ELSEVIER

BRIEF COMMUNICATIONS

Suppression or Exacerbation of Experimental Autoimmune Uveoretinitis in Lewis Rats by Pretreatment With or Without an Autoantigenic Peptide in Aluminum Hydroxide

Akemi Ozaki, Kazuyo Fukata, Atsuki Fukushima, Michiko Yasuoka, Osamu Yoshida and Hisayuki Ueno

Department of Ophthalmology, Kochi Medical School, Kochi, Japan

Purpose: Aluminum hydroxide (Al) is an adjuvant to induce Th2 immune responses. The aim of this study is to investigate the effects of pretreatment with Al on experimental autoimmune uveoretinitis (EAU), a Th1 disease model.

Methods: Lewis rats were pretreated with an uveitogenic peptide (#29)/Al, phosphate-buffered saline (PBS)/Al or PBS. EAU was then induced by immunization of #29 in complete Freund's adjuvant (CFA) with intravenous injection of *Bordetella pertussis*. Three weeks later, EAU was evaluated histologically and antigen-specific cellular immune responses were assessed.

Results: EAU was exacerbated in the PBS/Al group and attenuated in the #29/Al group, compared to the control PBS group. Antigen-specific cellular proliferation and interferon-γ production were augmented in the PBS/Al group and suppressed in the #29/Al group.

Conclusions: Suppression or exacerbation of EAU by the pretreatment in this study is related to inhibition or augmentation of antigen-specific Th1 immunity. **Jpn J Ophthalmol 2003;47:102–106** © 2003 Japanese Ophthalmological Society

Key Words: Autoimmunity, cellular activation, cytokine, Th1/Th2, tolerance/suppression.

Introduction

Differentiated $CD4^+$ T cells were recently classified into either Th1 or Th2 type by their cytokine profiles.[1] Th1 cells produce interferon-γ (IFN-γ), which inhibits Th2 cell development, whereas Th2 cells produce interleukin (IL)-4 and IL-10, which counteract Th1 cell development.[1] Induction of antigen-specific Th1 cells is important for the efficient induction of experimental autoimmune uveoretinitis (EAU), either by active or passive immunization systems.[2] Therefore, the idea that EAU could be inhibited by the induction of antigen-specific Th2 responses has been investigated, and the successful inhibition of EAU by inducing Th2 immune responses was demonstrated.[3] Th1/Th2 immunity is mainly controlled by cytokines, and several factors, including antigen dose, costimulatory molecules, and adjuvants also affect Th1/Th2 development.[1]

Adjuvants are usually used for augmentation of immune responses and it is well known that an appropriate adjuvant should be chosen for the induction of desired immune responses.[1] For example, to induce antigen-specific IgE by Th2 immune responses, antigen should be injected in emulsion with aluminum hydroxide (Al), while complete Freund's adjuvant (CFA) should be chosen for the induction of IFN-γ production by Th1 immune responses.[1] On the basis of this evidence, Al is usually used for ex-

Received: October 31, 2001

Correspondence and reprint requests to: Atsuki FUKUSHIMA, MD, PhD, Laboratory of Immunology, Department of Ophthalmology, Kochi Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

Published by Elsevier Science Inc.

0021-5155/03/$–see front matter
PII S0021-5155(02)00625-1

perimental allergic disease models and CFA is used for experimental organ-specific autoimmune disease models. Because the genetic background of animals also affects Th1/Th2 outcome, it is still not fully accepted that changing adjuvants could inhibit the development of experimental disease models. Yet, Mattsson et al reported that pretreatment with collagen in Al suppressed the development of rat collagen-induced arthritis,[4] in which Th1 is dominantly involved.

Although several reports demonstrated that treatment with inhibitory cytokines for the induction of each subset of helper T cells suppressed the development and severity of diseases,[3] opposite results were demonstrated so that treatment with IL-4 aggravated EAU, which is a prototype of Th1-mediated disease. These facts led us to investigate the possible suppression of EAU by pretreatment with a promiscuous S-Ag peptide[5] in Al, a Th2-inducible adjuvant. Similar to the results in the arthritis system in Dark Agouti rats,[4] the data recorded here demonstrated that pretreatment of the antigen in Al suppressed the severity of EAU. Unexpectedly, pretreatment with Al itself without the antigen augmented both immune responses and the severity of EAU.

