行政院國家科學委員會專題研究計畫成果報告

計畫名稱：實驗性自體免疫前葡萄膜炎之基因治療

英　　文：Gene Therapy for Experimental Autoimmune Anterior Uveitis

計畫類別：個別型計畫

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中文摘要

關鍵詞：葡萄膜炎、疫苗、細胞間素、脂小體、自體免疫疾病。

葡萄膜炎一般認爲是一種自體免疫疾病，其確切的病因與致病機轉不明。反覆發作可因各種併發症如角膜病變、白內障、青光眼、網膜水腫或損傷而造成失明，是失明的重要原因之一。

一般認爲葡萄膜炎是一種延發型過敏反應 (Delayed type hypersensitivity) 所造成的，在人類葡萄膜炎的組織病理檢查許多實驗動物模型可以得到證實，而最近的許多證據顯示 DTH 是由可分泌 γ-INF、IL-2、IL-12 的 T helper 1 (Th1) 所引導，這相對於分泌 IL-4, IL6、IL-10 爲主的 T helper 2 (Th 2)。後者可能是導致 Type 1 hypersensitivity 如氣喘等的重要原因。 Th 1 與 Th 2 相關的細胞間素可互相抗衡。因此在局部環境中給予較大量的 IL-4 可使原來會形成 Th 1 的反應轉成 Th 2，而較多量的 IL-12 也可使原來會形成 Th 2 的反應轉成 Th 1。

本研究將使用帶有 Interleukin 4 plasmid 的脂小體 (Liposome) 與治病抗原牛的黑色素相關抗原 (Melanin Associated Antigen, MAA) 先後打入實驗動物路易士鼠 (Lewis Rat)的後腳。我們的假設是當脂小體匯流到腳附近的淋巴結，而被其中的抗原呈現細胞 (Antigen Presenting cells, APC) 所吞噬之後，便可以在細胞附近的微細環境(Microenvironment)中有較多的 IL-4，這將有助於 Th2 免疫反應。於是在其後吸取並呈現抗原給 T 細胞時便能引導該 T 細胞成 Th 2 型。因而抑制 Th1 的免疫反應與延發型過敏反應，從而抑制或防止實驗性自體反應前葡萄膜炎的發生。而且在其後數週至數月當中，當有相同的抗原進入體內時，便能以 Th 2 而不是 Th 1 的方式反應，於是以 Th 1 反應爲主的實驗性自體免疫葡萄膜炎 (Experimental Autoimmune Anterior Uveitis，EAAU) 便能得到預防。而脂小體可以使 IL-4 plasmid 在組織間滯留時間延長，並增加 APC 的攝取，有助於增長 '疫苗' 的效期和效價。

本法所用的只是微量的核甘酸，可避免全身大量投予細胞介質而達到免疫調整的目的。而注射黃甘酸與致病抗原於同一地方則可使該免疫調節較具抗原專一性。本法應可稱爲'治療性疫苗'。如能應用在葡萄膜炎病人而能在數月或數年之中防止葡萄膜炎的發作或再發，當是葡萄膜炎患者的一大福音。

ABSTRACT

Keywords : **Uveitis, autoimmune disease, liposome, interleukin, vaccine**

Experimental autoimmune anterior uveitis (EAAU) is an inflammatory autoimmune disease of anterior uveal tract, which serves as a model for human acute anterior uveitis. It has been shown that EAAU is mediated by CD4+ T cells and delayed type hypersensitivity. We have shown that intraocular injection of soluble melanin associated antigen can prevent the development of primary onset and recurrence of EAAU.

To test the possibility of gene therapy for EAAU, we will inject IL-4 plasmid entrapping liposome and bovine MAA (uveitogenic antigen in the model of EAAU) into the hind footpad of Lewis rat. Our rational is the injected liposome-entrapped IL-4 plasmid will be taken to regional lymph node and transfect the lymphoid cells and antigen presenting cells over there. This will develop an IL-4 rich microenvironment that favors T helper 2 (Th2) response when antigen is presented to and activate specific T cell clones. T helper 2 pattern of cytokine secretion will downregulate T helper 1 (Th1) response and delayed type hypersensitivity and therefore prevent and/or attenuate the severity of the autoimmune inflammatory disease. The liposome entrapped plasmid will stay longer in tissue and also easier to be taken along with lymph to regional lymph node, and will make this gene therapy last longer and more effective.

