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Experimental immune-mediated blepharoconjunctivitis in rats induced by immunization with ragweed pollen

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Abstract *Background:* A study was performed to compare the effects of immunization with ragweed pollen (RW) in two different adjuvants on the characteristics of a previously described model of experimental immune-mediated blepharoconjunctivitis (EC) in rats. *Methods:* Lewis or Brown Norway (BN) rats were immunized with 100 µg of RW in emulsion with aluminum hydroxide [Al(OH)₃] or complete Freund's adjuvant (CFA). Three weeks later, the animals were challenged with eye drops containing RW in PBS. Twenty-four hours after topical challenge, eyes, blood, and lymph nodes were obtained for histology, measurement of antigen-specific antibodies, and proliferation or cytokine assays, respectively. In addition to active immunization, recipients of RW-primed lymph node cells were challenged and evaluated as above.

Results: RW in both adjuvants in-

duced infiltration with predominantly mononuclear cells in Lewis rats and eosinophils in BN rats. As well as active immunization, eosinophils were detected only in BN rats by adoptive transfer of cells. Lymphocyte proliferative responses to RW were high in immunized Lewis rats when CFA was used as an adjuvant. In contrast, proliferative responses in BN rats were higher when Al(OH)₃ was used. RW-specific IgE was detected only in BN rats. There were no significant differences in RW-specific IgG1/ IgG2a ratio among the four groups. Lewis rats had higher level of RW-specific interferon-γ in the culture supernatant.

Conclusions: The characteristics of EC are different in Lewis and BN rats, dependent on the genetic background of the rat strains. The response to RW was similar to other previously used antigens, such as ovalbumin.

Introduction

Experimental animal models for allergic conjunctivitis provide insight into disease pathogenesis and allow pre-clinical design of drugs before human clinical trials. In experimental allergic disease models including conjunctivitis, ovalbumin (OVA) is the most commonly used model antigen [15, 17, 18], mainly because it is a known and extensively analyzed food allergen [21]. Although OVA induces allergic conjunctivitis in animal models, it is not a common allergen in patients with allergic conjunctivitis [3].

In contrast, airborne plant allergens such as ragweed pollen (RW) are widespread and cause seasonal allergic conjunctivitis [3, 9]. Many people in the USA are allergic to RW [12]. Therefore, it is useful to employ RW as the model allergen in experimental allergic conjunctivitis. Foster and colleagues demonstrated that topical application of RW to guinea pigs led to the induction of conjunctivitis [10]. Recently, we reported the induction of experimental allergic conjunctivitis in SWR/J mice by systemic immunization followed by topical challenge [8].

Our group also analyzed the induction mechanism of experimental immune-mediated blepharoconjunctivitis (EC) [4] in rats by using OVA as the antigen. In Lewis rats, OVA-induced EC was mediated through CD4⁺ Th1 cells [4, 5, 19]. In general, Th1 cells regulate the function of Th2 cells [7], which includes production of cytokines such as IL-4 and IL-5 [11]. These cytokines are important for production of IgE and infiltration of eosinophils [4, 20]. Therefore, neither IgE in serum nor eosinophils in the conjunctiva could be induced in this strain. In contrast, OVA-specific IgE as well as eosinophil infiltration in the conjunctiva were present in Brown Norway (BN) rats with EC [20]. From these results, we established two different types of EC having different characteristics of immune responses. To further investigate the immune mechanisms of EC, we immunized Lewis and BN rats with RW and compared the results to OVA-induced EC.

Materials and methods

Rats

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

Antigens

Ragweed pollen (RW) was purchased from ICN Biomedicals Inc. (Aurora, Ohio). The pollen was stored at 4°C until use. Concanavalin A (Con A; Sigma Immunochemicals, St. Louis, Mo.) was used for a mitogen *in vitro*.

