成果報告

NSC 89-2314-B002-506

題目：

口服可溶性黑色素抗原以初級與次級預防實驗性自體免疫前葡萄膜炎

Oral Administration of Soluble Melanin Associated Antigen to Suppress Experimental Autoimmune Anterior Uveitis

林昌平

台大醫院眼科

中文摘要

葡萄膜炎一般認爲是一種自體免疫疾病。因反覆發生，造成角膜病變，白內障，青光眼或視網膜病變，而導致失明。其治療到目前止一直以局部或口服腎上類固醇或其它免疫抑制劑如環胞靈素爲主要方法。這類治療因爲是廣泛性，無選擇性的免疫抑制，而有各種相當的副作用。

口耐受性是指口服一種抗原，身體對該抗原的反應方式會有改變。以視網膜可溶性抗原爲例，在路易氏鼠口服視網膜可溶性抗原數週後再於腳上注射網膜可溶性抗原以引發實驗性自體免疫葡萄膜炎。發現口服網膜可溶性抗原的實驗組不會發作，而對照組口服其它不相關抗原則會如預期地發病，其抑制用具抗原選擇性。最近因各種自體免疫疾病的動物模型的建立與應用，而能進一步探討口耐受性在治療與預防自體免疫疾病的可能性。有些疾病在動物模型與小規模人體試驗中獲得令人鼓舞的結果，如類風濕性關節炎，多發性硬節症，難纏的葡萄膜炎等。

本報告中使用口服可溶性黑色素相關抗原來防止實驗性自體免疫前葡萄膜炎。因實驗性自體免疫前葡膜炎在發病三至六週可幾乎完全復原，再次施打黑色素相關抗原時可引起實驗性自體免疫前葡萄膜炎再發，因此可以研究在初次發病後讓路易士鼠口腹黑色素相關抗原，以研究口耐受性次級預防實驗自體免疫葡萄膜炎的可能性。（而後者正是臨床上最希望能應用的）

結果是口服本實驗中粗製的可溶性黑色素相關抗原無法抑制實驗性自體免疫前葡萄膜炎。本實驗中未能純化並大量製造可溶性黑色素相關抗原是最可能的原因。可溶性黑色素相關抗原可以純化並大量製造後，口耐受性抑制實驗性自體免疫前葡膜炎仍是值得研究的題目。

ABSTRACT

Uveitis is one of the leading causes of blindness. It was estimated that 10% of the blindness was caused by uveitis in USA. The treatment is mainly topical and systemic corticosteroid, and some of these patients may require the use of a variety of immunomodulatory, corticosteroid-sparing agents, such as cyclosporine or cytotoxic agents. To date, clinically oriented approaches have centered on the administration of pharmacologic substances that have a nonspecific effect on the immune response. The development of more effective treatment of organ-specific inflammatory disorders of putative autoimmune origin is an ongoing goal in many specialties of clinical medicine. Recently, alternative therapeutic strategies have been suggested based on our better understanding of immunologic mechanisms that lead to organ-specific inflammatory responses. The induction of immunologic tolerance, defined as a state of specific immunologic unresponsiveness to an antigen after exposure to that antigen, is one such approach that has gained attention recently. One effective method of inducing immunologic tolerance is through the oral administration of antigen. The tolerance induced is called oral tolerance. One feature of oral tolerance is that

"bystander" suppressive effect can be elicited to the organ or tissue that harboring the antigen. Oral tolerance has been tested in various animal models of autoimmune disorders, such as experimental autoimmune encephalomyelitis, collagen and adjuvant arthritis, experimental autoimmune diabetes, and experimental autoimmune uveitis. The effect of oral tolerance has also been tested in several clinical conditions in small scale with encouraging results, such as multiple sclerosis, rheumatoid arthritis, juvenile diabetes, Behcet's disease and other intractable uveites.

The most common disease entity of uveites in Taiwan is acute anterior uveitis (AAU). It is the recurrent nature of AAU that can result in blindness and socioeconomic loss though various complications, including glaucoma, cataract, and cystoid macular edema. Experimental autoimmune anterior uveitis (EAAU) has been established to simulate human AAU. It involves the use of melanin associated antigen extracted from bovine uveal tissue. We investigated the effect of oral tolerance in EAAU both as primary and secondary prevention, i.e. in unprimed and in primed animals. Unfortunately, the preparation of partially purified soluble melanin associated antigen in this report could not suppress experimental autoimmune anterior uveitis.

