REVIEW

Gene therapy in nonhuman primate models of human autoimmune disease

BA t'Hart^{1,4}, M Vervoordeldonk², JL Heeney³ and PP Tak²¹Department of Immunobiology, BPRC, Rijswijk, The Netherlands; ²Division of Clinical Immunology and Rheumatology, Department of Medicine, Academic Medical Centre, University of Amsterdam, The Netherlands; ³Department of Virology, BPRC, Rijswijk, The Netherlands; and ⁴ErasMS Centre for MS Patient Care, Research and Expertise, Rotterdam, Rijswijk, The Netherlands

Before autoimmune diseases in humans can be treated with gene therapy, the safety and efficacy of the used vectors must be tested in valid experimental models. Monkeys, such as the rhesus macaque or the common marmoset, provide such models. This publication reviews the state of the art in

monkey models for rheumatoid arthritis and multiple sclerosis and the (few) gene therapy experiments that have been performed in these models.

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Introduction

The impressive development in the biotechnology field has facilitated the generation of safer and more effective therapies for the treatment of autoimmune diseases in the human population. Examples of biotechnology-derived pharmaceuticals (biologicals) that have now reached the clinic include monoclonal antibodies or antagonist proteins for TNF- α (Infliximab, Etanercept) or interleukin-1 (IL-1) (Anakinra) for treatment of chronic inflammatory disorders like rheumatoid arthritis (RA) and inflammatory bowel disease¹ or cytokines, such as interferon- β for multiple sclerosis (MS).² Many more targets and diseases are being investigated in a variety of experimental models. The main lesson from all these studies is that experimentally induced autoimmune diseases can be safely treated with biological molecules. However, the *in vivo* half-life of most biological agents is relatively short. The repeated administration of high systemic doses required to achieve therapeutic levels in affected organs may lead to deleterious side effects. Furthermore, the therapeutic window of biological molecules is often limited by the induction of antibodies capable of blocking the therapeutic itself. Targeted gene transfer provides the possibility to achieve therapeutic effects while circumventing the above-mentioned complications.

A critical factor in the successful development of somatic gene therapy is the availability of valid animal models in which the safety and efficacy of constructs designed for treatment of patients can be accurately evaluated. In many cases, biological molecules that are designed to work in humans are insufficiently active in rodents and cannot be tested. Nonhuman primates with

their greater immunological and biological similarity to humans are in great demand as models for preclinical evaluation of highly specific biological therapeutics. Several immunomodulatory therapies seem to work less effectively in primates than in inbred rodents. This may be because of the fact that the immune system of a few months old inbred rat or mouse is less mature than that of an adult primate or human. The value of nonhuman primate disease models is, therefore, that they predict which experimental therapies that work well in rodents could also be effective in clinical trials.

At the time we wrote this article, we found in a Medline search combining the keywords 'gene therapy' and 'primate' about 400 hits; while combination of 'gene therapy' and 'monkey models' gave around 80 hits and combination of 'gene therapy' and 'autoimmune disease' gave 323 hits. In contrast, a focused search on the keywords 'gene therapy' and 'autoimmune disease' and 'primates' yielded only four titles. More frequently addressed disorders are eye and lung disease, transplant rejection, and immunodeficiency. It can thus be concluded that there is considerable research activity on the application of gene therapy as treatment of autoimmune disease and that also considerable experience exists with gene therapy in nonhuman primates. However, despite the promising results, gene therapy has only rarely been tested in nonhuman primate models of human autoimmune disease. Nevertheless, such models could provide crucial information on the suitability of gene transfer vector systems *in vivo* before trials in humans are undertaken.

The purpose of this article is to discuss established nonhuman primate models of human autoimmune disease, focusing on arthritis and MS. We will review data from our own studies showing that these models are fit for use as preclinical test systems for gene therapies.

Nonhuman primates as model of human autoimmune disease

The most widely used nonhuman primates in autoimmune disease research are Old World monkey species, in particular the rhesus and cynomolgus macaque (*Macaca mulatta* and *M. fascicularis*). More recently, disease models in New World monkeys have been established, such as common marmosets (*Callithrix jacchus*) or cottontop tamarins (*Saguinus oedipus*). The common ancestor of macaques and humans dates back to 35 million years, while New World monkeys are thought to have shared a common ancestor with humans 55 million years ago.^{3,4} This evolutionary proximity is reflected by a high degree of similarity at the level of polymorphic gene families, such as those encoding major histocompatibility complex or T-cell receptor molecules or immunoglobulins, and also of leukocyte surface molecules and immune regulatory molecules, such as cytokines and chemokines. Of importance for gene therapies that make use of virus-based constructs is the fact that both human and nonhuman primates can be infected with similar types of viruses and that infections often follow a similar course.

Certain autoimmune diseases are primarily caused by autoantibodies. While T cells do not seem to play a direct pathogenic role, they support autoantibody formation. Prevalent examples are autoimmune thyroiditis, myasthenia gravis (MG), systemic lupus erythematosus (SLE), and Guillain-Barré syndrome. Actively induced models of MG⁵ and SLE⁶ have been established. However, in view of the already mentioned high antigenic similarity of nonhuman primates and humans surprisingly few attempts have been made to induce such antibody-based autoimmune diseases in nonhuman primates by adoptive transfer of patient sera.

