

Gene Therapy-Mediated Modulation of Immune Processes in the Central Nervous System

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Abstract: Selective interference with immune processes in the central nervous system (CNS) is a very difficult task because of the limitations associated with the delivery of immuno modulatory molecules across the blood brain barrier. Systemic administration of immune-mediators, either by conventional routes or by intramuscularly or intravenous gene therapy, is hampered by severe side effects and alters immune-system functions also in peripheral organs. To overcome these problems, different gene therapy strategies have been developed to deliver immuno modulatory molecules directly within the central nervous system. The use of engineered CNS antigen-specific circulating cells as selective delivery vehicles, the direct injection of gene vectors into the brain parenchyma, or also the ependymal route, have been proposed as possible alternative gene therapy protocols to selectively interfere with immuno-pathological processes in the CNS. We will review the use of these CNS-targeted gene therapy protocols for the treatment of experimental autoimmune encephalomyelitis (EAE), the prototypical experimental immune-mediated disease of the CNS, and therefore discuss the relevance of these results for the therapy of multiple sclerosis (MS) the most common, immune-mediated, demyelinating disease of the CNS in humans.

Key Words: gene therapy, central nervous system, multiple sclerosis, experimental autoimmune encephalomyelitis, cytokines, and growth factors.

INTRODUCTION

Immunomodulatory therapies targeting the central nervous system (CNS) are a crucial challenge to future medicine. In fact, besides some pathogenetically-characterized immune-mediated diseases of the CNS that await more efficient therapeutic tools for a better management, such as multiple sclerosis (MS), other neuro degenerative and neuro pathological syndromes, have a recently recognized immune-mediated component that might represent a potential therapeutic target. Alzheimer's disease (AD) [1], amyotrophic lateral sclerosis (ALS) [2], other neuro-degenerative diseases [3], and even stroke [4] have recently been considered as diseases candidate for a novel immunomodulatory therapeutic approach, along with neuro-protective and if possible etiological treatments. However, the immune system and the CNS are in a very peculiar relationship, when compared to the rest of the body. In fact, the immune system surveys the CNS as any other district in the living organism. However, immune reactions in the CNS usually follow distinct rules because of anatomical and functional peculiarities that, collectively, have allowed the CNS to be defined as an immune-privileged organ. The anatomical, functional, and regulatory peculiarities of the immune system in the CNS are still under investigation and only partially understood. These studies are difficult also

because targeting molecules to the brain is extremely limited by the presence of the blood-brain barrier (BBB) [5]. To date, most of conventional neurotherapeutic agents are supposed to cross the BBB because of their smaller size. However, more than 98% of small molecules don't cross the BBB either [6]. We can then conclude that a specific delivery within the CNS of molecules able to modulate the local immune response would be therefore useful to investigate the features of the immune reaction in the brain, thus representing a novel therapeutic tool for several heretofore untreatable CNS-restricted inflammatory diseases. Unfortunately, the selective modulation of the immune system in the CNS by a conventional peripheral therapeutic approach seems, at present, an unreal task.

This review will focus on gene therapy as a possible alternative approach to target immunomodulatory molecules selectively to the CNS permitting an *in situ* regulation of immune reactions.

IMMUNOMODULATORY GENE THERAPY IN THE CENTRAL NERVOUS SYSTEM

Gene therapy has been recognized first as a tool for the correction of genetic disorders, and only in a second instance as an alternative drug delivery system. This might be the reason why immunomodulation in the CNS by gene therapy is a recent, although rapidly growing, field of investigation. The prototypical, chronic, immune-mediated demyelinating disease of the CNS is multiple sclerosis (MS). MS is of unknown etiology, and it is characterized by the presence of