Induction of EC by active immunization

Lewis and BN rats were injected subcutaneously with 100 µg of RW in either CFA (Yatoron, Tokyo, Japan) or Al(OH)₃ (Sigma). Three weeks later the rats were challenged with 2 mg of RW in 10 µl of PBS by eye drops as previously reported [10]. Twenty-four hours after challenge, rats were clinically evaluated and killed, and their eyes, blood, and lymph nodes were harvested for histological studies, measurement of antibody production, and proliferation assays or cytokine production, respectively. For histological studies, the eyes, including the lids, were fixed in 10% buffered formalin and embedded in paraffin. Sections 10 µm thick were stained with hematoxylin-eosin and May-Giemsa. As the bulbar conjunctiva has a lower area and a lower cellular component than the palpebral conjunctiva, the infiltrating cells were counted in the palpebral conjunctiva with an eye-piece grid of 400× magnification [4, 5, 19].

Induction of EC by adoptive transfer of RW-primed lymph node cells

EC was induced by adoptive transfer of RW-primed lymph node cells from rats immunized with RW in Al(OH)₃ as well as active immunization [4, 5, 19]. RW-primed lymph nodes were harvested

14 days after immunization, and 100×10⁶ cells were cultured in 25 cm² flasks (Nunc, Roskilde, Denmark) with RW at 50 µg/ml in a final volume of 10 ml RPMI 1640 medium supplemented with 5% fetal calf serum (FCS; ICN Biomedical Japan, Tokyo, Japan), 2-mercaptoethanol (2-ME; 5×10⁻⁵ M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Three days later, cells were harvested and washed 3 times with RPMI medium. Then, 50×10⁶ cells were intravenously injected into syngeneic recipient rats, and 4 days after transfer rats were challenged with RW topically. Twenty-four hours after challenge, rats were killed and evaluated as above.

Assessment for cellular immune responses of primed lymph node cells

Lymph nodes were combined and experiments were repeated 4 times. Lymph node cells (3×10⁵ cells/well) were cultured in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 5% FCS and 2-ME. Cells were stimulated with RW at final concentrations of 0.1, 1, 10, and 100 µg/ml. After incubation for 72 h at 37°C in a humidified atmosphere with 5% CO₂, cultures were pulsed for 16 h with 0.5 µCi/well of ³H-thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). 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).

Detection of RW-specific IgG by ELISA

Serum was collected from each rat via cardiac puncture at the time of death. Serum antibody levels against RW were measured by direct ELISA. RW (500 ng/well for total IgG and 5 µg/well for IgG1 or IgG2a) was absorbed to 96-well plates for 2 h at 37°C. The plates were washed with PBS-Tween, and serially diluted serum samples were added to the wells. Bound antibody was detected by peroxidase-conjugated anti-IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) or peroxidase-conjugated anti-IgG1 or anti-IgG2a (Pharmingen, San Diego, Calif.). The plates were developed by using 3,3',5,5'-tetramethylbenzidine base (TMB-ELISA, Gibco BRL, Gaithersburg, Md.) and the optical density (OD) of each well was read at 610 nm. Data were presented as the ratio (IgG1/IgG2a) of the OD 610 nm values of the 1:160 dilution.

Detection of RW-specific IgE by passive cutaneous anaphylaxis

Male SD rats were used to determine the skin-sensitizing capacity of serum from immunized Lewis or BN rats [20]. Dorsal surfaces of unsensitized SD rats were shaved, and serially diluted serum was injected intradermally. Forty-eight hours later, each rat received an intravenous injection of 1 mg RW and 2.5 mg Evans' blue dye in 1 ml of PBS. Rats were killed after 30 min, the dorsal skin was removed, and reactions were evaluated on the inner skin surface [20].

Detection of IFN-γ in the culture supernatant using ELISA

Lymph nodes from RW-primed rats were combined for culture. Cells were prepared as described above; 1×10⁶ cells were cultured for 48 h in 96-well flat-bottom plates with RW at 50 µg/ml in a final volume of 200 µl RPMI 1640 medium containing 2-ME and 5% FCS. Culture supernatants were harvested and assayed by commercial cytokine ELISA (BioSource International, Camarillo, Calif.) for interferon-γ (IFN-γ) following the recommended method.