T-cell-dependent autoimmune diseases are much more widely investigated in nonhuman primates, RA and MS in particular. In this article, we will primarily focus on these two diseases in which the feasibility of gene therapy has been tested in the past several years.

Nonhuman primate models of arthritis

Rhesus monkey model of collagen-induced arthritis

RA is the most prevalent immune-mediated arthritic disease in the human population. The pathological hallmarks of RA are the hyperproliferation of synovial tissue (synovitis) and the progressive destruction of joint cartilage and bone. The hyperplastic synovium becomes infiltrated with blood-borne T cells and macrophages already at an early stage, before warmth and swelling of the joints are observed.⁷ The infiltrated T cells are thought to contribute significantly to the pathophysiological cascade of reactions that gives rise to the disease and are, therefore, among the favored targets of immunotherapy.

Spontaneous cases of arthritis and osteoarthritis have been found in free-ranging rhesus monkeys at an incidence of around 20%, but are rare in animals kept under laboratory conditions. For this reason, actively induced models have been developed that can be established when needed. By immunization with collagen type II (CII) extracted from bovine cartilage in

complete Freund's adjuvant (CFA), a reproducible RA-like autoimmune polyarthritis could be induced in rhesus^{8,9} and cynomolgus macaques.¹⁰ The time of disease onset and the pattern of clinical signs may vary, probably reflecting the outbred character of this species. Bacterial antigens that have proven to be arthritogenic in susceptible strains of rats and mice fail to produce arthritis in rhesus monkeys.⁹

Collagen-induced arthritis (CIA) is a useful experimental model of human RA, which is widely used to investigate how pathogenic autoimmune mechanisms evoke joint inflammation and is used for the preclinical testing of novel intervention strategies.¹¹ The rhesus monkey model of CIA was originally designed as a preclinical model of human rheumatic disease for efficacy testing of biotechnology-derived pharmaceuticals, which by their species-specificity cannot be tested in rodents. The model shares several features of the joint pathology with human arthritic disease, and also shows some important differences. RA is a chronic progressive disease, which may affect multiple organs. Autoimmunity to CII is not consistently found in RA patients, but autoantibodies to self-IgG molecules (rheumatoid factors) are frequently detected. In contrast, CIA is a spontaneously remitting autoimmune disease that lacks rheumatoid factors, and inflammation is confined to the synovial joints and spinal column. Whereas in RA women are two to three times more often affected than men, a female prevalence has been reported in cynomolgus monkeys but was found to be absent in rhesus monkeys.¹⁰

A single immunization with bovine CII in CFA induces a heterogeneous pattern of joint inflammation in a random sample of rhesus monkeys from the outbred colony kept at the Biomedical Primate Research Centre in Rijswijk (The Netherlands).¹² Between 10 and 20% of the monkeys develop fulminant polyarthritis necessitating euthanasia within a few days after disease onset. At the other side of the spectrum there are about 30% nonresponders, which remain completely asymptomatic even after several booster immunizations. In the largest group of monkeys, the disease follows a more or less chronic course of variable severity and duration, which wanes spontaneously. In its most typical presentation, arthritis starts with warm and swollen ankles, and/or wrists (Figure 1). At a later stage, inflammatory swelling in one or more of the interphalangeal, (meta)carpal, and (meta)tarsal joints is observed. Finally, arthritis develops in knees, hips, and elbows. Occurrence of spondylitis is only rarely observed. The large body of data from rodent models indicates that CIA induction depends on synergy of delayed-type hypersensitivity and immune complex-mediated inflammatory mechanisms.^{13–15} This also seems to be the case in the rhesus monkey model of CIA.

A systematic comparison of responder versus non-responder monkeys and therapeutic intervention studies revealed genetic and immunological factors associated with CIA susceptibility. The fact that our Indian origin rhesus monkey colony has been pedigreed on the basis of segregation of major histocompatibility complex (MHC) class I and II polymorphisms has facilitated the identification of the MHC class I serotype *Mamu-A26* as a dominant resistance marker of CIA. In a large cohort of young adult monkeys, 95% of the CIA-resistant animals were *Mamu-A26* positive, whereas the marker was absent

Figure 1 Macroscopic and microscopic aspects of the rhesus monkey CIA model. The bottom figure (c) shows a rhesus monkey hand with prominent inflammatory swelling of the interphalangeal joints and wrist. PAS staining of proximal interphalangeal finger joint from a healthy (a) and severely arthritic (b) rhesus monkeys shows the dramatic erosion of joint cartilage in this model.

