Table 1 and 2). In general, there was a mononuclear cell meningitis present, particularly at day 10. This meningitis was more severe in animals given the vector via the IT route and the higher inoculum than it was in those given the virus via the IC route or the lower inoculum (Table 1 and 2).

IFN-γ concentrations: The IFN-γ concentrations in the CSF are presented in Fig. 3 and in the serum in Fig. 4. By day 5, in mice given either inoculum of virus IT, IFN-γ concentrations in the CSF increased over those in mice given PBS. Significant increases in IFN-γ concentration occurred at both day 5 and 10 for those animals given

Table 1: Histological assessment of mice given 2×10^7 (LD) or 2×10^8 (HD) PFU of Ad5E1-IFNγ or PBS, 5 or 10 days after IT inoculation

Treatments	Day post-inoculation	Tissue response
PBS	5	Normal
PBS	5	Normal
PBS	10	Normal
PBS	10	Normal
LD	5	Acute hemorrhage/needle stick lesion in cord/ otherwise normal
LD	5	Normal
LD	10	Mild meningitis (mononuclear cell)/ some acute hemorrhages in meninges
LD	10	Meningitis (mononuclear cell)
HD	5	Normal/ ? prominent meningeal cells
HD	5	Minimal meningitis / mostly normal
HD	5	Normal
HD	10	Normal/ ?mild white matter vacuolation/edema
HD	10	Meningitis (mononuclear cell)/ some edema/vacuolation
HD	10	Mild meningitis

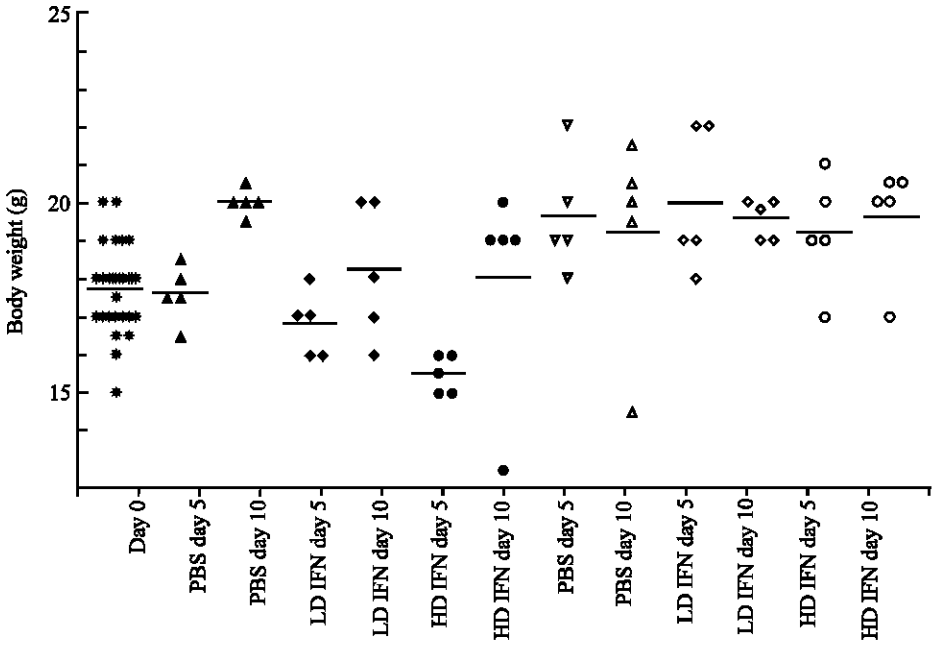

Fig. 1: Body weights of mice given $2x10^7$ (LD IFN) or $2x10^8$ (HD IFN) PFU of Ad5E1-IFNγ or PBS intrathecally (IT; solid markers) or intracerebrally (IC; open markers). Bar represents the mean