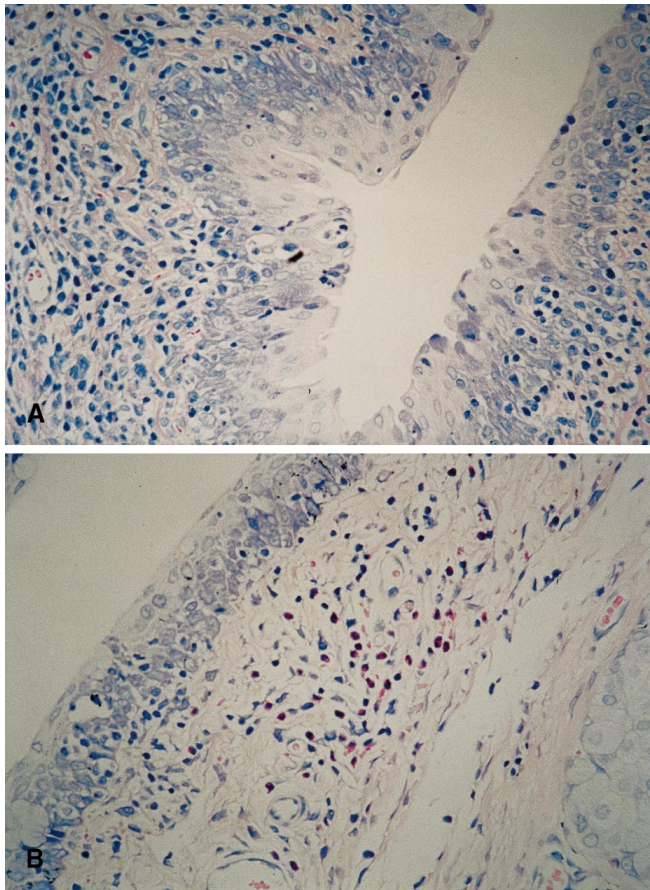


Fig. 1A, B Histology of the conjunctiva from a Lewis rat (**A**) and a BN rat (**B**) immunized with RW in $\text{Al}(\text{OH})_3$. The section was stained with May-Giemsa. Eosinophils were detected only in BN rat. Magnification $\times 320$

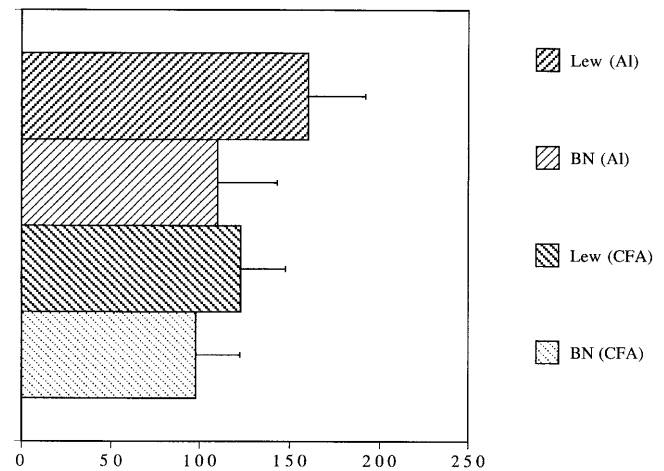
Statistical analysis

Statistical comparisons of the number of infiltrated cells, IgE titers, and IgG1/IgG2a ratios were performed by Mann-Whitney *U*-test.