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perivascular inflammatory infiltrates in the CNS containing T and B cells and activated macrophages, thus suggesting that MS is a T cell mediated, CNS-confined, chronic, inflammatory, demyelinating disease in which the ultimate effector cells are activated macrophages [7]. The inflammatory process, leading to multifocal patchy demyelination and axonal loss, is mainly sustained by pro-inflammatory cytokines that along with chemokines, adhesion molecules, and metalloproteinases modulate at different levels the immuno pathogenic process underlying MS [8]. Due to their central role in MS pathogenesis, "inflammatory" molecules might represent suitable therapeutic targets [9]. No currently available treatment for MS enables a satisfying control of disease evolution, and MS patients, usually young adults, accumulate various degrees of disability over several years of disease [10]. MS is modeled in animals by experimental autoimmune encephalomyelitis (EAE), which can be induced in rodents and non-human primates by active immunization with whole myelin, myelin proteins, myelin peptides, or non-myelin antigens [11-12; reviewed in 13]. Depending on the animal species, or strain, and the antigen used for active immunization, different disease courses (i.e. acute monophasic, relapsing-remitting or chronic), resembling clinical subtypes of human MS, can be obtained. Despite being performed on a more accessible model than the human CNS, the immunological studies on EAE in rodents have not produced conclusive data on the role of various immune mediators in the pathological process leading to lesion formation in the CNS [reviewed in 14]. However, numerous gene therapy protocols aimed at the delivery of immunologically active molecules in the brain have been published so far (Table 1). Three main approaches have been attempted: (i) genetically engineered cells, usually lymphocytes specific for CNS antigens, used as "Trojan horses" able to find their target and deliver the engineered therapeutic molecule; (ii) direct injection of biological or non-biological gene therapy vectors into the CNS parenchyma; (iii) injection of viral gene therapy vectors in the cerebrospinal fluid circulation, namely the "ependymal route". These three different approaches have been tested in different EAE models and gene therapy has been administered either at the time of disease induction (preventive treatment) or close to, or after, the disease onset (therapeutic treatment).

CNS GENE THERAPY USING CIRCULATING ENGINEERED CELLS

The perivascular inflammatory infiltrates in the CNS - the pathological hallmark of MS/EAE - are thought to be the cause of lesions formation and are renewed several times a day [15]. This florid traffic of lymphocytes from the circulation to the CNS can be exploited to take a therapeutic molecule selectively across the BBB. Several groups have used retroviruses as gene therapy vectors to permanently insert therapeutic genes into T lymphocytes that then have been re-infused to EAE mice. To ensure a specific delivery, the T cells engineered to release therapeutic molecules were specific for CNS antigens. The re-infused CNS-antigen specific T cells are supposed to home preferentially into the damaged area of the CNS, where inflammation is actively ongoing, and, therefore, to release the protective agent

