Subconjunctival Immunization of Mice for Inducing IgE Antibody Response in Parotic Lymph Node

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ABSTRACT—Subconjunctival immunization of mice with dinitrophenyl (DNP)-Ascaris plus alum led to the induction of a local anti-DNP IgE response in 8 days. Anti-DNP IgE was found to be secreted from isolated lymphocytes in the parotic lymph node neighboring the immunization site but not from those in the spleen and the mesenteric lymph node. The IgE response was also confirmed by the detection of Cε transcript in the parotic lymph node cells. Ocular topical application of betamethazone resulted in considerable suppression of the IgE response in the parotic lymph node, thus suggesting that this immunization protocol is useful for evaluating ocular topical anti-allergic drugs that are expected to suppress local IgE responses.

Keywords: Subconjunctival immunization, Anti-dinitrophenyl (DNP) IgE, Parotic lymph node

Investigations to elucidate the regulatory mechanisms of IgE antibody responses are of great importance for the development of technologies to treat various allergic diseases including asthma, atopic dermatitis and pollen-induced ocular and nasal allergy (1–3). It is also believed to be important for the development of experimental allergy models that can be elicited by appropriate sensitization routes that reflect the onset of clinically encountered allergic diseases. An ocular allergy like allergic conjunctivitis is thought to be triggered by the invasion of antigens through ocular mucosa. It has been reported that an experimental allergic conjunctivitis was induced in guinea pigs that were sensitized to fluoresceinyl ovalbumin (4) or ovalbumin (5) by repeated ocular topical application of the antigen. In these experimental models, however, it was found that conjunctival-associated lymphoid tissues produced the antigen-specific IgG1 and IgG2, but not IgE and IgA (5, 6). Peppard et al. have reported that both local and systemic IgA and IgM but not IgE antibody responses were found to be induced in rats following ocular topical immunization (7).

Mice are the experimental animals that have been most elaborately investigated genetically and immunologically. However, procedures that enable the induction of antigen-specific IgE responses by immunization via ocular routes have not been reported in mice. In the present report, we describe that subconjunctival immunization of mice resulted in the induction of an antigen-specific IgE response localized, at least, in parotic lymph nodes (PLN) neighboring the immunization site.

Female BALB/c mice were purchased from Charles River Japan, Inc. (Kanagawa), and were used at 8–12 weeks of age. Mice were immunized by subconjunctival injection with 10 μl saline containing 4 μg of dinitrophenyl-Ascaris (DNP-Asc) (Advance, Norwood, MA, USA) and 60 μg of alum. Four to 21 days after the immunization, PLN, mesenteric lymph nodes (MLN) and spleens were extirpated. Dissociated lymphocytes from each lymphoid tissue were cultured at 1 x 10⁶ cells/ml in 0.2 ml of the culture medium to estimate their anti-DNP IgE secretion. The cultures were carried out in triplicate for 3 days at 37°C under 5% CO₂ and 95% air in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), 1 mM sodium pyruvate, 2 mM l-glutamine, 1 x 10⁻⁵ M 2-mercaptoethanol, 100 U/ml penicillin G and 50 μg/ml streptomycin.

Anti-DNP IgE secreted into the medium was determined by an ELISA for the assay of anti-trinitrophenyl (TNP) IgE developed in our laboratory (2, 8). This assay method was equally effective for both anti-DNP and anti-TNP IgE due to the high cross-reactivity of anti-DNP IgE with TNP hapten. Briefly, flat-bottom, 96-well microplates (Nunc, Roskilde, Denmark) were coated with 2 μg/ml rat anti-mouse IgE monoclonal antibody (6HD5) (Seikagaku Kogyo, Tokyo) and blocked with Al buffer that contains 0.01 M sodium phosphate buffer (pH
0.1 M NaCl, 1 mM MgCl₂, 0.1% NaN₃ and 0.1% bovine serum albumin. 6HD5 has been shown to be highly specific for murine IgE with no significant cross-reactivities with other Ig isotypes (9). It was confirmed that the observed IgE levels in the following experiments were not due to the cross-reaction of coexisting IgG or IgM because these levels of authentic IgG and IgM did not give positive values in our IgE ELISA. A2 buffer that was prepared by dissolving 0.3 M NaCl in A1 buffer was used for the dilution of samples. Appropriately diluted samples were added into anti-IgE-coated wells at 50 µl/well and then incubated for 8–12 hr at 4°C. After washing the plates with A1 buffer, 50 µl of A2 buffer containing 2 µg/ml TNP-β-galactosidase was added in each well. TNP-β-galactosidase was prepared in our laboratory as reported previously (2). Plates were incubated at room temperature for 4 hr, and then extensively washed with A1 buffer. The β-galactosidase activity bound to the plate via adsorbed anti-TNP IgE was assayed by adding 100 µl of A1 buffer containing 25 µg/ml 4-methylumbelliferyl-β-D-galactoside (Sigma, St. Louis, MO, USA) followed by incubation at 37°C for 30 min. The reactions were stopped by the addition of 100 µl of 0.1 M glycine buffer, pH 10.3. The enzyme activities were measured fluorometrically using a fluorescence microplate reader MTP-32 (Corona Electric, Tokyo). The level of anti-DNP IgE was calculated from the calibration curve that was obtained using purified monoclonal anti-TNP IgE (IGELa2) (2). This procedure allowed the determination of 0.1–100 ng/ml the IgE. Data are presented as the mean values of triplicate assays. The experimental variation among triplicate measurements was usually less than 15% of the mean and was not shown in some figures.