This gene therapy will only involve the use of tiny amount of nucleic acid, and will avoid large amount of systemic administration of cytokines to modulate immune response. The injection of the mixture of antigen and nucleic acid at the same site can make this immunomodulation more antigen specific. This approach could be called 'therapeutic vaccine'. If it works and can be applied to uveitis patients, it will be of great benefit to them, who usually suffer from repeated recurrences and various complications that will lead to blindness.

INTRODUCTION

Uveitis is considered as a group of autoimmune disease. Recurrences and complications such as band keratopathy, cataract, glaucoma, macular edema and other retinal insult can cause blindness. The etiology and pathogenesis is not clear. Several animal models have been established to study the etiology and pathogenesis of uveitis, including experimental autoimmune uveitis, endotoxin induced uveitis, experimental phacoanaphylactoid uveitis, and experimental autoimmune anterior uveitis. Experimental autoimmune anterior uveitis (EAAU) is an inflammatory autoimmune disease of anterior uveal tract, which serves as a model for human acute anterior uveitis. It has been shown that EAAU is mediated by CD4+ T cells and delayed type hypersensitivity.

T lymphocytes have been implicated in a variety of autoimmune diseases, and therefore one potential therapeutic approach would be to tolerize the pathogenic self-reactive T cells. Cytokines may exert anti-inflammatory properties, allowing the hypothesis of their potential therapeutic use. Targeting the cytokines balance may modify the course of subsequent inflammatory events in an autoimmune disease. T helper 1 response, secreting mainly γ interferon, IL-2, and IL-12, favors delayed type hypersensitivity and augment autoimmune inflammatory reaction in various experimental autoimmune models which is mediated by delayed type hypersensitivity such as experimental rheumatoid arthritis, experimental autoimmune encephalomyelitis, EAU and EAAU. Microenvironment rich in Th1 patterns of cytokine can switch the original Th 2 response to Th 1 and vice versa. IL-4, IL-10 or IL-13 can switch Th1 response to Th2 and serve as a protection against several experimental autoimmune diseases mentioned above. . They are sometimes called anti-inflammatory cytokines. The very short half-life of cytokines makes them difficult and expensive to use directly; in such occurrence, high quantities have to be frequently injected. Gene therapy appears as an effective alternative solution. The transfection of cells with cytokines genes (e.g. either IL-4) and the engraftment of these vectors in animals, permit the in vivo secretion of high levels of Th 2 cytokines, and result in the protection of the animals from the development of Th1 and DTH related autoimmune diseases.

This gene therapy will only involve the use of tiny amount of nucleic acid, and will avoid systemic administration of large amount of cytokines to modulate immune response. The injection of the mixture of antigen and nucleic acid at the same site can make this immunomodulation more antigen-specific. This approach could be called 'therapeutic vaccine'. If it works and can be applied to uveitis patients, it will be of great benefit to them, who usually suffer from repeated recurrences and various

complications that will lead to blindness.