Materials and Methods

Rats

Six- to 8-week-old male Lewis rats (Seac Yoshitomi, Fukuoka) were maintained in a pathogen-free animal facility at Kochi Medical School. All animal procedures conformed to the ARVO Resolution on the Use of Animals in Research.

Reagents

The human S-Ag peptide #29[5] (TLTLLPLLANNRERRGIALD, Sawady Technology, Tokyo) was synthesized by *t*-butyloxycarbonyl derivatives of the amino acids and purified by high-performance liquid chromatography to at least 95% purity. CFA (Yatron, Tokyo), Al (Sigma, St. Louis, MO, USA), and *Bordetella pertussis* bacteria (PT; Wako Pure Chemicals, Osaka) were used for adjuvants.

Pretreatment and Induction of EAU

Rats were injected with 100 μL of PBS, PBS mixed with Al (10 mg), or #29 (50 μg) adsorbed to Al (10 mg) into left hind footpads (n = 12 per group). Three weeks later, all the rats were injected with 100 μL of #29 (50 μg) emulsified in CFA into the same hind footpad and PT (5×10^9) was additionally injected intravenously.

Evaluation of EAU

Three weeks after induction of EAU, rats were sacrificed after clinical evaluation and eyes were harvested for histology. Eyes were fixed in 10% buffered formalin and embedded in paraffin. Sections (3-μm thick) were cut and stained with hematoxylin-eosin. Histological EAU grading was scored as published elsewhere.[2,3]

Proliferative Responses

Draining lymph node (LN) cells were collected at the time of sacrifice and combined per group. Experiments were repeated six times and 2 rats per group were used in each set of experiments. LN cells (3×10^5) were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (ICN Biomedical Japan, Tokyo), 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) with or without #29 (0.1, 1, 10, 100 μg/mL) in a 96-well flat-bottomed plate for 96 hours. ^{3}H-TdR (0.5 μCi/well, Japan Atomic Energy Research Institute, Tokai) was added for the last 16 hours. Cultures were then harvested and the radioactivity was measured by standard techniques. Data were expressed as stimulation indices (mean cpm in stimulated cultures/mean cpm in unstimulated control cultures) or delta-cpm (mean cpm in stimulated cultures − mean cpm in unstimulated control cultures).

Cytokine Enzyme-Linked Immunosorbent Assay (ELISA)

For detection of cytokines, culture (#29 concentration at 10 μg/mL) was set up similar to that for the proliferation assay except for cell number (1×10^6/well). The supernatants were collected after 48-hour incubation and cytokine (IL-4, IL-10, and IFN-γ) contents were measured by commercial ELISA (BioSource International, Camarillo, CA, USA).

