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Intrasplenic electro-transfer of IL-4 encoding plasmid DNA efficiently inhibits rat experimental allergic encephalomyelitis

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Abstract

Most of the previous studies in which cytokine DNA plasmids were delivered by systemic administration exhibited only marginal therapeutic effects, if any, in the EAE model. One strategy to overcome this limitation would be to determine the optimal delivery route leading to significant beneficial effects both in early (prophylactic) and late (therapeutic) treatments. To address this issue, we directly compared the effects of intrasplenic (IS) and intramuscular (IM) electro-transfer of interleukin-4 (IL-4) DNA in the rat experimental allergic encephalomyelitis (EAE) model. In the preventive experiment, rats received IM (25 or 150 μg) or IS (25 μg) administration of IL-4 DNA followed by in vivo electroporation the day before MBP immunization. In the late treatment experiment, rats were treated with IM (150 μg) or IS (25 μg) administration of IL-4 DNA with electroporation 10 days after MBP immunization. As a control the same amount of vector DNA was used. Macroscopic analysis indicated that the onset of moderate to severe EAE in rats treated with IS transfer of 25 μg of IL-4 DNA was prevented on a significant level compared with IM 25 μg of the IL-4 DNA transfer group or the control group in the preventive experiments. More importantly, IS transfer of 25 μg of IL-4 DNA considerably suppressed the severity of EAE in late treatment experiments while IM transfer of 150 μg of IL-4 DNA had little effect. The MBP-specific expression of IFN-γ from stimulated splenocytes was considerably decreased by the IS IL-4 DNA transfer group both in the preventive and therapeutic experiments while IM transfer had this effect only in the preventive protocol. Histological analysis showed that spinal cord inflammation was considerably reduced in the IS IL-4 DNA transfer group. These data provide the first demonstration that IS electro-transfer of IL-4 DNA is more effective both in the prevention and modulation of EAE than IM transfer and that IS electro-gene transfer may present a new approach to cytokine therapy in autoimmune diseases.

Keywords: Experimental allergic encephalomyelitis; Gene therapy; Interleukin-4; In vivo electroporation

Experimental allergic encephalomyelitis (EAE) is an animal model for human multiple sclerosis (MS) [1]. It is a T-cell mediated demyelinating disease of the central nervous system (CNS) [2]. Pro-inflammatory cytokines (i.e., Th1 cytokines), such as IFN-γ, TNF-α, and TNF-β, are believed to play a crucial role in this pathogenic process since they can promote and sustain the development of myelin-specific T cells and promote the recruitment of peripheral myelinotoxic effector cells (i.e., monocyte/macrophages) [3,4]. Therefore, blockade or regulation of pro-inflammatory cytokines has been considered as an effective therapeutic strategy for EAE. Apart from direct blockade of IFN-γ/TNF by soluble receptor or blocking antibody, regulation can be achieved by modulatory cytokines such as IL-10, TGF-β, and IL-4 [5–8].

Owing to the chronic nature of the disease, gene therapy offers potentially unique advantages over previous protein

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therapy. When compared with various viral and non-viral techniques for gene transfer, naked DNA is probably the safest, simplest, and most inexpensive [9]. Recently, ourselves and others have shown that the gene transfer efficiency of naked DNA could be significantly enhanced by in vivo electroporation, resulting in beneficial effects in the animal model of autoimmune arthritis [10–13]. In this study, we applied this technology for the delivery of IL-4 DNA in the rat EAE model.

Most of the previous studies in which cytokine DNA plasmids were delivered by systemic administration exhibited little, if any, therapeutic effects in the EAE model [14–16]. One strategy to overcome this limitation would be to find the optimal delivery route that leads to significant therapeutic effects both in early (prophylactic) and late (therapeutic) treatments. Among the candidate organs to be targeted for electro-transfer of naked DNA, the spleen could be an appealing site. It is one of the most important lymphoid organs, involved in the initiation of immune responses. Because the spleen is enriched in lymphocytes, especially in the area of white pulp, it has been targeted for various immune modulation strategies such as vaccination [17]. Moreover, it has been reported that efficient intrasplenic (IS) gene transfer could be achieved by in vivo electroporation without impairment or over-activation of immune response [18]. The skeletal muscle could be another promising site for electro-gene therapy due to its large size, good capacity for protein synthesis, easy accessibility for intramuscular (IM) injection, and the possibility for repeat injections. Furthermore, the muscle has been shown to cause uptake of DNA and express it for a long time after IM administration [19–21].

In this study, we directly compared the beneficial effects of IS and IM electro-transfer of IL-4 DNA in the rat EAE model. A plasmid DNA encoding IL-4 gene was introduced into the spleens or skeletal muscles of EAE rats, and its effect on EAE was measured by clinical evaluation, histological examination, and in vitro analysis of splenocytes.

Materials and methods

Plasmid DNA. As a murine IL-4 expression vector, pCK-mIL4, which has been previously described in detail, was used [11]. The plasmids were purified using an EndoFree plasmid Maxi prep kit (Qiagen,Valencia, CA, USA), dissolved in 0.9% NaCl, diluted to 4 μg/μl, and stored at −20 °C prior to use.