For the detection of IgE mRNA by reverse transcription-polymerase chain reaction (RT-PCR), a 22 mer sequence, 5'-TCAAGGAAACCTCAGTCACC-3', corresponding to the murine JH₄ sequence was used for the sense primer. A 20 mer sequence, 5'-CTAGGATAGTCCTACTTTCG-3', from exon 1 of Cε was used for the antisense primer. Total RNA was extracted from 1×10⁶ PLN cells by the RNA Zol B method as described by Chomczynski and Sacchi (10). The extracted RNA was reverse-transcribed, and the resultant cDNA was amplified by PCR as described previously (11).

BALB/c mice were immunized with DNP-Asc subconjunctivally in both eyes. On days 4, 8, 14 and 21 after the immunization, PLN lymphocytes were isolated and cultured for 3 days. As shown in Fig. 1A, it was observed that the cells collected on day 8 or later secreted significant levels of anti-DNP IgE. The levels of secreted IgE varied between 4 and 8 ng/ml from experiment to experiment. The IgE secretion increased slightly until day 21. Although the data were not shown, the IgE secretion reached a plateau on day 2 of the culture. In parallel with the anti-DNP IgE secretion from PLN cells, the serum level of anti-DNP IgE also increased after 8 days (Fig. 1B). These IgE levels were less than 10% of those ob-

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**Fig. 1.** Kinetics of anti-DNP IgE response in mice that were immunized with DNP-Asc by subconjunctival injection. On days 0, 4, 8, 14 and 21 after the immunization, sera and PLN from a group of four mice were isolated. A: Secretion of anti-DNP IgE from PLN cells. Pooled PLN cells from each group were cultured in triplicate for 3 days at 1×10⁶ cells/ml in 0.2 ml of the culture medium. B: Serum level of anti-DNP IgE. Pooled sera from each group were assayed for the levels of anti-DNP IgE in triplicate. Each point in panels A and B represents the mean value of triplicate assays. C: Detection of IgE mRNA in PLN by RT-PCR. PLN isolated on day 8 (lane 2) and on day 18 (lane 1) were examined for the expression of Cε transcript. Molecular size markers, 396 and 298 bp are shown in the figure.
Fig. 2. Localized IgE response induced by the subconjunctival immunization. A: Secretion of anti-DNP IgE from various lymphoid organs. PLN, MLN and spleens were obtained from mice (N=4) on day 8 after the subconjunctival immunization. Dissociated lymphocytes from each organ were cultured as described in Fig. 1. B: Mice (N=3) were immunized in their right eyes. After 8 days, right and left PLN lymphocytes were compared for their secretion of anti-DNP IgE. Each bar represents the mean±S.D. from 4 and 3 samples in panels A and B, respectively.

Fig. 3. Suppression of anti-DNP IgE response in PLN by ocular topical application of betamethazone. The immunized mice (N=8) received ocular topical application of saline, betamethazone (0.1%) or DSCG (0.1%) daily as described in the text. Anti-DNP IgE secretion of PLN cells of each mouse was examined 14 days after the subconjunctival immunization. Each bar represents the mean±S.D. from 8 mice.
erved in mice that were immunized systemically by intraperitoneal injection of the antigen (12). We confirmed that these immunized mice showed increased vascular leakage when challenged with the antigen and Evans blue dye (data not shown). To further confirm the induction of the IgE response, the detection of IgE mRNA was carried out by RT-PCR as shown in Fig. 1C. A band corresponding to the Cε cDNA fragment was observed in the day 8 cells, but was undetectable in the day 18 cells. Although the reason is unclear why IgE secretion persisted after day 18, a plausible explanation is that the IgE secretion in this phase might be due to the release of preformed IgE from the lymphocytes.