INTRODUCTION

Wells first described the phenomenon of oral tolerance in 1911. Oral tolerance is a long recognized method to induce peripheral immune tolerance. The primary mechanisms by which orally administered antigen induces tolerance are via the generation of active suppression or clonal anergy. Low doses of orally administered antigen favor active suppression whereas higher doses favor clonal anergy. The regulatory cells that mediate active suppression act via the secretion of suppressive cytokines such as TGF β and IL-4 after being triggered by oral tolerogen. Furthermore, antigen that stimulates the gut-associated lymphoid tissue preferentially generates a Th2 type response. Because the regulatory cells generated following oral tolerization are triggered in an antigen-specific fashion but suppress in an antigen nonspecific fashion, that mediate "bystander suppression" when they encounter the fed autoantigen at the target organ. Thus it may not be necessary to identify the target autoantigen to suppress an organ-specific autoimmune disease via oral tolerance; it is necessary only to administer orally a protein capable of inducing regulatory cells that secrete suppressive cytokines at target organ. Orally administered autoantigens suppress several experimental autoimmune models in a disease- and antigen- specific fashion; the diseases include experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveitis (EAU), collagen- and adjuvant-induced arthritis, and diabetes in the NOD mouse. In addition, orally administered alloantigen suppresses alloreactivity and prolongs graft survival. Initial clinical trials of oral

tolerance in multiple sclerosis, rheumatoid arthritis, and difficult uveitis have demonstrated positive clinical effects with no apparent toxicity and decreases in T cell autoreactivity.

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 (hAAU), which, is the most common disease entity of uveitis in Taiwan. EAAU involves the use of insoluble extract from RPE and/or uveal tissue of bovine eyes. The exact components in this so-called melanin associated antigen (MAA) have not been clarified yet. It has been shown that EAAU is mediated by CD4+ T cells and delayed type hypersensitivity.

Various drugs and immunomodulation therapy have been tried in these 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.
In this study we investigated the effect of oral tolerance in EAAU both as primary and secondary prevention, i.e. in unprimed and in primed animals.

MATERIAL and METHODS

Animals. Female and male Lewis rats 200-250 gm of body weight were purchased from Experimental Animal Center, National Science Council, Taiwan.
Preparation of melanin associated antigen. Fresh bovine eyeballs were harvested and transferred to the lab within 3 hours of death. The iris tissue was excised and minced with PBS, and filtered through gauze. The filtrate was centrifuged at 10,000 g for 10 minutes. The pellet was resuspended with PBS and treated with 2% SDS at 75℃ for

10 minutes and the insoluble part was weighed , resuspended in PBS and stored at -70 ℃ for later use.

Soluble MAA Ten milligrams of MAA was digested with 100U of Staphylococcus aureus V8 protease (Sigma). The reaction was allowed to proceed in 75mM potassium phosphate buffer at 37℃ for 30 minutes to 1 hour in the presence of 4 M urea. After the digestion, samples were centrifuged at 1.2×10^5 g for 30 minutes at 4℃. The supernatant was dialyzed against water for 48 hours, lyophilized, and redissolved in PBS and stored in -70℃.

Immunizations of animals. One hundred microgram of MAA was resuspended in 0.015 ml of balanced salt solution (BSS) and emulsed with equal volume of Hunter's adjuvant (Sigma, St. Louis, MO). 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 was monitored daily with a slit-lamp biomicroscope for a period of 3 to 4 weeks. Upon onset of EAAU, the animals were sacrificed and the eyes were removed and processed for histopathologic evaluation using 4% glutaraldehyde and 10% buffered formaldehyde as fixative.

Recurrence of EAAU. The same protocol as 4.c was done after complete recovery from primary EAAU, which usually took 3-4 weeks.

Induction of oral tolerance. Lewis rats were fed a total of 20 mg melanin associated antigen and 40 mg of soybean trypsin inhibitor (STI; sigma, St Louis, MO, USA) administered in four feedings during 8 day period. STI (20 mg/ml) and MAA (5 mg/ml) was each dissolved or suspended in 0.15 mole/L sodium bicarbonate buffer (pH 8.0). Rats were deprived of food but not water for 12-18 h prior or each feeding of antigen. Rats were gently anesthetized with ether and fed MAA (1 ml) and STI (0.5 ml) by gastric intubation. In some experiments, panels of rats received only MAA (1 ml) suspended in bicarbonate buffer and no STI. Control rats were given four feedings of STI in bicarbonate buffer (vehicle control) or nothing (non-fed control). Three days following the last feeding, animals were injected with MAA plus CFA at footpad. These feedings were done in unprimed rats (as primary prevention) or previously primed rats (as secondary prevention). Standard protocols for clinical examination and histopathological examinations were done to detect the occurrence and severity of EAAU.

