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Possible participation of cyclooxygenase-2 in the recurrence of allergic inflammation in rats

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Abstract

In the recurrence of allergic inflammation in a rat air pouch model, pouch fluid volume, prostaglandin E_2 concentration in the pouch fluid, leukocyte infiltration into the pouch fluid, and granulation tissue weight were markedly increased by the antigen challenge. To clarify the role of cyclooxygenase-2 in the recurrence of allergic inflammation, the time-course of changes in protein levels of cyclooxygenase-1 and cyclooxygenase-2 in the granulation tissue and in the infiltrated leukocytes was examined by Western blot analysis. It was shown that cyclooxygenase-1 levels in the granulation tissue and in the infiltrated leukocytes were not changed by the antigen challenge, but cycoloxygenase-2 levels were increased. Furthermore, treatment with the selective cyclooxygenase-2 inhibitor, NS-398 ([*N*-2(cyclohexyloxy-4-nitrophenyl]-methanesulfonamide), suppressed the recurrence of allergic inflammation as did the non-selective cyclooxygenase-1/cyclooxygenase-2 inhibitor, indomethacin. The steroidal anti-inflammatory drug, dexamethasone, inhibited the induction of cyclooxygenase-2, and suppressed the allergic inflammation. These findings strongly suggested that cyclooxygenase-2 induced by the antigen challenge plays a role in the recurrence of inflammation induced by the allergic mechanism.

Keywords: Allergic inflammation, recurrence of; Granulation tissue; Prostaglandin E_2; Cyclooxygenase-1; Cyclooxygenase-2; NS-398; Indomethacin; Dexamethasone

1. Introduction

Several types of prostaglandins whose synthesis is mediated by cyclooxygenase (prostaglandin H synthase, prostaglandin endoperoxide synthase; EC 1.14.99.1) play significant roles in inflammatory reactions (Davies et al., 1984). Recently, two isoforms of cyclooxygenase, cyclooxygenase-1 and cyclooxygenase-2, have been described. In vitro studies have demonstrated that the expression of the cyclooxygenase-2 gene is enhanced by several kinds of extracellular stimuli, although cyclooxygenase-1 is constitutively expressed (Rosen et al., 1989; Xie et al., 1991; O'Banion et al., 1991, 1992; Kubuju et al., 1991; Hla and Neilson, 1992; Ryseck et al., 1992; O'Sullivan et al., 1992a,b; Lee et al., 1992). This suggests that the stimulation of arachidonic acid metabolism at the inflammatory site is due to the induction of cyclooxygenase-2 in the inflammatory cells by such stimuli. Several trials have been carried out to examine whether cyclooxygenase-2 is induced at the inflammatory site in the experimental inflammation models (Masferrer et al., 1994; Seibert et al., 1994; Appleton et al., 1995) and in patients with rheumatoid arthritis (Crofford et al., 1994). Although high levels of immunoreactive cyclooxygenase were found in the synovia from patients with arthritis (Sano et al., 1992), it remains unknown whether the disease activity of rheumatoid arthritis correlates with the induction of cyclooxygenase-2 in the synovial tissue. Using experimental inflammation models, Masferrer et al. (1994) demonstrated that carrageenan administration to a subcutaneous air pouch of rats increased the protein levels of cyclooxygenase-2 in the inner layer of cells within the pouch lining and in macrophages obtained from the pouch fluid. In addition, Appleton et al. (1995) demonstrated that the injection of croton oil in Freund's complete adjuvant into the air pouch made on the dorsum of mice induced cyclooxygenase-2 in

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fibroblast- and macrophage-like cells in the granulomatous tissue, and endothelial cells in the loose connective tissue of the dermis. Recently, Anderson et al. (1996) have reported that cyclooxygenase-2 mRNA and cyclooxygenase-2 protein were increased in the hind footpad by the injection of *M. butyricum* in mineral oil. In such experimental inflammation models, the inflammatory responses were induced by foreign substances such as carrageenan, croton oil, or adjuvant, and did not reflect the mechanism of the antigen-induced allergic inflammation. Therefore, in the present investigation, we examined the role of cyclooxygenase-2 in antigen-induced inflammation.