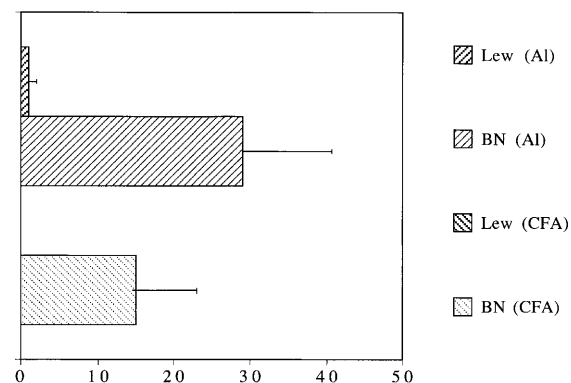
Results

Histological differences of EC between Lewis and BN rats

As shown in Fig. 1, mononuclear cells were the dominantly infiltrating cells in the conjunctiva of Lewis rats, whereas eosinophils were prominent in BN rats. The infiltrating cell number in the conjunctiva was counted following the method detailed elsewhere [4, 5, 19]. Lewis rats immunized with $\text{Al}(\text{OH})_3$ had statistically significantly higher infiltrating cell numbers (average=160) in the conjunctiva than did BN rats immunized with CFA (average=98, $P=0.008$) or with $\text{Al}(\text{OH})_3$ (average=110,



A



B

Fig. 2A, B Comparison of infiltrating total cell number (**A**) and eosinophil number (**B**) in the conjunctiva among the four different groups (two different strains with two different adjuvants). The x-axis indicates the number of infiltrating cells

$P=0.01$) (Fig. 2A). In contrast, more eosinophils were detected in the conjunctiva of BN rats than in Lewis rats (average: BN, CFA=15; Lew, CFA=0; BN, $\text{Al}(\text{OH})_3=29$; Lew, $\text{Al}(\text{OH})_3=1$; $P<0.005$, Fig. 2B). A comparison of the effects of adjuvant on the infiltration of eosinophils in each strain showed that more eosinophils were detected in BN rats using $\text{Al}(\text{OH})_3$ ($P=0.03$), while no significant difference was observed in Lewis rats ($P=0.25$). Furthermore, eosinophils were detected in the conjunctiva from BN rats (Fig. 3B) received with RW-primed lymph node cells. In contrast, no eosinophils were demonstrated in the conjunctiva from Lewis rats (Fig. 3A, and data not shown) ($P<0.0001$). There were no statistically significant differences on total infiltrated cell number in recipients of RW-primed lymph node cells between Lewis (average=95) and BN (average=88) rats ($P=0.002$).

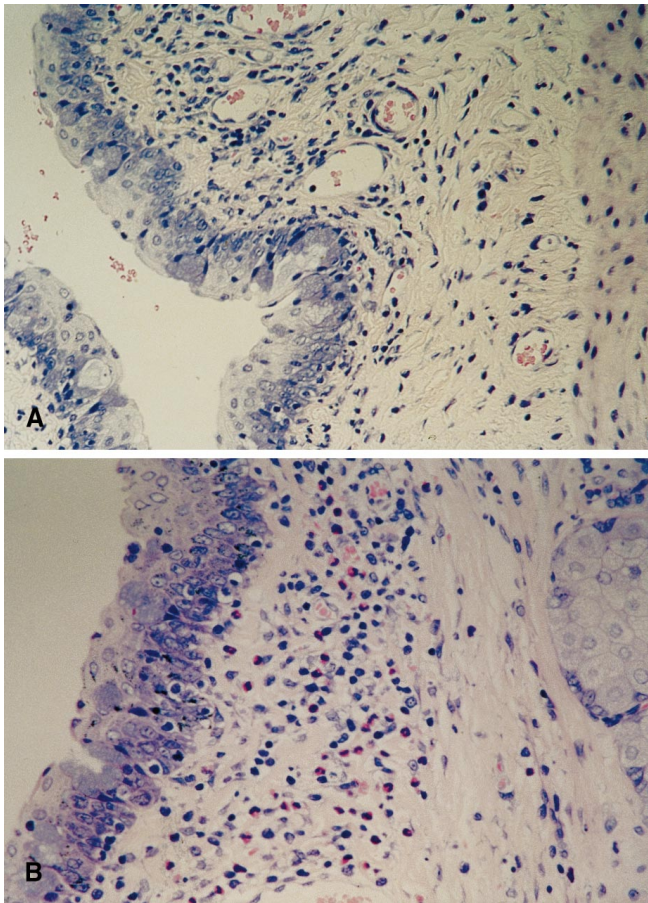


Fig. 3A, B Histology of the conjunctiva from a Lewis rat (A) or a BN rat (B) passively immunized by adoptive transfer of RW-primed in vitro stimulated lymph node cells. The section was stained with May-Giemsa. Similar to active immunization, eosinophils were detected only in BN rat. Magnification $\times 320$

Cellular proliferative responses of lymph node cells against RW

Using CFA as the adjuvant, cellular proliferative responses against RW in Lewis rats are stronger than those in BN rats (Fig. 4A). In contrast, lymph node cells from BN rats exhibited stronger proliferative responses against RW than those from Lewis rats by using $\text{Al}(\text{OH})_3$ as the adjuvant (Fig. 4B).