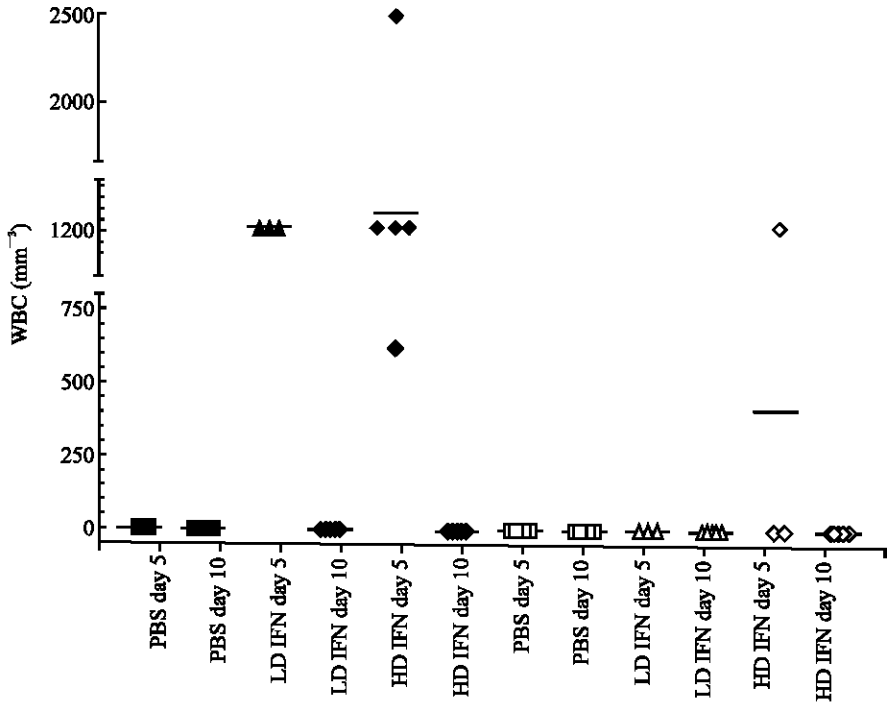

Fig. 2: WBC (mm^{-3}) in the CSF of mice given $2x10^7$ (LD IFN) or $2x10^8$ (HD IFN) PFU of Ad5E1-IFNγ or PBS intrathecally (IT; solid markers) or intracerebrally (IC; open markers). Bar represents the mean

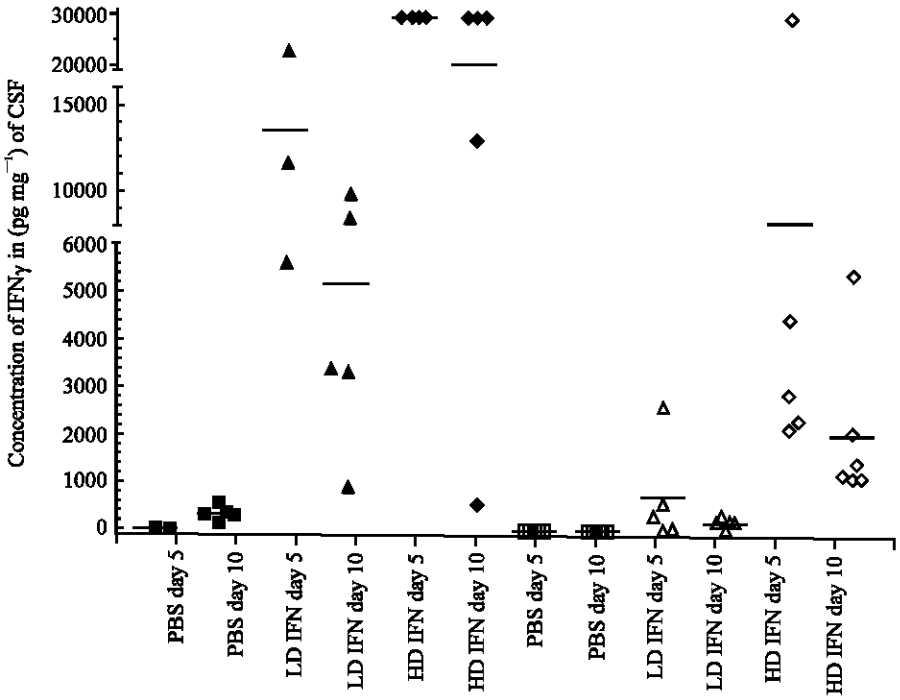

Fig. 3: Concentration of IFN-γ in the CSF of mice given $2x10^7$ (LD IFN) or $2x10^8$ (HD IFN) PFU of Ad5E1-IFNγ or PBS intrathecally (IT) (solid markers) or intracerebrally (IC) (open markers). Bar represents the mean