First, to determine whether the protein level of cyclooxygenase-2 at the site of allergic inflammation is increased by the antigen challenge, we employed the experimental animal model of allergic inflammation. Second, to clarify the role of cyclooxygenase-2 in allergic inflammation we examined the anti-inflammatory effect of the selective cyclooxygenase-2 inhibitor, NS-398 ([*N*-2-(cyclohexyloxy-4-nitrophenyl)]-methanesulfonamide) (Futaki et al., 1994). We used an air pouch-type allergic inflammation model in rats that had been described previously in our laboratory (Tsurufuji et al., 1982). In this model, the first antigen challenge into the air pouch of the immunized rats induces prominent local anaphylactic reactions followed by delayed-type hypersensitivity reactions (Ohuchi et al., 1985; Hirasawa et al., 1986). Five days after the first antigen challenge, vascular permeability, the number of leukocytes infiltrated into the pouch fluid and prostaglandin E_2 contents in the pouch fluid are decreased although a granulation tissue capsule holding about 10 ml of the pouch fluid is formed (Watanabe et al., 1987). Injection of the antigen into the pouch at this stage induces recurrence of the allergic inflammation (Watanabe et al., 1987). Therefore, we examined the time-course of changes in the protein levels of cyclooxygenase-1 and cyclooxygenase-2 in the granulation tissue and in the infiltrated leukocytes in the pouch fluid collected at various times after the second antigen challenge. Furthermore, the effects of NS-398, a selective cyclooxygenase-2 inhibitor (Futaki et al., 1994), on the recurrence of allergic inflammation were examined and compared with those of indomethacin, a non-selective cyclooxygenase-1/cyclooxygenase-2 inhibitor, characterized by using intact cells, broken cells, and purified enzyme preparations (Mitchell et al., 1993), and of dexamethasone, a steroidal anti-inflammatory drug.

2. Materials and methods

2.1. Immunization and induction of allergic inflammation

Male rats of the Sprague-Dawley strain, 39–41 days old and weighing 150–160 g (Charles River Japan, Kanagawa, Japan) were immunized with the antigen, azobenzenearsonate-conjugated acetyl bovine serum albumin, with the aid of Freund's complete adjuvant (Tsurufuji et al., 1982). Briefly, 5 mg of the antigen dissolved in 250 μl saline was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA), and each 100 μl of the emulsion was injected intradermally at 2 nuchal and 3 lumbar sites of each rat. Nine days after immunization, 10 ml of air was injected subcutaneously in the dorsum, under light diethylether anesthesia, to make an ellipsoid shaped air pouch. Twenty-four hours after the injection of air, 2 mg of the antigen dissolved in 4 ml of sterile 2% (w/v) solution of sodium carboxymethylcellulose (Cellogen F3H, Dai-ichi Kogyo Seiyaku, Niigata, Japan) in 0.9% NaCl solution supplemented with antibiotics (0.1 mg penicillin G potassium and 0.1 mg dihydrostreptomycin sulfate per 1 ml of the solution) was injected into the air pouch as the first antigen challenge (Tsurufuji et al., 1982). The animal experiments were done in accordance with procedures approved by the Animal Ethics Committee in the Faculty of Pharmaceutical Sciences, Tohoku University, Japan.

2.2. Induction of recurrence of allergic inflammation

Into a granulation capsule that had been formed during a period of 5 days after the first antigen challenge, holding about 10 ml of exudate in the capsule, 3 mg of the antigen dissolved in 0.5 ml of sterile 0.9% NaCl solution was injected as a second antigen challenge to induce recurrence of allergic inflammation (Watanabe et al., 1987). At an appropriate time, pouch fluid volume, total number of leukocytes in the pouch fluid, granulation tissue weight, and amount of prostaglandin E_2 in the pouch fluid were determined as described below.