in 99% of the CIA-susceptible monkeys.¹⁶ As no influence of *Mamu-A26* on susceptibility to autoimmune encephalomyelitis exists, we hypothesized that the resistance mechanism is specific for the disease-inducing antigen.¹⁷ Molecular analysis revealed that the *Mamu-A26* serotype may represent a set of *Mamu-B* alleles (unpublished observations). However, the mechanism of the *Mamu-A26*-associated resistance to CIA still remains an enigma. The only immunological feature of *Mamu-A26*-positive monkeys that could be correlated with disease resistance was their incapacity to produce anti-CII IgM antibodies, while the absolute levels and epitope specificity of anti-CII IgG antibodies did not differ between *Mamu-A26*-positive and -negative monkeys.^{12,18} This could be relevant for disease induction, since previous work has clearly shown the important role of IgM antibodies in the initial autoimmune attack on the cartilage surface.^{19,20}

The parallel analysis of CII-immunized *Mamu-A26*-positive and -negative monkeys yielded a set of useful disease parameters that allow a more objective monitoring of the disease process. Predictably, the loss of

bodyweight is a reliable indicator of disease onset and remission. Moreover, the two main pathological processes in CIA, synovial inflammation and joint destruction, can be measured independently using plasma C-reactive protein levels and urinary excretion rates of collagen crosslinks, respectively.²¹

The beneficial effect of prophylactic T-cell-directed therapy with cyclosporin A²² or daclizumab, a humanized anti-CD25 antibody,²³ suggests that T cells play an important role in the onset of arthritis. Consistent with this view, we observed infiltration by T cells in synovial biopsies obtained before the development of clinical signs of arthritis.⁷ However, it is difficult to prove the pathogenic role of synovial T cells with adoptive transfer, since suitable syngeneic recipients are lacking in outbred species.

Towards gene therapy for RA

The potential benefits of gene therapy for the treatment of RA have been discussed in several recent reviews.^{24–27} Preclinical studies have revealed that therapeutic genes can be efficiently delivered to the synovium using either intra-articular injection of certain viral vectors or local delivery of cells that were transduced *ex vivo*. In addition, the systemic delivery of genes by either *ex vivo* or *in vivo* gene therapy has therapeutic effects on the onset or progression of disease in different animal models of RA.^{28–30} Fibroblast-like synoviocytes (FLS) have become a major target for *ex vivo* gene therapy in RA. For systemic approaches also lymphocytes and, more recently, dendritic cells have been used successfully.^{31,32}

In order to facilitate the uptake and expression of the gene of interest, several types of vectors are used. The most efficient vectors are modified/adapted naturally occurring viruses. The characteristics of the various viral vectors differ in terms of efficiency, cytotoxicity, antigenicity, size of exogenous DNA they will accept, as well as their ability to infect nondividing cells and integrate. The most commonly used viral vectors are derived from retroviruses, adenoviruses, adeno-associated virus (AAV), and to a somewhat lesser extent herpes simplex virus (HSV). Replication-deficient adenoviruses, which have high transduction efficiency, are currently the most widely used in preclinical and clinical studies. The commonly used adenoviral vector for gene therapy is based on adenovirus type 5. However, most humans already carry antibodies against this type of adenovirus and there is evidence that a single administration of a therapeutic useful dose of adenovirus generates an immune response that is sufficiently strong to prevent successful readministration of the same vector. In addition, there was dose-dependent liver and skeletal muscle toxicity as well as coagulation abnormalities and a decrease in platelet counts after use of a first-generation adenoviral vector in rhesus macaques.³³ Therefore, it is unlikely that these first-generation adenoviral vectors will be used in a clinical setting. Interestingly, helper-dependent ('gutless') adenoviral vectors, which do not induce an immune response and mediate prolonged transgene expression, are currently under development and these may be less toxic.

HSV-based vectors, which have been used in a rabbit model of arthritis, are able to transduce a wide variety of cells, but have inflammatory capacity because of the expression of viral proteins. Further development is

necessary before administration into human joints can be studied. Another potentially interesting vector is AAV, since the parental wild-type AAV has never been shown to have any pathogenic effect. Recent advances in methods for generating large-scale, adenovirus-free preparations have brought wider interest in the use of this vector system, also for RA. AAV2 has been used in different animal models of RA for systemic³⁴ and local gene therapy.³⁵ A nonhuman primate study involving endotracheal delivery of AAV to specific segments of the macaque lung did not show any sign of inflammation induced by the vector.³⁶ AAV vectors are currently being used in several clinical gene therapy trials, but AAV has as yet not been used in trials with RA patients. Few studies have investigated the potential of different serotypes of AAV, which may result in additional vectors capable of efficient gene transfer. The majority of retroviral vectors are based on the Moloney murine leukemia virus (MMLV). These vectors are usually applied for *ex vivo* gene therapy in clinical trials. Moreover, this vector has been used in the first gene therapy clinical trial in RA patients (see below). Studies with nonviral vectors have also been reported, but therapeutic efficiency has as yet not been demonstrated in RA models.