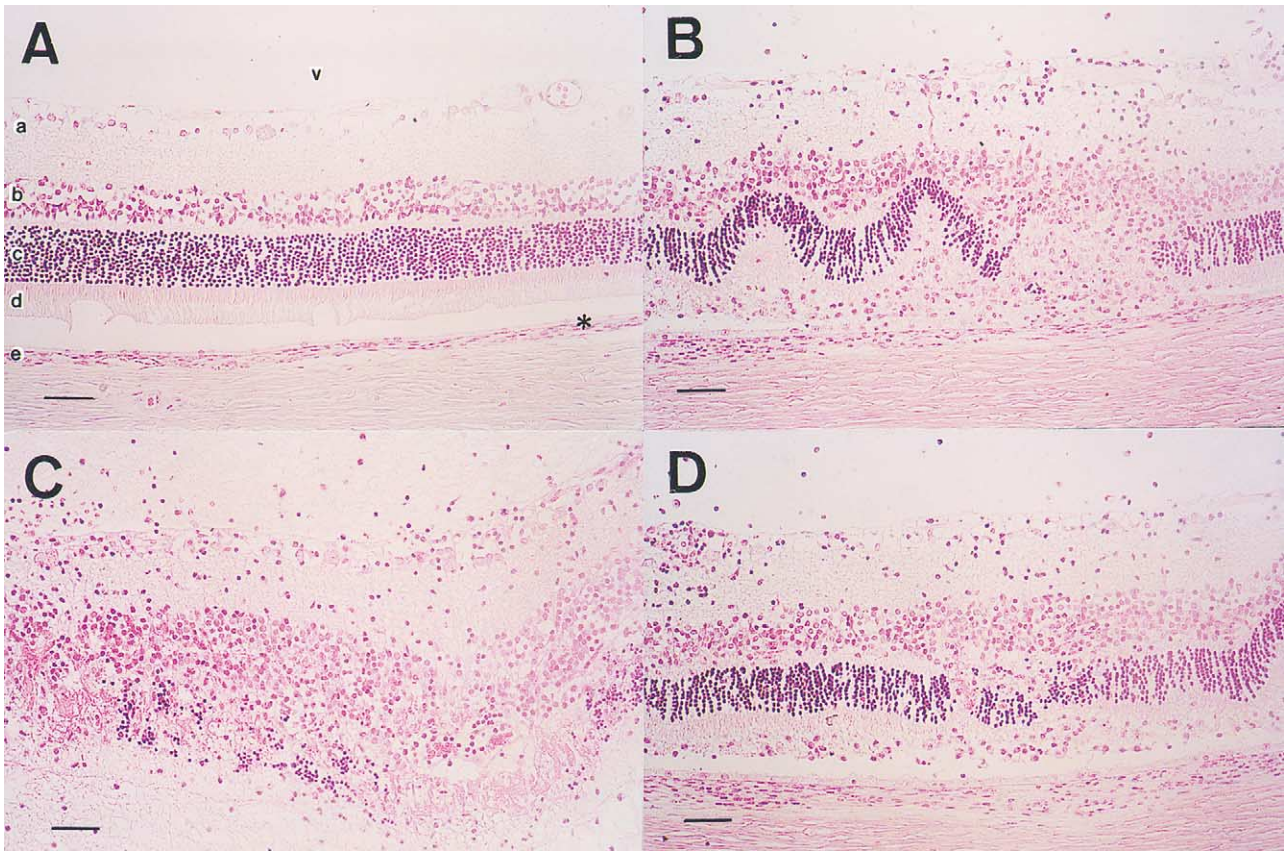
Statistical Analysis

Statistical comparisons of the severity of EAU and cytokine content in the culture supernatant were performed with the Mann-Whitney *U*-test. Results were mean ± SEM for the indicated number of rats. Statistical significance was assessed at the 95% confidence level.

Results

Incidence of EAU (number of rats developing EAU/number of EAU-induced rats) was 11/12 in

Jpn J Ophthalmol
Vol 47: 102–106, 2003



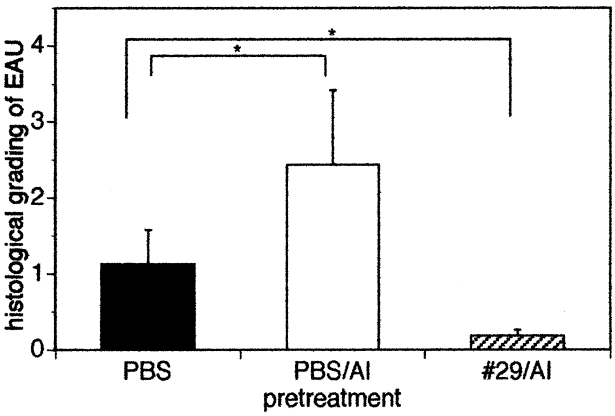


Figure 1. Histopathology of experimental autoimmune uveoretinitis (EAU) in rats pretreated with phosphate-buffered saline (PBS) (**B**), PBS/Al (**C**), or #29/Al (**D**). (**A**) Histology of retina in control Lewis rats. a: Ganglion cell layer, b: inner nuclear layer, c: outer nuclear layer, d: photoreceptor cell layer, e: choroid. etinal pigment epithelium, v: vitreous. Pretreatment was performed by subcutaneous injection of PBS, PBS/Al, or #29/Al. Three weeks later, EAU was induced by subcutaneous injection of #29/CFA together with intravenous injection of *Bordetella pertussis*. Eyes were harvested 3 weeks after EAU induction. Very severe histological changes occurred in all groups. Note that the histological findings in 10 out of the 12 rats in #29/Al group were the same as in control rats (**A**). Inflammatory cellular infiltration and destruction of retinal structure were most prominent in the PBS/Al group. Hematoxylin and eosin stain. Bar = 50 μm. (**E**) Comparison of histological grading of experimental autoimmune uveoretinitis (EAU) among the three groups. Significantly more severe EAU was noted in the phosphate-buffered saline (PBS)/Al group compared to the PBS group ($P < .05$), while significantly less severe EAU was induced in the #29/Al group compared to the PBS group ($P < .05$, n = 12 rats per group). Black bar: PBS group, white bar: PBS/Al group, hatched bar: #29/Al group. *$P < .05$.