2.3. Drug treatment

NS-398 ([*N*-2 (cyclohexyloxy-4-nitrophenyl)]-methanesulfonamide, Funakoshi Co., Tokyo, Japan), indomethacin and dexamethasone (Sigma, St. Louis, MO, USA) were dissolved in ethanol and appropriately diluted in saline. The final ethanol concentration in saline was adjusted to 2% (v/v). NS-398 (100 μg), indomethacin (100 μg) or dexamethasone (10 μg) in 0.5 ml saline was injected into the pouch just after the second antigen challenge, and 1 and 2 days after the second antigen challenge. Control rats received the same amount of saline. The rats were killed by cutting the carotid artery 3 days after the second antigen challenge under diethylether anesthesia, and the entire pouch fluid was collected and measured. The granulation capsule was dissected and weighed, and a portion of the granulation tissue was used for histochemical analysis and the rest was frozen at −80°C.

2.4. Determination of total number of leukocytes in the pouch fluid

The pouch fluid was centrifuged at $1000 \times g$ and 4°C for 5 min to precipitate the cells. The cells were suspended

in phosphate-buffered saline (PBS, pH 7.4) and the total number of cells was counted using a hemocytometer. In some cases, differential counting of the infiltrated leukocytes was performed after May-Giemsa staining.

2.5. Determination of prostaglandin E_2 concentrations in the pouch fluid

One milliliter of the supernatant fraction of the pouch fluid, into which 5×10^{-4} μCi of [^{3}H]prostaglandin E_2 (160 Ci/mmol, [5,6,8,11,12,14,15-^{3}H(N)]prostaglandin E_2, Dupont NEN, Wilmington, DE, USA) had been added, was applied to a SEP-PAK C_{18} cartridge (Waters Associated, Milford, MA, USA). The lipophilic fraction containing prostaglandin E_2 was eluted with methanol, evaporated and dissolved in an aliquot of 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1% (w/v) gelatin, 0.15 M NaCl, and 10 mg/ml sodium azide. A portion of the solution was used for radioimmunoassay of prostaglandin E_2 (Watanabe et al., 1987). Another portion was used for the measurement of radioactivity for the calculation of recovery over the entire procedure (Watanabe et al., 1987). Prostaglandin E_2 antiserum was purchased from Seragen (Boston, MA, USA).

2.6. Western blot analysis of cyclooxygenase-1 and cyclooxygenase-2

The granulation tissue was homogenized in ice-cold solubilization buffer (50 mM Tris-HCl containing 1 mM *N,N*-diethyldithiocarbamate, 10 mM Na_2EDTA, 1% (v/v) Tween-20, 10 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride, pH 8.0) and centrifuged at $10\,000 \times g$ and 4°C for 10 min. The protein concentration in the supernatant fraction was determined (Wang and Smith, 1975), and was adjusted to 2 μg/μl of the solubilization buffer. 30 μl of the solution was applied into each well of a 10% (w/v) sodium dodecylsulfate-polyacrylamide slab gel (140 × 140 × 1 mm), electrophoresed at 25 mA for 4 h and transferred onto a nitrocellulose membrane. The infiltrated leukocytes were sonicated 5 times using a Handy Sonic Disrupter (UR-20P, TOMY, Tokyo, Japan) at 90% maximum power for 10 s at a time in 1 ml of ice-cold solubilization buffer, and centrifuged at $10\,000 \times g$ and 4°C for 10 min. The supernatant fraction from an aliquot containing 10^6 leukocytes was poured into each well of the slab gel, electrophoresed as described above, and transferred onto a nitrocellulose membrane. To detect cyclooxygenase-2, the membrane was incubated with polyclonal rabbit anti-mouse prostaglandin H synthase 2 antibody (1:100 dilution, Oxford Biomedical Research, Oxford, MI, USA) for 2 h at room temperature, followed by biotinylated anti-rabbit immunoglobulin G antibody (1:2000 dilution, Vector Laboratories, Burlingame, CA, USA) for 3 h at 4°C, and with avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min at room temperature. To detect cyclooxygenase-1, polyclonal goat-anti-sheep cyclooxygenase-1 (1:25 000 dilution, Oxford Biomedical Research) and biotinylated anti-goat immunoglobulin G antibody (1:2000 dilution, Vector Laboratories) were used. The reaction product was detected with the enhanced chemiluminescence detection system (ECL system, Amersham, Arlington Heights, IL, USA). The membrane was exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA) at room temperature for 20 to 30 s and photographed.