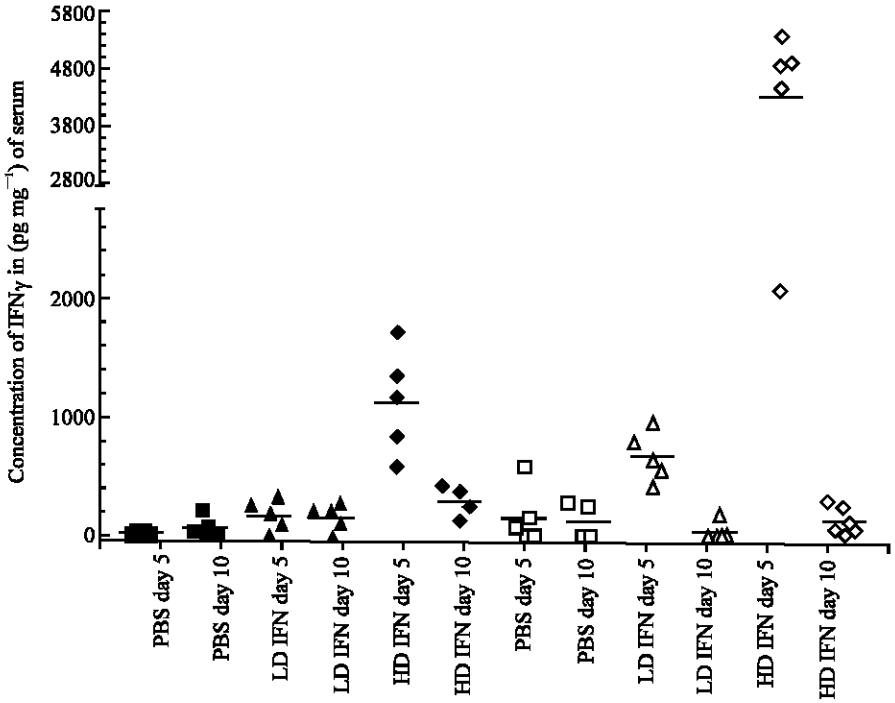

Fig. 4: Concentration of IFN-γ in the serum of mice given $2x10^7$ (LD IFN) or $2x10^8$ (HD IFN) PFU of Ad5E1-IFNγ or PBS intrathecally (IT) (solid markers) or intracerebrally (IC) (open markers). Bar represents the mean

Table 2: Histological assessment of mice given 2 x 10^7 (LD) or 2 x 10^8 (HD) PFU of Ad5E1-IFNγ or PBS, 5 or 10 days after IC inoculation

Treatments	Day post-inoculation	Tissue response
PBS	5	Normal
PBS	5	Normal
PBS	10	Normal
PBS	10	Normal
LD	5	Normal
LD	5	Minimal meningitis
LD	10	Normal
LD	10	Moderate meningitis
HD	5	Normal
HD	5	Minimal meningitis / mononuclear cells
HD	5	Minimal meningitis / mononuclear cells
HD	10	Minimal meningitis / mononuclear cells
HD	10	Minimal meningitis / mononuclear cells
HD	10	Minimal meningitis / mononuclear cells

$2x10^8$ virus IT ($p<0.05$); other comparisons were not significantly different. Concentrations of IFN-γ decreased from day 5 to 10 in both inoculum groups given virus IT; the decreases were not significant. However, IFN-γ concentrations increased from day 5 to 10 in mice given PBS IT, which may reflect injection related inflammation. Those animals given virus IC also showed increased concentrations of IFN-γ in the CSF, but these increases were lower than for IT administration. Similar to the results for IT inoculation, $2x10^8$ virus given IC caused a significant increase in IFN-γ concentration in the CSF compared with diluent administration ($p<0.05$). Those given the lower inoculum of $2x10^7$ virus IC showed lower IFN-γ concentrations in the CSF than did those given the virus IT (Fig. 4).