the PBS group, 11/12 in the PBS/Al group, and 2/12 in the #29/Al group. Histology results in 10 of the 12 rats pretreated with #29 in Al were the same as in Figure 1A (normal Lewis rats). Histological examination demonstrated that pretreatment with #29 in Al suppressed the severity of EAU (Figures 1D, 1E, average histological score = 0.19, $P = .043$), while pretreatment with PBS in Al augmented the severity of

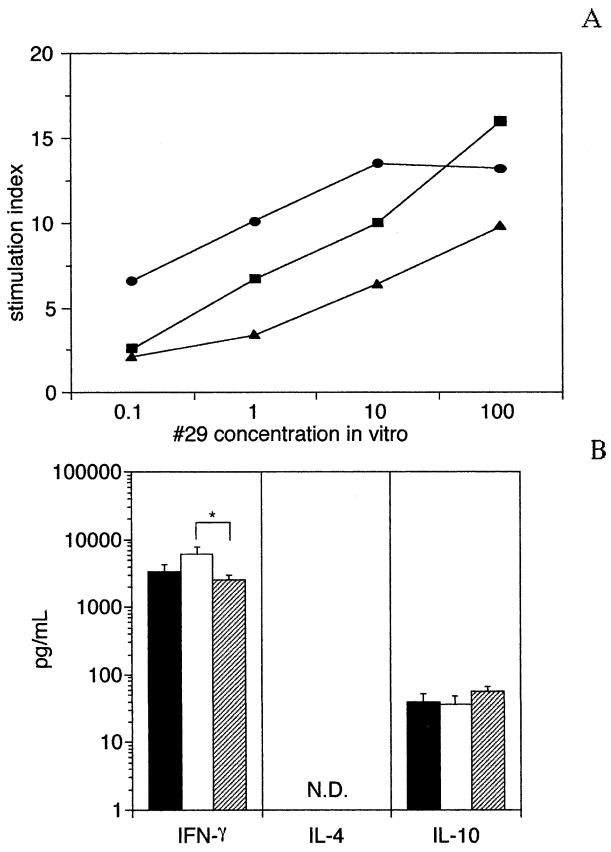


Figure 2. Cellular immune responses of lymph node (LN) cells in rats pretreated with phosphate-buffered saline (PBS), PBS/Al, or #29/Al. LN cells were harvested 3 weeks after induction of EAU, and LN cells were combined per group in each experiment. Experiments were repeated four to six times. (**A**) Proliferative responses of LN cells against #29 after 96-hour culture. PBS/Al group exhibited vigorous proliferation even at low concentrations, whereas #29/Al showed attenuated proliferation even at the highest concentration. The data are representative of six experiments with similar results. X-axis depicts #29 concentration in vitro (μg/mL). Background cpm without #29 in vitro were 137 ± 4 in the PBS group, 128 ± 13 in the PBS/Al group, and 149 ± 9 in the #29/Al group. Significant differences were noted at 1 and 10 μg/mL among the three groups. ■ PBS group, • PBS/Al group, ▲ #29/Al group. (**B**) interferon (IFN)-γ, interleukin (IL)-4 and IL-10 production of LN cells after 48-hour culture. Significantly higher amount of IFN-γ was produced in the PBS/Al group compared to the #29/Al group ($P < .05$). No difference in IL-4 and IL-10 production was observed among the three groups. Mean values ± SD are shown for four experiments. Black bar: PBS group, white bar: PBS/Al group, hatched bar: #29/Al group. *$P < .05$.