2.7. Histochemical staining of vascular endothelial cells

The granulation tissue was fixed in 99% (v/v) methanol for 2 min at 4°C, and incubated with 0.3% (v/v) H_2O_2 solution in methanol for 30 min at room temperature to reduce endogenous peroxidase activity. To improve the quality of the staining, the tissue was incubated with trypsin solution (50 mM Tris-HCl containing 0.1% (w/v) trypsin, 0.1% (w/v) $CaCl_2$, pH 7.4) for 35 min at 37°C. Non-specific binding was blocked with blocking solution (20 mM Tris-HCl containing 0.1% (v/v) Tween-20, 3% (w/v) bovine serum albumin, pH 7.5) for 1 h at room temperature. The tissue was immersed for 30 min at room temperature in PBS (pH 7.4) containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.1 mg/ml peroxidase-labeled *Griffonia simplifolia* agglutinin-I (Sigma) that specifically binds to rat vascular endothelial cells and macrophages. The reaction products were visualized by using 3,3′-diaminobenzidine intensified with $NiCl_2$. The tissue was counterstained with Meyer's hematoxylin, mounted and photographed. The number of small vessels in the same area was then counted under light microscopy in 4 different fields of each granulation tissue, and the mean number with S.E. was obtained from 6 to 7 rats in each group.

2.8. Statistical analysis

The results were analyzed for statistical significance by Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

3. Results

3.1. Recurrence of allergic inflammation

Injection of the antigen into the pouch 5 days after the first antigen challenge provoked the recurrence of allergic inflammation as revealed by the increase in pouch fluid volume, leukocyte infiltration into the pouch fluid, and granulation tissue weight (Fig. 1). Before the second antigen challenge, the pouch fluid volume was about 10 ml, and was significantly increased 8 h after the second antigen challenge. Thereafter, the pouch fluid volume increased with time, reaching about 25 ml at 48 h. Both

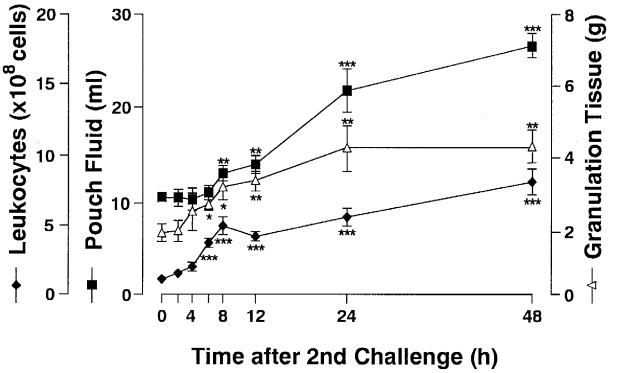

Fig. 1. Time-course of the pouch fluid volume, number of leukocytes in the pouch fluid, and the granulation tissue weight after the second antigen challenge. Five days after the first antigen challenge, 0.5 ml of saline containing 3 mg of the antigen was injected into the pouch. Rats were killed at the times indicated after the second antigen challenge, and the entire pouch fluid volume (■), number of leukocytes in the pouch fluid (◆), and the granulation tissue weight (△) were determined. Values are the means ± S.E. from 5 to 6 rats. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. corresponding control.

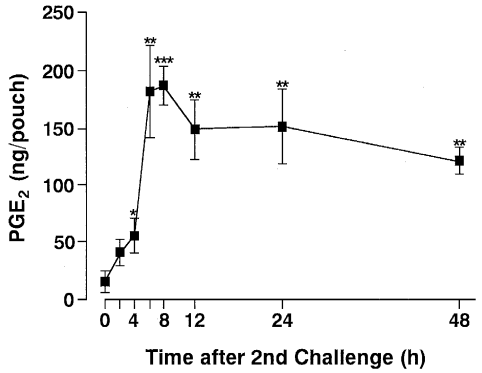

Fig. 2. Time-course of prostaglandin E_2 contents in the pouch fluid after the second antigen challenge. The entire pouch fluid was centrifuged at 1000 × *g* and 4°C for 5 min to remove infiltrated leukocytes, and the supernatant fraction was applied to a SEP-PAK C18 cartridge. The lipophilic fraction containing prostaglandin E_2 was eluted with methanol, evaporated, and reconstituted in the isogel Tris-buffer. Contents of prostaglandin E_2 were radioimmunoassayed, and the total amounts of prostaglandin E_2 (PGE_2) in the pouch fluid are shown. Values are the means ± S.E. from 5 to 6 rats. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0-time control.