MATERIALS AND METHODS

1. IL-4 plasmid. Dr. Bor-Luen Chiang, Graduate Institute of Clinical Medicine, National Taiwan University, will kindly provide the plasmid. The IL-4 cDNA from ATCC (ATCC37561) was cloned into the pcDNA3 (Invitrogen Corp., San Diego, CA, USA) expression vector (pcD/3-IL4) under the control of a CMV promoter. This construct was grown in DH5 α cells, and the plasmid was subsequently purified by double $CsCl_2$ gradient ultracentrifugation.
2. Liposome entrapping IL-4 plasmid. Dr. Yu, Department of Pharmaceutics, National Taiwan University will kindly manufacture this liposome. Small unilamellar vesicles prepared from 16 μmol phospatidylcholine and 8 μmol dioleoxyl phosphatidylcholine in the absence or presence of 4 μmol of sterylamine (SA), 1,2-Dioleoxyloxy-3- (trimethyl-ammonium) propane or carbamyl cholesterol (cationic liposomes), were mixed with 10-100 μg DNA and freeze-dried overnight. Following rehydration under controlled conditions, the generated dehydrated-rehydrated vesicles were washed by centrifugation and suspended in 0.15 M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7.4 (PBS). DNA entrapment values were 77-83% of the amount used. DNA immunization.
3. DNA Vaccination. Female Lewis rats will be injected into left hind footpad with 50 μl PBS containing 1-10 μg empty liposomes or liposomes entrapping IL-4 plasmid.
4. Experimental autoimmune anterior uveitis.
   a. MAA (Melanin Associated Antigen): MAA will be extracted from bovine uveal tissue as described in the literature. Briefly, the anterior segment of the bovine eye was removed by cutting along the latitude of 4 mm behind limbus. The iris tissue was cut off, collected and ground in minimal amount of phosphate buffered saline (PBS). Big chunks were filtered off through gauze. The filtrate will be washed twice with PBS and centrifuged at 10,000 g. The insoluble part will be incubated with 2% SDS at 75°C for 10 minutes and centrifuged again at 10,000g and the insoluble part will be dried and weighed and frozen at -80°C for later use as MAA after resuspension with PBS.
   b. Animals. Female Lewis rats (150 to 200 g) will be purchased from Experimental Animal Center, NSC, Taiwan. Five rats will be housed per cage. They will be allowed food pellets and water ad libitum. The animals will be kept in a constant diurnal, (50:50) dark/light cycle.
   c. Immunizations of animals. 100 μg MAA were resuspended in 0.05 of balanced salt solution (BSS) and emulsed with equal volume of Complete Freund's

Adjuvant. The emulsion was inoculated into footpad of Lewis rats. One milliliter of BSS containing 100 μg of pertussis toxin and 100 μg of MAA was injected into peritoneum at the same time. One week after injection, clinical signs of EAAU will be monitored daily with a slit-lamp biomicroscope. For a period of 3 to 4 weeks. Upon onset of EAAU, the animals will be sacrificed and the eyes will be removed and processed for histopathologic evaluation using 4% glutaraldehyde and 10% buffered formaldehyde as fixative.

5. Experimental design. Panels of 5 rats for empty and plasmid-entrapping liposome will be used in the following experiment.
   a. Pharmacodynamics of liposome. Interval between injection of luciferase plasmid liposome into hind footpad and the expression of luciferase popliteal lymph node will be determined. The lymph node will be harvested at various time point: 1 min, 10 min, 1, 6, 12 hours, and 3, 7, 14, 21 days, 1 3 and 6 months. They will be ground and the substrate will be added to determine the expression and presence of luciferase.
   b. Interval between injection of IL-4 plasmid liposome and MAA in primary prevention (privation of the occurrence of EAAU). Immunization with MAA will be done on day 0. Liposomes will be injected on day –21, -14, –7, 0(concurrently), and 3.

RESULTS

Expression of luciferase could be noted as early as 2 days after the injection of liposome bound plasmid and lasted for 5 to 7 days.

However, the injection of various amounts of free or liposome-bound IL-4 at various of time before or after the induction of experimental autoimmune anterior uveitis could not suppress the development of EAAU.

DISCUSSION

Various drugs and immunomodulation therapy have been tried in experimental autoimmune uveitis animal models. Cyclosporine, FK-506, and Rapamycin are among those drugs that were first demonstrated effective in animal models and then applied to human uveitis. For immunomodulation therapy, most are still in the status of animal study. Systemic injection of monoclonal antibody to class II molecule or adhesion molecule (such as ICAM-1) has been tried with success. Intraocular injection of antigen involves the so called anterior chamber associated immune deviation (ACAID) have been shown to be effective in the suppression of the onset of

EAU, using retinal S antigen or IRBP. In previous study, we have demonstrated intraocular injection of soluble melanin associated antigen (MAA) can suppress the development of primary EAAU and the recurrence of the disease. The application of oral tolerance to prevent and/or treat autoimmune disorders has recently been of great interest to many investigators. Some encouraging effect have been reported in human multiple sclerosis, rheumatoid arthritis and difficult uveitis.