Humoral immune responses against RW

There was no statistical significant difference among the four groups concerning the IgG1/IgG2a ratio, although $\text{Al}(\text{OH})_3$ immunization was inclined to induce higher RW-specific IgG1 antibody ($0.06 < P < 0.22$, Fig. 5A). BN rats immunized with either adjuvant produced high RW-specific IgE, while no IgE was detected in Lewis rats by

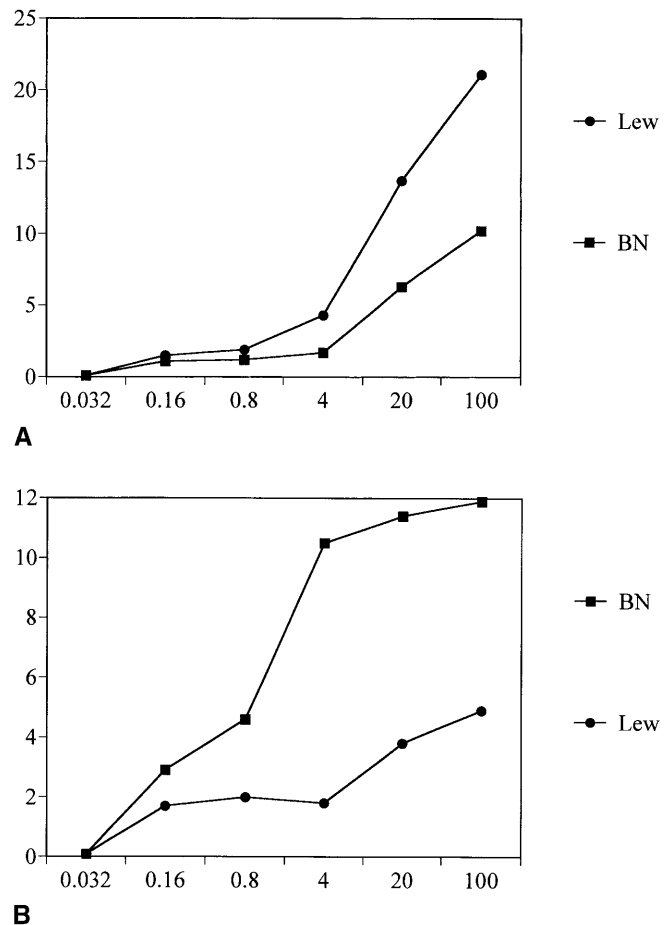


Fig. 4A, B Proliferative responses of RW-primed lymph node cells from Lewis and BN rats immunized with CFA (A) or $\text{Al}(\text{OH})_3$ (B). The x-axis indicates RW concentration in vitro ($\mu\text{g/ml}$) and the y-axis indicates stimulation index (mean cpm in stimulated cultures/mean cpm in unstimulated control cultures)

PCA method (Fig. 5B). In BN rats, $\text{Al}(\text{OH})_3$ induced more RW-specific IgE production than CFA ($P=0.007$, Fig. 5B).

IFN- γ production in the culture supernatant

IFN- γ production in the culture supernatant was tested by ELISA as described before [5, 19]. As shown in Fig. 6, similar to the data with OVA, more IFN- γ was produced in Lewis rats regardless of adjuvants used (CFA=1589 pg/ml, $\text{Al}(\text{OH})_3$ =1243 pg/ml), whereas lower amount of IFN- γ was detected in BN rats (CFA=312 pg/ml, $\text{Al}(\text{OH})_3$ =286 pg/ml).