leukocyte infiltration into the pouch fluid and granulation tissue weight increased with time, and significant increases were observed after 6 h. Before the second antigen challenge, the population of leukocytes in the pouch fluid consisted of 53.6% macrophages, 31.8% neutrophils, 14.5% lymphocytes and 0.1% eosinophils. After the second antigen challenge, the percentage of neutrophils increased, but that of macrophages and lymphocytes decreased. At 48 h, the leukocytes consisted of 23.4% macrophages, 72.2% neutrophils, 4.1% lymphocytes and 0.3% eosinophils (means of 5–6 rats).

3.2. The amount of prostaglandin E_2 in the pouch fluid

The total amount of prostaglandin E_2 in the pouch fluid increased markedly from 2 to 6 h after the second antigen challenge, and remained high until 48 h (Fig. 2).

3.3. Time-course of changes in the protein levels of cyclooxygenase-1 and cyclooxygenase-2 in the granulation tissue

Cyclooxygenase-1 was detected in the granulation tissue before the second antigen challenge (Fig. 3). After the second antigen challenge, no prominent changes in the amount of cyclooxygenase-1 in the granulation tissue were observed (Fig. 3). In contrast, cyclooxygenase-2 was not detected in the granulation tissue before the second antigen challenge (Fig. 3). However, after the second antigen challenge, the amount of cyclooxygenase-2 in the granulation tissue increased with time up to 6 h, and was constant up to 48 h (Fig. 3). These findings suggest that cyclooxygenase-2 in the granulation tissue is induced by the antigen challenge, and contributes to the production of prostaglandin E_2 at the inflammatory site.

3.4. Time-course of changes in the protein levels of cyclooxygenase-1 and cyclooxygenase-2 in the leukocytes in the pouch fluid

The amount of cyclooxygenase-1 in 10^6 leukocytes was similar before and after the second antigen challenge (Fig. 4). In contrast, cyclooxygenase-2 was barely detectable in 10^6 leukocytes in the pouch fluid before the second antigen challenge, but its levels increased after the second antigen challenge up to 6 h, and remained at a similar level up to 48 h (Fig. 4). These findings suggest that cyclooxygenase-2 is induced in the leukocytes infiltrated into the pouch fluid by the antigen challenge.

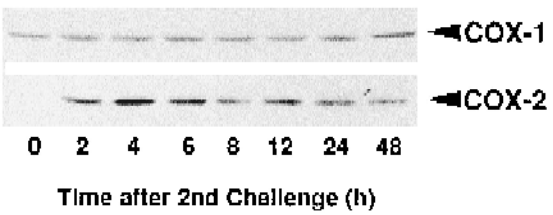

Fig. 3. Western blot analysis of cyclooxygenase-1 and cyclooxygenase-2 levels in the granulation tissue. The granulation tissue was obtained at the times indicated after the second antigen challenge, homogenized, and centrifuged at 10000 × *g* and 4°C for 10 min. The supernatant fractions (each 30 μg protein/30 μl) were poured into each well of a 10% SDS-polyacrylamide slab gel, electrophoresed, and transferred onto a nitrocellulose membrane. To detect cyclooxygenase-1, the membrane was incubated with polyclonal goat anti-sheep prostaglandin H synthase 1 antibody, biotinylated anti-goat immunoglobulin G, and avidin-biotin-peroxidase complex, in turn. Cyclooxygenase-2 was detected with polyclonal rabbit anti-mouse prostaglandin H synthase 2 antibody and biotinylated anti-rabbit immunoglobulin G. The reaction products of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) were detected with the ECL system. Similar results were obtained in five separate experiments with different rats.