T lymphocytes have been implicated in a variety of autoimmune diseases, and therefore one potential therapeutic approach would be to tolerize the pathogenic self-reactive T cells. Cytokines may exert anti-inflammatory properties, allowing the hypothesis of their potential therapeutic use. Targeting the cytokines balance may modify the course of subsequent inflammatory events in an autoimmune disease. T helper 1 response, secreting mainly γ interferon, IL-2, and IL-12, favors delayed type hypersensitivity and augment autoimmune inflammatory reaction in various experimental autoimmune models which is mediated by delayed type hypersensitivity such as experimental rheumatoid arthritis, experimental autoimmune encephalomyelitis (EAE), EAU, and EAAU. T helper 2 response, secreting mainly IL-4. IL-5, IL-6, IL-10, and IL-13, favors non-complement fixing antibody reaction and therefore some type 1 hypersensitivity diseases, such as bronchial asthma. Microenvironment rich in Th1 patterns of cytokine can switch the original Th1 response to Th2 and vice versa. IL-4, IL-10 or IL-13 can switch Th1 response to Th2 and serve as a protection against several experimental autoimmune diseases mentioned above. . They are sometimes called anti-inflammatory cytokines. The very short half-life of cytokines makes them difficult and expensive to use directly; in such occurrence, high quantities have to be frequently injected. In this context, gene therapy appears as an effective alternative solution: the transfection of cells with cytokines genes (e.g. either IL-4 or IL-13) then the engraftment of these vectors in animals, permit the in vivo secretion of high levels of cytokines, and result in the protection of the animals from the development of the disease.

Gene therapy as therapeutic vaccine for autoimmune inflammatory diseases has been tried in animal models for rheumatoid arthritis, experimental autoimmune encephalomyelitis(EAE). Shaw et al demonstrated that encephalitogenic T cells, transduced with a retroviral gene construct to express interleukin 4, could delay the onset and reduce the severity of EAE when adoptively transferred to myelin basic protein-immunized mice. He suggested that T lymphocytes transduced with retroviral vectors can delivered 'regulatory cytokines' in a site-specific manner and may represent a viable therapeutic strategy for the treatment of autoimmune disease. Gene therapy in rheumatoid arthritis is presently in an experimental phase. Genes encoding for anti-inflammatory proteins can be transfected in joint cells. Therefore gene

encoding for interleukin-1 receptor antagonist has been transfected into synovial cells or into chondrocytes. Others have reported that clinical and histopathological parameters of collagen-induced arthritis (an animal model of rheumatoid arthritis) can be reduced by engraftment of CHO cells transfected with genes encoding for anti-inflammatory cytokines such as interleukine-4 or interleukine-13.

Gregoriadis et al reported that intramuscular immunization of mice with plasmid entrapped into cationic liposome lead to greatly improved cellular and humoral immune response. He speculated that this involves antigen-presenting cells locally or in the regional lymph node.

Cytokines are often difficult to detect in serum and tissue, due to their liability, to their short half-lives, and to the available detection systems. We will use luciferase plasmid as reporter gene in pilot study for the pharmacodynamics of transfection of lymphoid tissue in regional lymph node when liposome entrapped plasmid is injected into hind footpad of Lewis rats.

The induction of traditional EAAU involves the use of Complete Freund's Adjuvant and pertussis toxin. These substances are of great pro-inflammatory effect and may be difficult to suppress using the experimental desgin in this report. A milder form of EAAU was reported recently. By using the melanin-associated antigen only, Bora could induce EAAU. This may be related to the adjuvant effect of melanin pigment itself. This milder form of EAAU may serve the animal model to test the experimental design of this report in the future.

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