exactly at the site and time where it is needed. Myelin basic protein (MBP)-specific T cells have been retrovirally transduced with the interleukin (IL)-4, IL-10 or tumor necrosis factor (TNF) gene and have been transferred into syngeneic (PL/J x SJL/J)F1 mice affected by chronic-relapsing, MBP-induced, EAE. IL-4-engineered cells, re-infused after disease onset, were able to ameliorate the disease course, whereas IL-10-transduced cells were ineffective, and TNF- α -engineered cells worsened the clinical signs of EAE [16, 17]. In a different study, a MBP-specific BALB/c T helper 1 clone has been retrovirally transduced with the latent form of transforming growth factor (TGF) β , and transferred into (SJL x BALB/c)F1 mice affected by acute monophasic EAE induced by immunization with the proteolipid protein (PLP) peptide 139-151. This treatment was effective when administered both at the time of immunization and of the disease onset, although only partially beneficial when engineered T cells were infused 3 days after EAE onset [18]. Activated PLP139-151-specific T cells have also been retrovirally transfected with an expression cassette in which the IL-2 promoter drives murine IL-10. In this way, transcription of the transgene is induced only when the IL-2 promoter is active - at the time of cell activation - supposedly when the cell is on the inflammatory site. These cells, transferred into PLP139-151-immunized (SW x SJL)F1 mice affected by relapsing-remitting EAE, were able to ameliorate both clinical and neuropathological signs of the disease [19]. Growth factors have also recently been engineered into myelin antigen-specific T cells with the aim to foster endogenous repair mechanisms and considering in parallel their possible activity on immune cells. In a first study, PLP139-151-specific T lymphocytes were engineered with an antigen-inducible transgene coding for platelet-derived growth factor (PDGF)-A. The transfer of these cells in PLP139-151-immunized (SW x SJL)F1 mice was able to ameliorate ongoing relapsing-remitting EAE [20]. In a second study, MBP-specific CD4⁺ T cells were transduced with a recombinant retrovirus coding for nerve growth factor (NGF). These myelin antigen-specific T cells, when re-infused in Lewis rats, were unable to mediate EAE and, furthermore, inhibited acute monophasic EAE when co-transferred with non-transduced MBP-specific T cells. This latter result was associated not only with the induction of tissue repair, but also with the reduction of inflammatory infiltrates in the CNS, and especially of infiltrating monocytes, thus suggesting an unexpected immunomodulatory role for NGF [21]. An original and recent variation of this approach has been proposed lately. Using engineered B cells, EAE susceptible mouse strains were made resistant by specific down-regulation of the pathogenic auto-reactive T cell clone. Starting from the concept that antigen presentation from B cells is usually tolerogenic, normal B cells were retrovirally transduced to express PLP139-151 fused to a lysosomal target sequence, to ensure loading on MHC-II molecules. Transformed B cells transferred into PLP139-151-immunized (SJL x BALB/c)F1 mice were able to prevent relapsing-remitting EAE in the majority of mice, and to delay onset and reduce severity in the affected ones [22]. In a second recent study, on the same lines, B cells were retrovirally transformed to express an IgG-MBP fusion protein. Transformed cells were then co-transferred with MBP-specific encephalitogenic T cells into syngenic

Table 1. Experimental Gene-Therapy Trials in Rodent and Non-Human Primate Models of EAE^a

Delivery route	Gene vector	Therapeutic gene	Animal strain	Immunization	Administration schedule ^b	Clinical efficacy ^c	Refs
Engineered cells							
MBP-specific T cells	Retrovirus	IL-4	(PL/JxSJL)F ₁ mice	MBP	Therapeutic	+	16
MBP-specific T cells	Retrovirus	IL-4	(PL/JxSJL)F ₁ mice	MBP	Therapeutic	+	17
MBP-specific T cells	Retrovirus	IL-10	(PL/JxSJL)F ₁ mice	MBP	Therapeutic	-	16
PLP-specific T cells	Retrovirus	IL-10	SWXJ mice	PLP139-151	Therapeutic	+	19
MBP-specific T cells	Retrovirus	TNF	(PL/JxSJL)F ₁ mice	MBP	Therapeutic	worsening	17
B cells	Retrovirus	PLP100-154	(SJLxBALB/c)F ₁ mice	PLP139-151	Preventive	+	22
B cells	Retrovirus	MBP-IgG1	(PL/JxSJL)F ₁ mice	bMBP + MBP1-17	Preventive	+	23
B cells	Retrovirus	MBP-IgG1	(PL/JxSJL)F ₁ mice	bMBP + MBP1-17	Therapeutic	+	23
B cells	Retrovirus	MBP-IgG1	(PL/JxSJL)F ₁ mice	PLP139-151	Therapeutic	-	23
B cells	Retrovirus	MBP-IgG1	(PL/JxSJL)F ₁ mice	bMBP + PLP139-151	Therapeutic	-	23
PLP-specific T cells	Retrovirus	PDGF-	SWXJ mice	PLP139-151	Therapeutic	+	20
MBP-specific T cells	Retrovirus	TGF-	(SJLxBALB/c)F ₁ mice	PLP139-151	Therapeutic	+	18
MBP-specific T cells	Retrovirus	NGF	Lewis rats	MBP	Preventive	+	21
Direct injection							
Intracerebral	Liposomes	IL-4	Biozzi AB/H mice	SCH	Therapeutic	+	27
Intracerebral	HSV-1 134.5	IL-4	BALB/c	SCH	Preventive	+	31
Intracerebral	HSV-1 134.5	IL-10	BALB/c	SCH	Preventive	-	31
Intracerebral	Liposomes	IL-10	Biozzi AB/H mice	SCH	Therapeutic	+	27
Intracerebral	Retrovirus	IL-10	Biozzi AB/H mice	SCH	Therapeutic	+	29
Intracerebral	Adenovirus	IL-10	Biozzi AB/H mice	SCH	Therapeutic	-	29
Intracerebral	Liposomes	IFN	Biozzi AB/H mice	SCH	Therapeutic	+	28
Intracerebral	Liposomes	p55 TNFR-Ig	Biozzi AB/H mice	SCH	Therapeutic	+	27
Intracerebral	Liposomes	p75 dTNFR	Biozzi AB/H mice	SCH	Therapeutic	+	28
Intracerebral	Retrovirus	p75 dTNFR	Biozzi AB/H mice	SCH	Preventive	+	30
Intracerebral	Liposomes	TGF-	Biozzi AB/H mice	SCH	Therapeutic	+	27
Ependymal route							
Intracisternal injection	HSV-1	IL-4	Biozzi AB/H mice	MOG40-55	Preventive	+	34
Intracisternal injection	HSV-1	IL-4	Biozzi AB/H mice	SCH	Therapeutic	+	35
Intracisternal injection	HSV-1	IL-4	Macaca mulatta	Whole myelin	Therapeutic	+	36
Intraventricular inject.	Adenovirus	IL-10	CSJLF ₁ mice	SCH	Therapeutic	+	43
Intracisternal injection	HSV-1	IFN	C57BL/6 mice	MOG35-55	Preventive	+	37
Intracisternal injection	HSV-1	IFN	C57BL/6 mice	MOG35-55	Therapeutic	+	37
Intracisternal injection	HSV-1	FGF-II	C57BL/6 mice	MOG35-55	Therapeutic	+	38