The number of possible therapeutic genes that may be used for gene therapy of arthritis is still growing. Blockade of the effects of IL-1 and TNF- α by gene therapy has shown prevention of disease progression and joint destruction in several animal models of arthritis.^{35,37–39} The combination of TNF blockade and IL-1 blockade by adenoviral gene therapy in arthritic rabbits was shown to be even more effective than blockade of either cytokine alone.³⁹ The transcription factor nuclear factor κ B (NF- κ B), which induces activation of many proinflammatory genes including TNF and IL-1, may be a potential target for local gene therapy. However, its widespread role could cause toxicity if used systemically. Blocking NF- κ B activity by direct intra-articular gene therapy with an adenoviral vector containing a dominant-negative I κ B kinase β (IKK) mutant clearly ameliorated rat adjuvant arthritis.⁴⁰ Other strategies include the transfer of genes encoding anti-inflammatory cytokines, which may suppress and counterbalance Th1-driven responses and inhibit pro-inflammatory cytokine production. Examples include IFN- β ,³⁰ IL-4,⁴¹ IL-10,³⁷ and IL-13,⁴² which have all been used successfully in animal models of RA.

Several factors may account for the increase in cellularity of rheumatoid synovial tissue, including inadequate apoptosis. Therefore, gene therapy might be used to induce apoptosis in the inflamed joints. Since the death factor Fas and its ligand FasL play pivotal roles in the induction of apoptosis of immune cells, adenovirus-mediated FasL gene transfer was examined in arthritic joints and was shown to reduce inflammation and ameliorate murine CIA.⁴³ Another way to induce apoptosis in synoviocytes would be by delivering the herpes thymidine kinase (TK) gene followed by administration of ganciclovir. This approach was used in rhesus monkeys with CIA⁴⁴ and will be discussed below in more detail.

Angiogenesis, the formation of new blood vessels, is one of the earliest histopathologic findings in RA and

appears to be necessary for pannus development. The efficacy of local expression of an antiangiogenic gene by *ex vivo* gene therapy using a retroviral vector was investigated in the murine CIA model.⁴⁵ Local production of angiostatin in the knee was able to prevent the onset of CIA not only in the knee injected with genetically engineered cells, but also in uninjected ipsilateral joints. The injection of an endostatin-expressing lentiviral vector directly into the joints of human TNF-transgenic mice resulted in reduced vessel density in the synovial tissue associated with a decrease in the overall mean arthritis index.⁴⁶

Two phase I studies have been performed in RA patients, one in the US and one in Germany.^{47,48} In these studies, a gene encoding human IL-1 receptor antagonist (IL-1RA) was transferred into the metacarpophalangeal (MCP) joints of female RA patients. Autologous synoviocytes were transduced *ex vivo* with a retroviral vector containing IL-1RA and the transduced cells were injected back into the MCP joints. All nine patients included in the US trial have completed the protocol, and the preliminary data confirmed that it is possible to transfer genes safely to human, rheumatoid joints and to express those genes intra-articularly. This was not only the first application of human gene therapy in arthritis patients, but also the first use of gene therapy approved for nonlethal disease. Based on these results a phase II trial has been planned.

Synovectomy by adenovirus-encoded thymidine kinase and ganciclovir in CIA in rhesus monkeys

To date the only published gene therapy study in a nonhuman primate model of arthritis was on removal of the hyperplastic synovium in CIA-affected rhesus monkeys.⁴⁴ Although this study addressed a specific question, it also covered important basic aspects of gene therapy in RA, including the feasibility of *in vivo* gene transfer to tissue cells, the biodistribution of the vector, and finally the overall safety of the procedure. The experimental strategy in the gene therapy efficacy trial was to transduce specifically synovial cells *in vivo* using intra-articular injection of replication-defective adenovirus harboring the herpes simplex virus TK gene (Ad.TK). TK gene therapy made the synovial cells sensitive to intravenously administered ganciclovir, resulting in apoptotic cell death. On the basis of experiments with reporter genes (LacZ and luciferase), we concluded that the intra-articularly injected adenovirus only infected superficial intimal macrophages and FLS without penetration into the underlying tissue layers (Figure 2). Interestingly, although several of the monkeys had detectable serum titers of antiadenovirus antibodies, this did not appear to affect transgene expression nor was synovial inflammation enhanced. In light of the safety concerns one might have, it is important to note that there was no vector spill outside the injected joints.

A second study from the same group addressed the influence of the chosen promoter on transgene expression in the tissues. It was shown that the expression of reporter transgenes (LacZ/luciferase) in human and rhesus monkey synoviocytes is several fold higher when driven by a cytomegalovirus promoter than by an adenovirus major late promoter.⁴⁹

Figure 2 Transgene expression in an arthritic knee joint. Anatomical view of an arthritic knee joint from a rhesus monkey affected by CIA 3 days after injection of 10^8 IU AdLacZ that was stained for β -Gal. The blue area indicates the presence of the protein product of the lacZ gene.