3.5. Effects of indomethacin, NS-398 and dexamethasone on the recurrence of allergic inflammation

Intrapouch injection of indomethacin (100 μg), NS-398 (100 μg), or dexamethasone (10 μg) at the time of the second antigen challenge, and 1 and 2 days after the second antigen challenge suppressed the recurrence of allergic inflammation when evaluated 3 days after the second antigen challenge. As shown in Fig. 5, these drugs significantly inhibited pouch fluid accumulation, leukocyte infiltration, and granulation tissue formation. The inhibitory effect of the selective cyclooxygenase-2 inhibitor, NS-398, was similar to that of the non-selective cyclooxygenase-1/cyclooxygenase-2 inhibitor, indomethacin. The effect of the steroidal anti-inflammatory drug, dexamethasone, was the most potent. The populations of each type of cell in the leukocytes after indomethacin treatment were not significantly different from those after NS-398 treatment. These drugs significantly decreased the number of neutrophils. In contrast, dexamethasone treatment significantly decreased the number of all types of cells (data not shown).

The number of blood vessels in 1 mm^2 of granulation tissue was 383.4 ± 59.5, 228.6 ± 25.4, 258.4 ± 34.9 and 65.1 ± 11.9 in the control, indomethacin, NS-398 and dexamethasone groups, respectively (means ± S.E. from 6–7 rats). These findings indicate that treatment with indomethacin, NS-398, or dexamethasone significantly inhibited blood vessel formation in the granulation tissue, and that dexamethasone showed the most potent effect.

Injection of 30 μg of indomethacin or NS-398, or 3 μg of dexamethasone at the time of the second antigen challenge, and 1 and 2 days after the second antigen challenge, significantly inhibited the pouch fluid accumulation, leukocyte infiltration, granulation tissue formation, and microvascular formation in the granulation tissue at day 3. The inhibition by each drug at such a dose was less than half of that obtained with 100 μg for indomethacin and NS-398, and 10 μg for dexamethasone (data not shown).

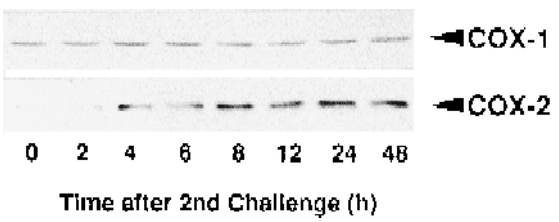

Fig. 4. Western blot analysis of cyclooxygenase-1 and cyclooxygenase-2 levels in the leukocytes infiltrated into the pouch fluid. The infiltrated leukocytes were collected at the times indicated after the second-time antigen challenge, sonicated, and centrifuged at $100\,000 \times g$ and 4°C for 10 min. The supernatant fraction from each aliquot (10^6 cells) was poured into each well of the slab gel. Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) were detected as described in the legend to Fig. 3.

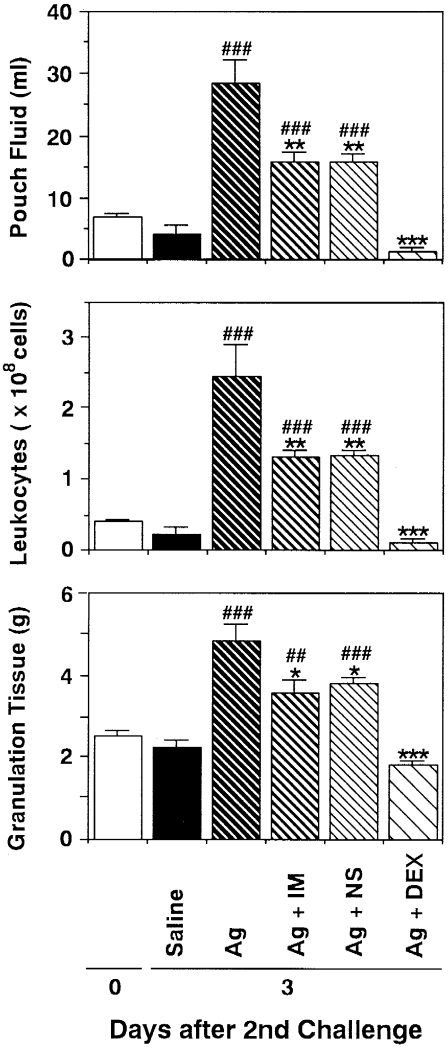

Fig. 5. Effects of indomethacin, NS-398 and dexamethasone on the recurrence of allergic inflammation. Indomethacin (IM, 0.1 mg), NS-398 (NS, 0.1 mg), or dexamethasone (DEX, 0.01 mg) in 0.5 ml saline was injected into the pouch just after the second antigen challenge, and 1 and 2 days after the second antigen challenge. Control rats received the same amount of saline. Three days after the second antigen challenge, the rats were killed, the entire pouch fluid was collected, and the granulation tissue was dissected. Values are the means ± S.E. from 7 to 8 rats. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. corresponding control. $^{\#\#\#}P < 0.001$ vs. the antigen-free control.