^aAbbreviations: p55 TNFR-Ig, p55 TNF receptor-Ig fusion protein; p75 dTNFR, p75 TNF dimeric receptor; SCH = spinal cord homogenate; bMBP = bovine myelin basic protein.

^bPreventive, at the time of immunization or shortly after; Therapeutic, close to or after disease onset. ^c +, disease amelioration; -, no effect.

(PL/J x SJL)F1 mice, preventing relapsing-remitting EAE, even when injected after the disease onset. The effect was antigen-specific, since MBP-Ig transformed B cells were unable to prevent EAE induced by co-immunization with PLP and MBP [23]. Thus, several different approaches of cellular gene therapy can be used to modulate immune-mediated pathological processes in the CNS injecting engineered cells as "trojan horses". Unfortunately, several drawbacks limit the applicability of this approach to MS. While pathogenic auto-antigens are known in the experimental model, the epitopes driving the immune response in human MS are still unknown. What should, therefore, be the proper antigenic specificity if the T cell approach is taken in humans? Furthermore, T cells need to be activated *in vitro* in order to properly home to inflammatory sites in the CNS. This may lead somehow to disease exacerbation if the balance between beneficial effects of the delivered therapeutic molecule and the detrimental contribution of the myelin antigen-specific T cell to the pathogenic process is unfavorable. Human trials with altered peptide ligands have already demonstrated that undesired activation of T cells with myelin antigens may lead to disease worsening [24, 25]. Furthermore, studies using engineered peripheral lymphocytes have failed to demonstrate, in a definitive way, that transformed cells are able to travel to the site of lesion within the CNS and there release the active molecule. It could well be that the beneficial effects may derive from the release of the putative protective molecule in the periphery, therefore raising again the issue of peripheral side effects. The above-described B cell approach, on the other hand, is also novel and very interesting, but, again, it is difficult to envisage antigen-specific therapies in MS where the putative auto-antigen is still unknown.