Nonhuman primate models of MS

Monkey models of MS

MS is a chronic inflammatory disease of the central nervous system (CNS). The pathological hallmark of MS and the most likely cause of the progressive neurological deficit is the CNS white matter lesion. Lesions are focal areas of inflammatory demyelination with a variable degree of axonal damage and gliotic scar formation. Although the etiologic trigger of MS is not known, most experts believe that autoreactive T- and B cells with specificity for components of CNS myelin contribute significantly to the disease. Similar to RA, MS is a clinically and pathologically heterogeneous disease. In a recently published broad survey of MS pathology at least four major types of lesions could be distinguished, which probably also represent four different disease mechanisms.⁵⁰ The authors found evidence for an autoimmune-based cause in only two of the subtypes, namely type I formed by cellular autoimmune reactions and type II by synergy of cellular and humoral autoimmune mechanisms.

The two established nonhuman primate models of experimental autoimmune encephalomyelitis (EAE), in the rhesus monkey and the common marmoset, each resemble a subtype of MS. Acute EAE in rhesus monkeys resembles postinfectious forms of acute MS with based type I lesions, whereas the chronic EAE model in marmosets rather resembles the more common chronic form of MS. This remarkable species difference stays intact when EAE is induced in both species with the same protocol, including complete adjuvant emulsions

Figure 3 T_2 -weighted brain MR-images of a rat and a common marmoset. In T_2 -weighted brain MR-images of naïve animals, the white matter of a rat and a common marmoset appear as dark gray areas (arrow-head). The picture illustrates that the common marmoset brain contains much more white matter than the rat brain.

with human myelin or recombinant human myelin/oligodendrocyte glycoprotein (rhMOG).⁵¹

A particularly useful aspect of both monkey models of MS is the fact that the ratio of white *versus* gray matter is much higher than in rodent models (Figure 3). Hence, magnetic resonance imaging (MRI) can be used to examine the development and spatial distribution of brain white matter in intact animals at different time points during the EAE course, comparable to its use in MS patients (Figure 4).

Common marmoset model of experimental autoimmune encephalomyelitis

Immunization with MS patient brain myelin emulsified in complete adjuvant induced chronic EAE in 100% of common marmosets in random samples from outbred colonies.^{52,53} The disease course in myelin-immunized marmosets varied among individual monkeys; cases with acute, primary progressive or relapsing-remitting/secondary progressive disease were found. Pathologically this model is characterized by a heterogeneous pattern of lesions in the white matter of the brain and spinal cord. In a single monkey MS-like early active, late active, chronic inactive, and remyelinating lesions can be found at the same time.⁵⁴ The pathomorphological aspect of the MS-type II-like lesions indicates that they are formed by specific inflammatory demyelination with sparing of axons.

It has become increasingly clear that MOG-reactive T- and B cells play a central role in the immunopathogenesis of this model. The 100% disease incidence in the outbred colony at the BPRC could be explained by the presence of a monomorphic MHC-DR molecule (Caja-DRB*W1201) in each tested monkey, which functions as a major restriction element for activation of MOG24-36 specific CD4+ T cells.^{55,56} Disease susceptibility was similar when EAE was induced with rhMOG or the encephalitogenic MOG peptide (14–36) that encompasses the specific CD4 cell epitope. Essentially, the same results were obtained by Genain *et al.*⁵⁷ The beneficial effect of the phosphodiesterase inhibitor rolipram on myelin-induced EAE supports the view that the pathogenic CD4 cells are of the Th1 type.⁵⁷ While autoreactive T cells were sufficient for the initiation of CNS inflammation, specific demyelination was dependent on the presence of anti-

Figure 4 MS-like brain white matter lesion in marmosets. T₂-weighted brain MR-image was made of a common marmoset immunized with human myelin during clinical EAE. The MR-image (a) reveals a hyperintense region (arrow) in the right hemisphere. The histological staining (b; Klüver Barrera) shows that this region (arrow) is a demyelinated lesion. A magnification of the lesion in the bottom panel shows the extent of myelin loss (c; myelin staining for 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP-ase)) of macrophage infiltration (d; MRP-14 antibody staining of infiltrating activated macrophages) and of axonal pathology (e; Bielschowski silver impregnation).

MOG antibodies, in particular those binding to conformational epitopes present in the extracellular domain of the MOG molecule.^{58–60} This might explain the observation that expression of clinical EAE in monkeys immunized with a chimeric protein of human proteolipid protein (PLP) and myelin basic protein MBP is associated with spreading of the autoimmune reaction to MOG.⁶⁰

The dominant role of MOG in the etiopathogenesis of marmoset EAE has prompted us to investigate in further detail the autoimmune mechanisms operating in the course of MOG-induced EAE. Marmosets immunized with rhMOG, which is a recombinant protein representing the 125 amino-acids long N-terminal extracellular domain of human MOG, developed in all cases severe demyelinating EAE. Most monkeys displayed primary progressive EAE after an asymptomatic period of variable length. Monkeys with a relapsing-remitting disease course were only rarely found. As in MS there was marked discrepancy between the lesion load and the severity of neurological deficit. Using serial MRI, the presence of many large confluent brain white matter

lesions could be demonstrated weeks before clinical signs developed.⁶¹

Therapeutic intervention studies shed some light on the immunopathogenic mechanisms underlying lesion formation and neurological deficit. It was shown that treatment with an antagonist mouse antibody to human CD40 (5D12) protected myelin-immunized marmosets against clinical EAE. However, clinical signs emerged as soon as antibody treatment was discontinued or when a neutralizing immune response developed.⁶² The mechanism of action of anti-CD40 therapy was investigated in the rhMOG-induced EAE model.⁶³ Early administration of humanized 5D12 protected monkeys against the development of clinical EAE, but did not completely suppress inflammatory demyelination of the CNS. The proliferative response of axillary and inguinal lymph node cells towards MOG was at the same level in antibody-treated and placebo-treated monkeys. However, there was marked suppression of the anti-MOG autoantibody response.