EAU (Figures 1C, 1E, average histological score = 2.44, $P = .043$), compared to the control group that was pretreated with only PBS (Figures 1B, 1E, average histological score = 1.13). To study the correlation between severity of EAU and antigen-specific cellular immune responses, antigen-specific proliferation assay and cytokine production were measured. The PBS/Al group exhibited a level of proliferative response similar to the #29 group at 10 times lower concentration in vitro compared to the PBS group, while 10 times higher concentration in vitro was necessary for the #29/Al group to induce the same level of proliferation as the PBS group (Figure 2A). Significant difference was noted at 1 and 10 μg/mL among the three groups. IFN-γ production by stimulation with #29 in vitro was in accord with that of proliferative responses against #29. The PBS/Al group produced a higher amount of IFN-γ compared to the other two groups (Figure 2B). A smaller amount of IFN-γ was noted in the #29/Al group than in the PBS group (Figure 2B). A statistically significant difference in IFN-γ production was noted between the PBS/Al group and the #29/Al group ($P = .048$). Data on these two immunological parameters were in parallel with the data of EAU severity (Figures 1D, 2A, and 2B). In addition, the number of lymphocytes per lymph node was significantly higher in the PBS/Al group compared to the PBS group (data not shown). These differences might be related to the fact that EAU was more severe in the PBS/Al group than in the PBS group.

Furthermore, Th2 type cytokine production such as IL-4 and IL-10 was investigated. IL-4 was not detected in the culture supernatant from any group (Figure 2B). A small amount of IL-10 was produced in all groups, yet no apparent differences among the three groups were demonstrated (Figure 2B). Reverse transcriptase-polymerase chain reaction using lymph node cells further demonstrated that similar levels of IL-4 and IL-10 were expressed among the three groups (data not shown). Thus, the difference in EAU severity may be attributed to the difference between in vitro antigen-specific cellular proliferative responses and IFN-γ production. Although there were reports mentioning the dissociation of proliferative responses and severity of EAU, the importance of these parameters for the induction of EAU has been generally accepted.[2] The reason for suppression of EAU severity as well as proliferation and IFN-γ production by #29/Al pretreatment is not likely to depend on the augmentation of Th2 immune responses, because no difference in IL-4 and IL-10 production in the culture supernatant was noted among the three groups (Figure 2B). Similar to the previous report concerning the arthritis model,[4] pretreatment of the antigen with Al suppressed the severity of EAU. Although augmented antigen-spe-

Jpn J Ophthalmol
Vol 47: 102–106, 2003

cific Th2 immunity was induced in the previous report, Th2 immunity was not augmented by the treatment in our system. We used Th1-prone Lewis rats, while DA rats were used in the previous report.[4] Differences in the strains of rats used in the experiments may account for the differential induction of antigen-specific Th2 immunity by Ag/Al pretreatment. It is also of note that we used an autoantigenic peptide whereas an autoantigenic protein was used in the previous report. In addition, we used pertussis as an additional adjuvant. It was reported that injection of pertussis in addition to antigen plus CFA further augmented Th1 immunity.[2] Therefore, Th2 immunity could not be expected to be induced in our system.

In summary, a Th1-related disease, EAU, could be suppressed by pretreatment of the antigen and using Al as an adjuvant, whereas pretreatment with Al without the antigen exacerbated EAU. Pretreatment affected antigen-specific Th1 immune responses, but minimally affected Th2 immunity. Although mechanisms for the suppression of EAU have not been elucidated, data on Th2 cytokine production together with preliminary results suggest that Th2 shift, anergy and activation-induced cell death are less likely to be involved. A combination of these mechanisms or an unknown mechanism might be involved in the control of this pretreatment.

This work was partially supported by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, Sports, Culture and Technology, Japan (No. 0281737), and a Kochi Shinyokinko Anshintomonokai Award.

References

1. Constant SL, Bottomly K. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu Rev Immunol 1997;15:297–322.
2. Caspi RR, Silver PB, Chan CC, et al. Genetic susceptibility to experimental autoimmune uveoretinitis in the rat is associated with an elevated Th1 response. J Immunol 1996;157: 2668–2675.
3. Kezuka T, Sakai J, Yokoi H, et al. Peptide-mediated suppression of experimental autoimmune uveoretinitis in mice: development of a peptide vaccine. Int Immunol 1996;8:1229–1235.
4. Mattsson L, Lorentzen JC, Svelander L, et al. Immunization with alum-collagen II complex suppresses the development of collagen-induced arthritis in rats by deviating the immune response. Scand J Immunol 1997;46:619–624.
5. Fukushima A, Lai JC, Chanaud NP III, et al. Permissive recognition of immunodominant determinants of the retinal S-antigen in different rat strains, primates and humans. Int Immunol 1997;9:169–177.