GENE THERAPY THROUGH THE DIRECT INJECTION IN THE CNS PARENCHYMA

To overcome the above-mentioned concerns, many groups have taken a different approach by injecting gene vectors directly into the CNS parenchyma. Retroviral vectors, which are able to transform only cycling cells, and are preferred for *ex vivo* gene therapy protocols, cannot be employed for direct injection in the brain because the very low number of replicating CNS resident cells would lead to a negligible efficiency of gene transfer. Among the different vectors used in the following studies, lentiviral vectors, able to permanently insert their genome into resting cells, are missing. No doubt that in the near future lentiviral vectors – already used for other experimental CNS gene therapy protocols [26], will be also used to modulate CNS-confined immune-mediated diseases. Naked plasmids, containing expression cassettes coding for IL-4, TGF β , interferon (IFN) γ , p55TNF receptor-Ig, p75TNF receptor-Ig, or IL-10, have been injected intracerebrally into the right frontal lobe of Biozzi AB/H mice affected by relapsing-remitting EAE induced by immunization with spinal cord homogenate. This approach proved completely ineffective on the disease course. If, however, the same plasmids were complexed with lipofectin (DNA-liposome complexes), then IL-4, IFN γ , TGF β , p55TNF receptor-Ig, and p75TNF receptor-Ig,

injected three days before disease onset, were able to ameliorate EAE, while IL-10 remained ineffective [27, 28]. Also an adenoviral vector, engineered to contain an IL-10 expression cassette, and injected intracerebrally in mice affected by the same EAE model, failed to inhibit the disease [29]. On the contrary, intracranial injection of fibroblasts, retrovirally transformed to release high amounts of IL-10, inhibited spinal cord homogenate-induced EAE in Biozzi AB/H mice. This latter treatment was surprisingly associated with increased recruitment of B cells, and CD8 $^{+}$ T cells in CNS inflammatory infiltrates, rather than with a decrease of leukocyte trafficking [29]. CNS intraparenchymal injection of retroviral-transformed fibroblasts was therapeutically effective, ameliorating relapsing-remitting EAE in Biozzi AB/H mice also when the delivered molecule was p75TNF receptor-Ig [30]. Finally, replication attenuated herpes simplex virus type-1 (HSV-1) vectors, depleted for the gamma 34.5 gene, have been used for direct intracerebral injection. These depleted herpetic vectors, despite being partially replication competent, are supposed to be of attenuated neuropathogenicity. HSV-1-derived vectors coding for IL-4, but not for IL-10, induced protection from acute monophasic EAE obtained in BALB/c mice by immunization with spinal cord homogenate. Disease inhibition was associated with decreased leukocyte infiltration, demyelination, and axonal loss [31].

Injecting gene therapy vectors directly in the CNS is a promising approach too. However, injection of naked DNA or DNA-liposome complexes leads to a short-term (i.e., for a few days) gene expression, a relevant limitation in a chronic disease like MS. The use of partially replicating viruses, such as HSV-1 gamma 34.5 deletion mutants, is interesting in experimental settings, but raises unaffordable safety issues if transferred in human protocols. Finally, direct CNS intraparenchymal injection of the gene therapy vectors employed in these studies allows only limited spreading from the injection site, another major issue in MS, a disease multifocally affecting the whole CNS.

THE EPENDYMAL ROUTE FOR CNS GENE THERAPY

Recently, a novel strategy has been established for the delivery of genes into the CNS. Based on the injection of non-replicative viral vectors, this approach employs the cerebrospinal fluid (CSF) as the driving force to deliver therapeutic molecules to the entire CNS. Viral vectors delivered into the CSF circulation, either through injections into the cisterna magna (i.c.) or by stereotaxic intracerebroventricular (i.c.v.) injections, will infect the ependymal and leptomeningeal cells facing liquor spaces. Ependymal and leptomeningeal cells, infected with the viral vector, will then produce the heterologous protein and, in case the transgene codes for a secreted protein, release it in the CSF circulation. From the CSF, the secreted protein can travel within the CNS parenchyma and exert there its beneficial effects. HSV-1-derived vectors engineered with cytokine genes have been especially successful [32, 33]. HSV-1-derived vectors were used to deliver IL-4 in mice with EAE, either before or after the disease onset. I.c. injection of an IL-4-coding HSV-1 vector was able to inhibit