IL-12, by which CD40 exerts in part its effects, is another interesting therapeutic target.^{64,65} This cytokine

promotes Th1 responses to autoantigens, and is clearly expressed in the EAE-affected marmoset brain in early lesions.⁶⁶ Antibody-mediated neutralization of IL-12 had a strong protective effect on both the clinical and neuropathological expressions of EAE in marmosets.⁶⁷ Of special note in both studies was the observation that molecules with the size of an antibody could penetrate lesions and modify local immune responses.

Rhesus monkey model of experimental autoimmune encephalomyelitis

In its characteristic clinical course and destructive pathomorphological presentation of brain white matter lesions, the rhesus monkey model of EAE resembles acute forms of MS, such as disseminated encephalomyelitis and acute hemorrhagic encephalomyelitis. Recent unpublished data indicate that the MS type I-like lesions in this model are formed by a complex pathogenic process involving at least the synergy of anti-self and antiviral immune reactions.⁶⁸ The influence of MHC class II genes on disease susceptibility,¹⁷ the suppressive effect of anti-HLA-DR and anti-CD4 antibodies,⁶⁹ and the induction of brain white matter inflammation with CSF pleiocytosis by autologous transfer of myelin-reactive T-helper 1 cell lines⁷⁰ all support a central role of autoreactive Th1 cells in the EAE pathogenesis. Auto-antibodies do not seem to play a direct pathogenic role in this model.⁶⁸ This concept provided the basis for a gene therapy trial aimed at intra-CSF delivery of IL-4, a potent inhibitor of Th1 cell functions. This experiment will be discussed below.

Modulation of experimental autoimmune encephalomyelitis by gene therapy

The pathogenesis of autoimmune encephalomyelitis in primates involves cognate interaction between T cells and APC at multiple levels, namely in peripheral lymphoid organs where autoreactive T cells are primed, at the blood-brain barrier (BBB), within the brain parenchyma, and within the cervical lymph nodes and spleen where macrophages containing phagocytosed myelin drain to.^{71,72} Proinflammatory cytokines are formed as a result of T cell/APC interaction and play a central role in the orchestration of the immunological mechanisms that drive the pathogenesis of MS and are, therefore, considered important targets for immunotherapy. Systemic administration of neutralizing reagents (including antibodies and soluble receptors) and counter-regulatory cytokines has proven efficacy in rodent EAE models. However, several anticytokine therapies that worked well in EAE in mice and rats were less effective or even had detrimental effects in patients. This might be explained as owing to the timing of the treatment, the route of drug administration, and the fundamental immunological differences between rodents and primates.

Biological molecules that are considered to act within the brain and spinal cord have to take several hurdles on their way to the affected target organ. First, the molecule has to pass the BBB, which under nonpathological conditions separates the brain from the peripheral circulation. When CNS inflammation occurs, the permeability of the BBB increases under the influence of inflammatory mediators, such as TNF- α , IL-1 β , and

matrix metalloproteinases. Under these conditions, large biological molecules, such as antibodies, could extravasate into the lesions^{62,67} (Figure 5). On the other hand, a significant proportion of active demyelinating lesions in the EAE-affected common marmoset brain did not show contrast enhancement at T1-weighted MRI, suggesting that the BBB is impermeable in these lesions.⁵⁴ This might explain why therapeutic antibodies, such as anti-human CD40 or anti-human IL-12, are only partially effective when administered in a therapeutic fashion.⁶² Second, there is poor diffusion of larger (biological) molecules within the CNS white matter. The diffusion coefficient of a 20 kDa protein in CNS tissue is in the order of 10^{-6} cm²/s.⁷³ This implies that it takes more than 3 days for a molecule of that size to diffuse 1 mm into the CNS tissue.