Discussion

Our previous reports indicated that the difference of genetic background between Lewis and BN rats determines

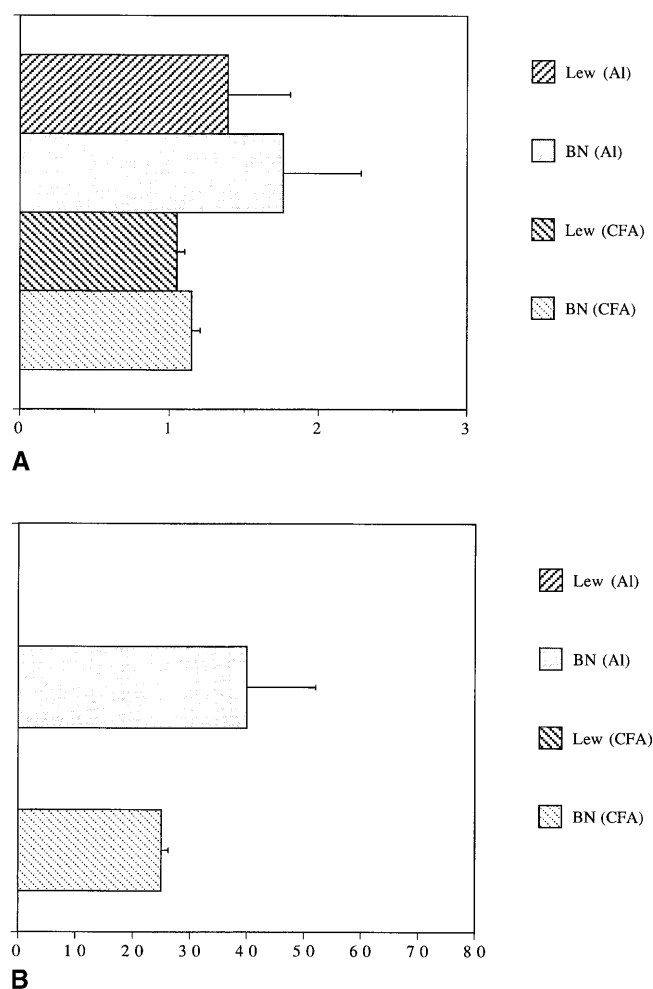


Fig. 5 **A** Comparison of RW-specific IgG1:IgG2a ratio among the four different groups. RW-specific IgG titers were measured by ELISA and data are expressed as the ratio of ODs (IgG1/IgG2a). The x-axis indicates the ratio. **B** Comparison of RW-specific IgE among the four different groups. RW-specific IgE titers were measured by passive cutaneous anaphylaxis (PCA). The x-axis indicates the maximal dilution factor of the serum for positive reaction. IgE was below the detectable level in Lewis rats immunized with either adjuvant

the characteristics of immunity, leading to different types of EC when OVA was used as a model antigen [20]. RW should be better suited as the antigen in conjunctivitis models, since it is a common antigen for seasonal allergic conjunctivitis in the USA [12]. Therefore, we used RW as the antigen for EC in rats to compare the capability of induction of EC and immunity with OVA.

In a study using *Dermatophagoides pteronyssinus* (Der p 1) as an allergen, it was demonstrated that Der p 1 has the ability to affect immune responses toward Th2, probably due to its protease activity [2]. As aeroallergens such as RW often have proteolytic activities similar to Der p 1 [1], we sought to test possible induction of Th2