chronic-relapsing EAE development in Biozzi AB/H mice immunized with the myelin oligodendrocyte glycoprotein (MOG) 40-55 peptide [34]. Disease prevention was associated with a decreased recruitment within the CNS of monocyte/macrophages from the peripheral circulation. The i.c., HSV-1-mediated, IL-4 delivery was able also to ameliorate ongoing relapsing-remitting EAE in spinal cord homogenate-immunized Biozzi AB/H mice, determining, in this case, a significant modulation of the local cytokine milieu, leading to down-regulation of pro-inflammatory cytokines and chemokines [35]. This latter approach has been tested also in non-human primates affected by a very acute, invariably fatal, form of EAE induced by immunization with whole myelin. Sixty percent of monkeys i.c.-injected at the time of disease onset with an HSV-1 vector engineered with the human IL-4 gene, were completely protected from EAE signs and symptoms [36]. The ependymal route using HSV-1 derived vectors has been employed also to deliver the IFN gene, which was able to both inhibit or treat MOG35-55-induced chronic EAE in C57BL/6 mice, through the induction of *in situ* apoptotic death of encephalitogenic T cells [37]. Finally, HSV-1-mediated intracisternal delivery of the fibroblast growth factor (FGF)-II gene was able to induce oligodendrocyte precursors proliferation and migration, thus ameliorating ongoing chronic EAE in MOG35-55-immunized C57BL/6 mice [38]. HSV-1-derived vectors, however, have a low chance to be employed in a human clinical setting due to: a) the short term transgene production (i.e., up to 4 weeks); b) their possible immunogenicity; and c) their derivation from a virus potentially very dangerous for its selective neurovirulence. Other vectors may be, therefore, more suitable for the "ependymal route" of administration of the transgene. Previous data have shown that intraventricular injection in naive mice and rats of first generation replication-defective recombinant adenoviral vectors (Ad.Sv gal, Ad-1AT, and AxCAHBG) leads to the expression of reporter genes in ependymal cells [39, 40], and to production into the CSF of proteins coded by the transgenes, such as β -glucuronidase and 1-antitrypsin [40, 41]. Moreover, a recent report confirmed that the injection into the CSF space, either sub-occipitally (within the cisterna magna) or more caudally by lumbar puncture, of an adenoviral vector containing the β -gal reporter gene determined a widespread infection of ependymal cells surrounding the CSF space in non-human primates [42]. Adenoviral vectors were validated also in EAE for the ependymal delivery of IL-10, through i.c.v. injection, in this case resulting in the amelioration of an acute monophasic disease induced in CSJLF₁ mice by immunization with spinal cord homogenate [43].

The viral vector-mediated CNS delivery of cytokine and growth factor genes through the ependymal route presents the following major advantages: (a) availability of high cytokine and growth factor levels virtually in all areas of the CNS, thus representing an ideal approach for multifocal diseases, such as MS; (b) persistent therapeutic effect for over 4 weeks after a single vector administration, irrespective of the injection site into the CSF circulation (i.e. ventricular route or lumbar route); and (c) absence of CNS or peripheral unpredictable and undesirable side effects. This

latter point has been especially addressed from an immunological point of view. Neither the delivery of IL-4 [34-36], nor of IFN- γ [37], affected the functions of antigen-specific encephalitogenic T cells in the periphery, and non-human primates, followed for one month after i.c. injection of an HSV-1 vector engineered to produce the human IL-4 gene, did not display any sign of CNS and peripheral toxicity [36].