These hurdles could theoretically be circumvented by the use of gene therapy. Delivery of Th2 'protective' cytokine genes that are able to counteract the detrimental activity of Th1 cytokines has been studied in various EAE models. Both viral vectors and plasmids containing cytokine genes have been delivered systemically, either in the blood stream or using circulating encephalitogenic T cells. An alternative approach is direct administration of the construct into the CNS. By transferring cytokines

Figure 5 Localization of intravenously injected anti-CD40 antibody (blue) within the CNS. We injected 1 mg of the mouse-anti-human CD40 antibody 5D12 in 0.5 ml phosphate-buffered saline (PBS) into the vena saphena of an EAE-affected common marmoset 1 h before the monkey was killed. Cryosections of lesions were double-stained with anti-laminin antibody to demarcate the Virchow-Robin space (red) and with anti-mouse IgG antibody to visualize localization of injected anti-CD40 antibody. The pictures show prominent localization of anti-CD40 within the perivascular space, but also within the surrounding white matter.

in encephalitogenic T cells, 'suppressor' T cells are generated that will home to the sites of inflammation and demyelination in the CNS. After crossing the BBB, these cells could be reactivated to produce Th1-antagonizing molecules *in situ*, thereby inhibiting neighboring Th1 encephalitogenic T cells. Previous work has shown that IL-4 gene therapy ameliorated murine EAE, whereas TNF- α exacerbated the disease, and IL-10 was ineffective.^{74,75} An MBP-specific Th1 clone transduced with a retroviral vector containing the TGF β gene delayed and ameliorated EAE development in mice when administered systemically before or at EAE onset.⁷⁶ MBP-specific CD4⁺ T cells transduced with the IL-12 p40 gene were also able to reduce disease activity in EAE.⁷⁷ Transduced CD4⁺ T cells were able to traffic into the CNS, as shown by the detection of bioluminescent lymphocytes, and exhibited long-term transgene expression. MBP-specific T cells were also successfully used in EAE in Lewis rats, where a gene encoding nerve growth factor was shown to suppress the induction of EAE.⁷⁸

The systemic injection of viral vectors has been reported in only one study.⁷⁹ Viral vectors derived from Vaccinia virus were injected intravenously into mice affected by EAE. Vectors containing the genes encoding IL-6, TNF- α , IL-1, IL-2, and IL-10, but not IL-4 or IFN- γ ameliorated the disease. The data are limited, however, since clinical parameters were evaluated only during a 1-week period and no histopathological evidence for a disease-protective effect was provided.

Plasmid constructs engineered with cytokine genes, which were injected intramuscularly or intracerebrally, have also been used in mice with EAE. Intracerebral administration of naked cytokine DNAs was not successful in the treatment of EAE. However, intramuscular injection of naked DNA coding for TGF β or IL-4-IgG chimeric protein into mice affected by MBP-induced EAE did reduce clinical and histopathological signs and symptoms.⁸⁰ In these protocols, a pronounced down-regulation of proliferating activity of MBP-specific T cells was observed. In contrast, cDNA coding for IL-4, TGF β , IFN- β , p75TNF-, and p55TNF receptor-Ig complexed with lipofectin-ameliorated EAE when injected intracerebrally 3 days before disease onset.⁸¹

Different therapeutic outcomes in EAE dependent upon the mode of delivery of IL-10 were found. IL-10 encoding DNA-liposome complexes were ineffective, and an adenoviral vector encoding murine IL-10 failed to inhibit EAE when injected into the parenchyma of the CNS, despite producing high levels of IL-10 protein. In contrast, intracerebral injection of fibroblasts transduced with a retroviral vector expressing IL-10 inhibited murine EAE.⁸² Overall, the results pointed to a limited effect of direct injection of genes encoding cytokines into the parenchyma of the CNS. Interestingly, an IL-4 encoding HSV-vector injected intrathecally did not only have a beneficial effect in murine EAE when administered before the development of clinical signs, but also in established disease.^{83,84} The same approach was therefore evaluated in nonhuman primates with EAE.

IL-4 gene therapy in rhesus monkeys with experimental autoimmune encephalomyelitis

By its close proximity to chronic MS, the common marmoset model of EAE provides in general a very

useful model to test new therapies for chronic MS. However, vector administration in a clinically relevant manner, for instance via the occipital or lumbar route, is hampered by the small size of the common marmoset. For this reason, we investigated *in vivo* gene therapy in myelin-induced acute EAE in rhesus monkeys using an intrathecally administered gene construct.⁸⁵ HSV deleted of genes encoding infected cell proteins (ICP) was used, because previous work has shown that this provides a safe and effective vector for gene transfer into the CNS in nonhuman primates.⁸⁶ These vectors can accommodate multiple foreign genes, can be grown at high titer and, importantly, infect postmitotic cells including most resident cells within the CNS. The construct was a replication-deficient HSV type I encoding human IL-4 (abbreviated as TH4). In controls, the IL-4 gene was replaced by LacZ (THZ). The vectors were directly injected into the CSF, either into the cisterna magna or via lumbar puncture to mimic a possible future administration route in humans.

Tests with the THZ vector in naïve rhesus monkeys, killed 3 days after the vector injection, revealed widespread distribution of the virus in all ventricular and meningeal spaces, in association with limited penetration. Only the superficial cell layers directly facing the CSF were infected, namely ependymal, choroidal, and leptomeningeal cells. We did not observe signs of direct toxicity, apart from a transient and nonspecific inflammatory reaction because of the vector injection. IL-4 levels of 0.1 and 0.4 pg/ml CSF, produced by vector-infected choroidal and ependymal cells, were measured in CSF collected 30 days after intrathecal injection of the TH4 construct in two naïve monkeys. Interestingly there was no vector leakage into peripheral blood, induction of anti-HSV-1 antibodies, or skewing of peripheral cytokine production.