In the following experiments, we investigated which lymphoid organs elicit anti-DNP IgE production in response to subconjunctival immunization. Only a small number of lymphocytes were obtained from conjunctival-associated lymphoid tissues and thus were insufficient to examine the IgE response. Massive hyperplasia was not observed in peripheral lymph nodes other than PLN after the immunization. The sizes of the spleen and MLN did not change significantly. As shown in Fig. 2A, only the PLN cells but not those in MLN and spleens were found to secrete anti-DNP IgE. On the other hand, when mice received the subconjunctival immunization, only in their right eyes, the IgE secretion was observed only in PLN on the immunized side (right), but not on unimmunized side (left) as clearly shown in Fig. 2B. These findings suggest that this subconjunctival immunization induces the IgE response localized in the neighboring lymph nodes. Localized IgE responses appeared to be of clinical significance in some allergic diseases. For instance, it has been reported that Th2-like CD4+ T cells involved in the augmentation of IgE responses were found to accumulate in the conjunctiva of vernal conjunctivitis patients (13).

Figure 3 shows the effects of topically applied anti-allergic agents on the IgE secretion from PLN lymphocytes. Betamethazone or disodium cromoglycate (DSCG) was applied topically in the eyes of the mice four times a day for 20 consecutive days on day 5 to day 14 of the subconjunctival immunization. The IgE secretion was tested using PLN cells isolated on day 14. Application of betamethazone resulted in more than 90% suppression of the IgE response, consistent with the previous report showing that the steroid was effective for suppressing IgE production from human B cells (14). DSCG showed only a marginal inhibitory effect.

Taken together, the subconjunctival immunization procedure described here will be a useful experimental system in mice for evaluating pharmacological effects of topical anti-allergic agents against IgE-mediated ocular allergy.

REFERENCES

1. Coffman RL, Seymour BWP, Lebman DA, Hiraki DD, Christiansen JA, Shadrer B, Cherwinski HM, Savelkoul HFJ, Finkelman FD, Bond MW and Mosmann TR: The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol Rev 102, 5–28 (1988)

2. Ohmori H, Hikida M and Takai T: Prostaglandin E2 as a selective stimulator of antigen-specific IgE response in murine lymphocytes. Eur J Immunol 20, 2499–2503 (1990)

3. Ohmori H, Kanda T, Takai T and Hikida M: Induction of antigen-specific IgE response in murine lymphocytes by IL-10. Immunol Lett 47, 127–132 (1995)

4. Khatami M, Donnelly JJ, Haldar JP, Wei Z-G and Rockey JH: Massive follicular lymphoid hyperplasia in experimental allergic conjunctivitis. Local antibody production. Arch Ophthalmol 107, 433–438 (1989)

5. Hall JM and Pribnow JF: Ocular immunization of guinea pigs. Curr Eye Res 6, 817–824 (1987)

6. Haldar JP, Khatami M, Donnelly JJ and Rockey JH: Experimental allergic conjunctivitis: Production of different isotypes of antibody by conjunctival-associated lymphoid tissue in culture. Reg Immunol 1, 92–99 (1988)

7. Peppard JV, Mann RV and Montgomery PC: Antibody production in rats following ocular-topical or gastrointestinal immunization: kinetics of local and systemic antibody production. Curr Eye Res 7, 471–481 (1988)

8. Haruna K, Hikida M, Ohsugi Y and Ohmori H: The secondary antigen-specific IgE response in murine lymphocytes is resistant to blockade by anti-IL-4 antibody and an antisense oligodeoxynucleotide for IL-4 mRNA. Cell Immunol 151, 52–64 (1993)

9. Azuma M, Hirano T, Miyajima H, Watanabe N, Yagita H, Enomoto S, Furusawa S, Ovary Z, Kinashi T, Honjo T and Okumura K: Regulation of murine IgE production in SJL/J and nude mice. Potentiation of IgE production by recombinant interleukin 4. J Immunol 139, 2538–2544 (1987)

10. Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 156–159 (1987)

11. Ohmori H, Hase N, Hikida M, Takai T and Endo N: Enhancement of antigen-induced interleukin 4 and IgE production by specific IgG1 in murine lymphocytes. Cell Immunol 145, 299–310 (1992)

12. Hase N, Takai T, Hikida M and Ohmori H: Predominant suppression of anti-TNP IgE response in mice by monoclonal anti-TNP IgG1 antibody: Characterization of its mode of action by in vivo and in vitro studies. Int J Immunopharmacol 16, 787–794 (1994)

13. Maggi E, Biswas P, Del Prete G, Parronchi P, Macchia D, Simonelli C, Emmi L, De Carli M, Tiri A, Ricci M and Romagnani S: Accumulation of Th2-like helper T cells in the conjunctiva of patients with vernal conjunctivitis. J Immunol 146, 1169–1174 (1991)

14. Del Prete GF, Vercelli D, Tiri A, Maggi E, Rossi O and Romagnani S: Effect of in vitro irradiated and cell cycle-inhibiting drugs on the spontaneous human IgE synthesis in vitro. J Allergy Clin Immunol 79, 69–77 (1987)