CONCLUSIONS

MS is a chronic, multifocal, immune-mediated disease of the CNS. Thus, a non-etiological therapeutic approach has to be able to interfere with immune processes in the whole CNS for a prolonged time. Any peripheral route of administration has been to date limited by the difficulty to achieve active drug concentrations across the blood brain barrier, without causing peripheral side effects and without interfering with normal immune system functions. As a consequence, most clinical trials based on the systemic administration of immunomodulatory molecules in MS have, so far, failed [44-47]. Gene therapy offers the tools to overcome these limitations by directly targeting the brain. The different therapeutic approaches that we have discussed here, despite needing further development, deserve consideration as possible alternatives in the future MS therapeutic scenario.

Engineered cells re-infused in the blood stream of patients might represent a non-invasive approach, in which the *ex-vivo* genetic manipulation could allow a tight quality control of the gene transfer. However, along with the current lack of any indication of their fate *in vivo*, and the risks involved in the re-infusion of activated, potentially encephalitogenic, cells, they have a limited life span. At the moment, this approach satisfies the requirement for a multifocal, although not chronic, disease.

Direct injection into the CNS of gene therapy vectors, also is a promising approach. However, it requires the intervention of a neurosurgeon and carries the risks involved in any CNS surgery, such as bleedings or unwanted CNS lesions. Currently available gene therapy vectors, such as lentiviral vectors, would allow long-term expression of the therapeutic molecule from CNS resident cells, which have a very long life span. However, spreading from the injection site is limited to few millimetres and the diffusion range of the released molecule will necessarily be insufficient to target the whole CNS. Thus, this gene therapy approach satisfies the requirements for a chronic, although not multifocal, CNS disease.

The ependymal route is, at the moment, the only gene delivery protocol satisfying the requirements for a CNS disease, which is - like MS - at the same time chronic and multifocal. Ependymal cells, in fact, infected with helper-dependent adenoviral (HD-Ad) vectors, are able to produce an heterologous protein for over 5 months [33], and the CSF circulation will allow the released molecule to reach virtually all areas of the CNS. The demonstrated absence of peripheral side effects is an additional favourable feature of this approach. However, not all released molecules may be able to travel from the CSF circulation to the CNS parenchyma

with the same efficiency, thus limiting the nature of the transferred gene that can be employed with this approach.

Some of the above-mentioned limitations, may be overcome by future available technology. The development of our knowledge on hematopoietic stem cells, for example, may in future allow us to permanently genetically modify subpopulations of non-pathogenic lymphocytes - able to selectively home within the CNS - and to use them as vehicle for local drug delivery. On the other hand, some advantages will come from the development of innovative gene therapy vectors able to spread over larger areas of the CNS.

There are, however, some concerns - common to any delivery route for selective CNS gene therapy - that must be overcome before these protocols will be applied in human trials. Firstly, there is the immunogenicity of the gene therapy vectors and their products: repeated, or prolonged, exposure of foreign viral molecules to the immune system may lead to a specific immune reaction against the gene therapy vector, thus making infected cells the target of an undesired auto-immune reaction [48]. In this respect, the ependymal approach seems very safe, since neither with HSV-1 nor with first generation adenoviral vectors - both highly immunogenic - an immune reaction against the viral vector has been observed in non-human primates, up to one month after injection in the CSF circulation [36, 42]. Furthermore, genetic material originated from bacterial plasmids, contained in gene therapy constructs, is, *per se*, immunogenic and able to modulate immune and autoimmune responses [49, 50]. Secondly, there is the impossibility to regulate the level and timing of gene expression. Availability of regulatable promoters, suitable for the use in humans, would enhance the safety profile of this approach, thus allowing to produce the desired transgene only during specific phases of the disease (i.e., release of an anti-inflammatory agent during the clinical relapse or release of a neurotrophic growth factor during recovery phases). Despite it has been almost ten years from the first human gene therapy protocol, this technology has still to solve enormous problems. It represents, however, one of the most conceivable approaches to therapeutic challenges, such as those posed by MS, that appear difficult to face with conventional pharmacological tools.

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