Having demonstrated that vector administration had no overt negative side effects, we subsequently tested the potentially beneficial effect of TH4 on EAE. In all, 10 monkeys were randomly divided into two groups, immunized with human myelin in CFA, and followed up for 45 days. Four monkeys received 10⁸ PFU of the control construct THZ 11 days after immunization; these animals developed acute clinical EAE with severe inflammatory brain lesions, while the fifth one developed milder signs of encephalitis. In contrast of the five TH4-treated monkeys, three remained completely devoid of any neurological and pathological signs (Figure 6). The beneficial effect of the TH4 gene therapy treatment was reflected by a clear reduction in mRNA expression for mRNA levels in CSF cells of TNF- α and MCP-1 in CSF cells.⁸⁵

Taken together, these data indicate that intrathecal injection of an engineered herpetic vector is a safe and nontoxic approach for sustained delivery of IL-4 into the CSF. Moreover, treatment with the IL-4 encoding vector may clearly suppress clinicopathological signs of acute EAE in myelin-immunized monkeys.

Future developments

Selective targeting of specific molecules in affected organs by gene therapy remains a promising approach, which can be tested in nonhuman primate models of

Figure 6 Gene therapy in EAE-affected rhesus monkeys. In all, 10 randomly selected monkeys were immunized with human myelin in complete adjuvant to induce autoimmune encephalomyelitis. At day 11 after immunization, we injected into the CSF of five monkeys HSV-1 vector containing the human IL-4 gene (circles) and in the remaining five monkeys HSV-1 vector containing the lacZ gene (squares) (copied with permission from Poliani et al⁷³).

human disease. The site-specific delivery of 'regulatory proteins' that could correctly modulate the immune response holds promise as an innovative therapeutic strategy for immune-mediated diseases, including RA and MS.

Autoreactive T cells infiltrating the CNS white matter during the course of MS as well as antigen-specific T cells migrating into the inflamed joints in RA could represent attractive vehicles for therapeutic genes. Intriguing results have already been obtained in inbred rodent models of arthritis,⁸⁷ neuritis,⁸⁸ and encephalomyelitis.⁷⁸ However, it is still an open question whether this approach will be feasible in the genetically heterogeneous human population. It is not clear, for example, if each patient will need autologous T cells as vector or that perhaps a limited set of donor cells could be sufficient. This research question can be well investigated in an outbred population of nonhuman primates.

A second issue that may be addressed in nonhuman primate models is the species specificity of vectors. Stable transduction of haemopoietic stem cells resulting in lineage-specific expression of therapeutic genes is one of the goals in gene therapy for autoimmune diseases. However, high-level transduction of hemopoietic stem cells appears to be considerably more difficult in primates than was expected on the basis of the promising results obtained in mice. Nevertheless, the feasibility of gene transfer into bone marrow-derived hemopoietic stem cells and progenitor cells has now been shown in the rhesus monkey with adenoviral vectors^{89,90} and an amphotropic retroviral vector in the common marmoset.⁹¹ However, although much effort is put in the development of safety-modified lentiviral vectors that are now developed for transduction of human stem cells, safety remains the burning issue with all retroviral vectors.^{92,93} The recently reported occurrence of lymphoma with clonally integrated viral vector in a patient who received gene therapy for severe combined immunodeficiency seems a tragic illustration of the potential risks with integrating viral vectors (<http://sciencenow.sciencemag.org>; October 3, 2002).

Third, nonhuman primates, in particular the rhesus monkey, provide a useful animal model, in which adenoviral or other viral vector toxicity can be studied. Most risks involved in gene therapy are related to vector

systems or the genes they carry. Although viral vectors are genetically modified to minimize their ability to replicate and cause disease, an immune response to viral proteins may still occur and there is a possibility of recombination of the virus resulting in replication-competent viruses. The importance of the potential risks of the use of viral vectors is illustrated by the fatal death of a patient in a gene therapy trial. To understand the cause of this tragic event, rhesus monkeys were treated with high dosages of adenoviral vector, similar to the human trial.⁹⁴ From these experiments, evidence emerged that the protein coat of the vector possibly triggered a massive cytokine release by the recipient's immune system.

The use of live attenuated viral vectors has many opportunities and challenges for gene therapy of autoimmune diseases. Perhaps the greatest challenge is to overcome pre-existing immunity to vectors derived from viruses that are widespread in the human population, such as herpes- or adenovirus. Significant advances have been made to attenuate these vectors so that they do not cause disease in a naïve host. However, it remains to be seen if the immunological triggers induced by live vectors can be beneficially applied in an autoimmune setting. Clearly, the tissue-specific delivery and cell-specific expression of the therapeutic transgene are areas of current focus, which will provide us with important advances in the future. Taken together, it can be anticipated that nonhuman primates will increasingly be used to improve delivery systems for gene therapy and to understand and prevent toxicity.

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