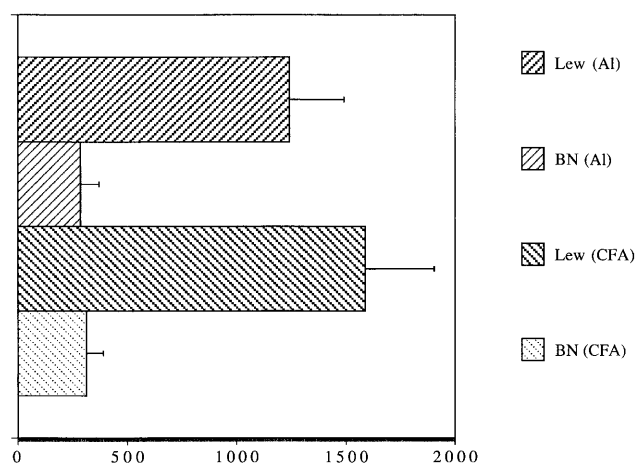


Fig. 6 Comparison of IFN- γ in the culture supernatant among the four different groups. Culture supernatants were harvested after 48 h culture with RW, and IFN- γ concentration was measured by ELISA. The x-axis indicates IFN- γ concentration (pg/ml)

immune responses in Lewis rats by immunization of RW instead of OVA. As shown in Figs. 1 and 2, infiltration of eosinophils was detected only in BN rats, similar to the previous report using OVA. At least 15% of infiltrated cells was composed of eosinophils in BN rats and Al(OH)₃ induced more eosinophilic infiltration in the conjunctiva (Figs. 1A, B, 2B). Concerning the number of infiltrating eosinophils, there were no apparent differences between RW and OVA in each strain by either adjuvant [15]. No eosinophils were detected in Lewis rats regardless of adjuvants as well as antigens, suggesting that RW was not able to induce Th2 immune responses in Lewis rats.

In addition to active immunization, adoptive transfer of RW-primed lymph node cells induced distinct types of inflammatory changes similar to those seen in active immunization. As transferred cells were composed of T cells (70–80%) and B cells (20–30%), it is conceivable that these transferred cells promoted the infiltration of eosinophils in the conjunctiva in BN rats. IL-4 has been demonstrated to be crucial for the migration of eosinophils to the lung in experimental asthma models, either by active immunization of IL-4 knockout (IL-4^{-/-}) mice [6] or transfer of IL-4^{-/-} Th2 cells into WT mice [13]. Taking the previous data concerning the OVA system into consideration, IL-4 produced by RW-primed lymph node cells in BN rats might have induced eosinophilic infiltration into the conjunctiva, although IL-4 was not tested in this study. It is also probable that very little production of IL-4 led to no eosinophil infiltration in Lewis rats. It is obviously important to investigate IL-5, which is the key cytokine for eosinophil growth and differentiation [16].

The most striking difference between RW and OVA concerning cellular proliferative responses was that BN

rats immunized with RW in $\text{Al}(\text{OH})_3$ exhibited vigorous proliferation against RW, while OVA/ $\text{Al}(\text{OH})_3$ immunization induced marginal proliferative responses against OVA in BN rats [20]. Although we do not have a good explanation for this difference, it may be due to differences between the antigens used. It is generally accepted that insoluble proteins have more immunogenicity than soluble ones [14]. As RW in the form used here is insoluble, this may cause higher immunogenicity, thus leading to higher proliferative responses against immunogen RW. In fact, the recovered cell number from BN rats immunized with RW in $\text{Al}(\text{OH})_3$ is 10 times higher than that with OVA in $\text{Al}(\text{OH})_3$ (data not shown).

RW-specific humoral immune responses evaluated by IgE were basically similar to those in the previous report using OVA, which demonstrated that BN rats produced antigen-specific IgE, while antigen-specific IgE was not detected in Lewis rats [20]. The data could be interpreted

as indicating that regardless of antigens used, BN rats are prone to production of IgE, while Lewis rats are not. Although there were no significant differences in IgG1/IgG2a ratios among RW-treated groups (around 1.5), OVA/ $\text{Al}(\text{OH})_3$ immunization induced higher ratios (around 3.0) than OVA/CFA immunization [20]. These data could be interpreted as showing that RW is less affected by adjuvants compared to OVA, similar to the data in cellular proliferation.

In conclusion, both active and passive immunization of RW in two different strains of rats were able to induce distinct types of immune responses (Th1 vs Th2), thus leading to different features of EC. Furthermore, in BN rats, eosinophilic infiltration could be induced by adoptive transfer of RW-primed lymph node cells.

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