Determination of the IFN-γ concentrations in the serum of mice showed that IT or IC administration of the vector also results in a rise of serum IFN-γ concentrations (Fig. 4). Lower concentrations were found in the animals given virus IT, yet these increases were significant on day 5 for those given $2x10^8$ virus in comparison with those given PBS ($p<0.05$). Similarly, mice given the virus IC showed substantial increases in the serum concentration of IFN-γ (Fig. 4). These increases were significant at day 5 ($p<0.05$) when comparing PBS-treated with those given $2x10^8$ virus. However, serum IFN-γ concentrations had decreased to near control levels by day 10 after inoculation.

DISCUSSION

The results of the current studies provide evidence that the administration of a viral vector carrying the murine IFN-γ gene directly into the CSF of normal animals results in up to 30,000-fold increases in concentration of IFN-γ in the CSF. We chose to use two different inocula of viral particles to determine whether there was a dose-escalation effect. This proved to be the case, with the animals given the higher dosage of viral particles having higher concentration of IFN-γ in their CSF, especially 5 days after injection. However, other parameters showed that this inoculum dosage might not be desirable since there were also higher numbers of WBC in the CSF, and the recipients also showed more histological evidence of mononuclear meningitis at both 5 and 10 days post-injection. Animals given the lower inoculum of virus showed a more normal histological appearance and fewer WBC in the CSF. One potential concern of administering too high a viral inoculum would be IFN-γ-induced encephalomyelitis or demyelination. Both of these responses have been reported after direct injection IFN-γ containing adenoviral vector to the brain parenchyma[23] or IFN-γ to the tissues of the CNS or by over expression[20,24,25], but these abnormalities were not observed in our study.

Interestingly, the use of two different routes of injection also resulted in different responses. Those animals given the vector intrathecally appeared to be more affected. Clinical observations indicated that mice given 2 x 10^8 PFU intrathecally became hypoactive by day 3 and that most animals had ruffled fur and were ataxic; by day 10, these animals showed improvement in appearance and activity. The clinical signs were much less severe in mice given the same dose intracerebrally and they appeared normal by day 10. Few animals given the 2 x 10^7 inoculum showed any clinical signs. Correlating with the clinical signs, mice given the virus intrathecally showed weight loss, greater numbers of WBC in the CSF and more severe histological abnormalities than did mice given the virus intracerebrally. Mice given virus by intracerebral injection gained weight and had fewer WBC in the CSF. However, moderate mononuclear cell meningitis was evident in most of the animals on day 10.

Our results corroborate and extend those of Fathallah-Shaykh *et al.*[22] on the appearance of IFN-γ in the serum after CNS administration of the IFN-γ containing adenoviral vector. We show here that this occurs by either route of administration and is maximal at

day 5 after transfection. The most notable difference in the route of injection was the concentration of IFN-γ in the CSF. Those mice given either inoculum via an intrathecal route had higher levels of IFN-γ than did those given the same inoculum via the intracerebral route. Whether this difference is due to the cell types involved in the uptake of the vector and subsequent production of the IFN-γ remains to be determined. However, one might speculate that there is a difference in the clearance of the viral vector, or the IFN-γ produced, from the CSF after each route of injection. This speculation is supported by the lower concentrations of IFN-γ in the CSF of the intracerebrally injected animals and the higher serum concentration at day 5 in the intracerebrally injected mice given the high dosage of virus. Taken together these data suggest that the vector or the IFN-γ is cleared rapidly from the CSF, and enters the lymph or blood stream when given intracerebrally. In contrast, the vector or the IFN-γ is cleared less rapidly when given intrathecally. This may be also due to the time necessary for the flow of CSF to reach the ventricles from the lumbar region or due to penetration and absorption (or deposition) of IFN-γ into parenchymal cells, after intracerebral injection, in the latter stages of the study.

Overall, present results clearly demonstrate that administration of the Ad5E1-IFNγ vector directly into the CSF results in significant production of IFN-γ that can be detected in the CSF up to 10 days after injection. Results also corroborate previous data, studying other routes of injection, indicating that the duration of IFN-γ production appears limited, with a decline between 5 and 10 days after inoculation of the virus. These data are encouraging and provide a basis for testing this therapeutic strategy in future studies of central nervous system infections. Based on our data it would appear that a dose of 2×10^7 PFU of vector would be desirable, as the one that produces significant IFN-γ with the least deleterious side-effects.

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