RESULTS

Similar incidence (around 80%) of experimental autoimmune anterior uveitis occurred in vehicle control (feed with STI), non-fed control group, and experimental

group (fed with soluble MAA and/or non-soluble MAA) with similar severity. No suppression of EAAU could be achieved by oral tolerance with the preparation of soluble MAA or insoluble MAA in this project.

DISCUSSION

In various experiments with oral tolerance in experimental models, purified protein was used. The amount used to induce oral tolerance was 40 mg of purified protein in four feedings during the period of one to two weeks. We planned this experiment with crude antigen (of which the exact amount was unknown) to take advantage of the "by-stander suppression" effect of oral tolerance. However, the melanin rich preparation of insoluble MAA was pro-inflammatoy and the soluble MAA was not a purified one, the actual amount fed could not be estimated and may actually far less than the amount needed. This may well be the reason why oral tolerance could not be induced to suppress the primary and secondary EAAU. This study should be performed again when the antigen is purified and relative larger amount of antigen can be prepared.

REFERENCES

1. Broekhuyse RM, Kunlmann ED, Winkens HJ, Van Vugt AHM. Experimental autoimmune anterior uveitis (EAAU), a new form of experimental uveitis. I. Induction by a detergent-insoluble, intrinsic protein fraction of the retinal pigment epithelium. Exp Eye Res. 1991; 52:465-74.
2. Broekhuyse, RM, Kunlmann, ED, Winkens HJ. Experimental autoimmune anterior uveitis (EAAU). II. Does-dependent induction and adoptive transfer using a melanin-bound antigen of the retinal pigment epithelium. Exp Eye Res. 1992;55,401-11.
3. Broekhuyse RM, Kunlmann ED, Winkens HJ. Experimental autoimmune anterior uveitis (EAAU). III. Induction by Immunization with purified uveal and skin melanins. Exp Eye Res. 1993;56,575-83.
4. Broekhuyse RM, Kunlmann ED. Experimental autoimmune anterior uveitis. The preparation of uveitogenic ocular melanin. Invest Ophthalmol Vis Sci. 1993;34, 698-700.
5. Broekhuyse RM, Kunlmann ED, Winkens HJ, Van Vugt AHM. Experimental autoimmune anterior uveitis (EAAU), a new form of experimental uveitis. I. Induction by a detergent-insoluble, intrinsic protein fraction of the retina pigment epithelium. Exp Eye Res. 1991;52, 465-74.
6. Chan CC, Hikita N, Dastgheib K, Whitcup SM, Grey I, Nussenblatt RB. Immunopathology of experimental autoimmune anterior uveitis (EAAU). Invest Ophthalmol Vis Sci. 1993;34:999.
7. Kabeer NH, Simpson SC, Gobleman CL, Kim MC, Tandhasetti MT, Kaplan HJ, Bora NS: Experimental allergic iritis, induction by immunization with bovine melanin associated antigen. Invest Ophthalmol Vis Sci. 1993;34:1482.
8. Nussenblatt RB, Igal G, Weiner H, et al. Treatment of Uveitis by oral

administration of retinal antigens: results of a phse I/II rendomiaed marked trial. Am J Ophthalmol 1997; 123:583-92.

9. Oral tolerance in a murine model of relapsing experimental autoimmune uveoretinitis (EAU): induction of protective tolerance in primed animals. Thurau SR, Chan CC, Nussenblastt RR, et al. Clin Exp Immunol 1997; 109:370-376.

10. Oral tolerance as therapy for experimental autoimmune encephalomyelitis and multiple sclerosis: demonstration of T cell anergy. Jewell SD, Gienapp IE, Cox KL, et al. Immunol and Cell Biology 1998; 76:74-82.

11. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-speicific autoimmune diseases by oral administration of autoantigens. Weiner HL, Friedman A, Miller A, el al. Annu Rev Immunol. 1994; 12:809-37.