DNA injection and in vivo electroporation. Rats were anesthetized with ketamine (6.75 mg/rat)/xylazine (330 μg/rat). For IM DNA transfer, aliquots of 50 μl or 300 μl of plasmid DNA (pCK-mIL4 or control pCK) at 0.5 μg/μl in 0.9% NaCl were injected into one or two sites in the gastrocnemius muscle of the hind legs (total amount of DNA was 25 or 150 μg per rat). Commercially available two-array needle electrodes (model 530, BTX, San Diego, CA) were used for electroporation. The needle electrodes were applied to the shaved skin on either side of the marked DNA injection point. Consecutively square-wave electrical pulses were administered eight times using an ECM830 pulse generator (BTX, San Diego, CA) at 400 V/cm and a rate of one pulse/s, with each pulse being 20 ms in duration.

For IS electro-transfer, after anesthesia, a 2 cm laparatomy was performed on the left flank and the spleen was drawn out from the peritoneal cavity. The vasculature of the spleen was clamped and a 30-gauge syringe was then deeply inserted into the spleen and the DNA was injected [18]. A total of 25 μg of plasmid DNA diluted in 50 μl of 0.9% NaCl was used for each rat. Immediately after DNA injection, the caliper electrodes (model 383, BTX) were placed on each side of the spleen, the electric pulses were applied using an ECM830 pulse generator, and the clamp was then removed. The electrical parameters for IS electro-transfer were eight pulses of 20 ms at 200 V/cm at a frequency of 1 Hz. The spleen was then placed back into the peritoneal cavity and the incision was sutured.

Induction of EAE and treatment protocol. Lewis rats (Charles River, MA, USA), weighed 200–250 g at the start of experiments, were immunized subcutaneously into the hind footpad with a mixture of purified guinea pig myelin basic protein (MBP) (25 μg/100μl of PBS; Sigma, MO, USA), emulsified in Freund's complete adjuvant containing *Mycobacterium tuberculosis* H37Ra (250 μg/100 μl; Sigma) in a final volume of 200 μl. In the preventive experiment, on the day prior to immunization, the rats were divided into six groups and individually treated with either IM electro-transfer of IL-4 or control DNA (25 μg or 150 μg per rat) or IS electro-transfer of IL-4 or control DNA (25 μg per rat). In the late treatment experiment, IM or IS electro-transfer of IL-4 or control DNA (25 μg per rat) was performed 10 days after MBP immunization. The treated rats were monitored for 20 days after immunization and were then sacrificed.

Macroscopic scoring of EAE. Clinical EAE scores were evaluated daily for 20 days post-MBP immunization. Clinical signs were scored on a scale from 0 to 4: 0, no clinical signs; 0.5, partial loss of tail tonus; 1, complete loss of tail tonus; 1.5, unsteady gait; 2, paresis of hind legs; 2.5, complete paralysis of the hind legs and/or lower part of the body; 3, paresis of the complete lower part of body up to the diafragma; and 4, death due to EAE [22]. Scoring of clinical EAE grade was carried out by two independent observers who were blinded with regard to the experimental groups.

Histopathology. The spinal cords of the rats were removed and fixed in 10% phosphate-buffered formalin for 2 days and then embedded in paraffin. Five micrometer slices of the spinal cord tissue were prepared and stained with hematoxylin and eosin. The tissue was then examined by light microscopy in a blinded manner by a pathologist, evaluated for the extent of inflammation, and then graded as previously described with a slight modification: 0, no inflammation; 1, a few mononuclear cells; 2, organization of inflammatory infiltrates around vessels; 3, extensive peri-vascular cuffing [23].

Measurement of cytokine levels in rat serum or cultured splenocytes. The levels of murine IL-4 in rat sera and the levels of rat IFN-γ, rat IL-4, and murine IL-4 in cultured primary spleen cells were measured using commercially available ELISAs for murine IL-4 (Endogen, IL, USA), rat IFN-γ (R&D Systems, Minneapolis, MN, USA), and rat IL-4 (R&D Systems), according to the manufacturer's recommendations. Briefly, the sera were obtained from sacrificed rats and directly subjected to mouse IL-4 ELISA without any pretreatment. In the case of rat IFN-γ and rat IL-4 levels, splenocytes (1×10^7 cells/ml), which were isolated from IL-4 DNA or control DNA-treated rats, were cultured for 24 h in 24-well plates either with medium containing MBP (10 μmol/ml) or concanavalin A (ConA; 50 μg/ml). Then the supernatants obtained from MBP- or ConA-stimulated splenocytes were directly subjected to ELISA. The MBP-specific induction of IFN-γ or IL-4 was calculated by dividing the level of INF-γ or IL-4 from MBP-stimulated splenocytes by that from ConA-stimulated splenocytes. For mouse IL-4 levels in supernatant from cultured splenocytes, splenocytes (1×10^7 cells/ml) were isolated from IL-4 DNA or control DNA-treated rats and were cultured for 72 h in 6-well plates, and then supernatant was used for mouse IL-4 ELISA.

Real-time quantitative PCR. The presence of transfected pCK-mIL4 DNA in splenocytes or PBMC from the IL-4 DNA or control DNA-treated rats was examined by real-time quantitative PCR using primers specific for pCK-mIL4. The primer sequences were as follows: pCK, 5′-TCT TTT CTG CAG TCA CC-3′; and mIL4, 5′-CTT CTC CTG TGA CCT CGT TC-3′. Real-time quantitative PCR was employed using the ABI Prism 7700 sequence detector system (PE Biosystems, Foster City, USA) as previously described [24]. The amount of fluorescence measured in a sample is proportional to the amount of specific PCR product