3.6. Effects of indomethacin, NS-398, and dexamethasone on the amount of prostaglandin E_2 in the pouch fluid

As shown in Fig. 6, treatment with indomethacin, NS-398, and dexamethasone significantly lowered the amount of prostaglandin E_2 in the pouch fluid 3 days after the second antigen challenge. There was no significant difference in the amount of prostaglandin E_2 in the pouch fluid between the group treated with the non-selective cyclooxygenase-1/cyclooxygenase-2 inhibitor, indomethacin, and that in the group treated with the selective cyclooxygenase-2 inhibitor, NS-398. There was also no significant

difference in the amount of prostaglandin E_2 between the two groups treated with 30 μg of indomethacin and NS-398 (data not shown). These findings suggest that cyclooxygenase-2 participates in the production of most of the prostaglandin E_2 in the pouch fluid at 3 days. The amount of prostaglandin E_2 in the group treated with dexamethasone was significantly lower ($P < 0.001$) than that in the group treated with indomethacin or NS-398.

3.7. Effects of indomethacin, NS-398, and dexamethasone on the protein levels of cyclooxygenase-1 and cyclooxygenase-2 in the granulation tissue

In the granulation tissue collected 3 days after the second antigen challenge, cyclooxygenase-1 and cyclooxygenase-2 were detected by Western blot analysis (Fig. 7). Intrapouch injection of indomethacin or NS-398 (each 100 μg) at the time of the second antigen challenge, and 1 and 2 days after the second antigen challenge did not influence the levels of cyclooxygenase-1 and cyclooxygenase-2 in the granulation tissue at 3 days (Fig. 7). However, treatment with dexamethasone (10 μg) lowered the level of cyclooxygenase-2 in the granulation tissue at 3 days, but did not affect the level of cyclooxygenase-1 (Fig. 7). Treatment with 30 μg of indomethacin or NS-398 also did not affect the levels of cyclooxygenase-1 or cyclooxygenase-2 at 3 days (data not shown). Dexamethasone, 3 μg, also did not affect the level of cyclooxygenase-1 but decreased cyclooxygenase-2 (data not shown).

In the leukocytes infiltrated into the pouch fluid at 3 days, the cyclooxygenase-1 level was not changed by treatment with indomethacin, NS-398, or dexamethasone, while the level of cyclooxygenase-2 was lowered by dexamethasone treatment but was not affected by treatment with indomethacin or NS-398 (data not shown).

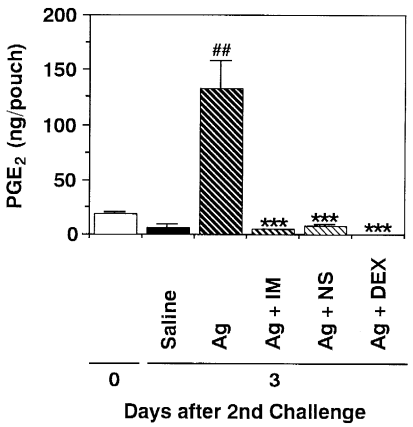

Fig. 6. Effects of indomethacin, NS-398, and dexamethasone on prostaglandin E_2 contents in the pouch fluid 3 days after the second antigen challenge. The entire pouch fluid was collected as described in the legend to Fig. 5, and centrifuged at 1000 *g* and 4°C for 5 min. The supernatant fraction was applied to a SEP-PAK C18 cartridge. The lipophilic fraction was eluted with methanol, evaporated, and reconstituted in isogel Tris buffer. Contents of prostaglandin E_2 (PGE_2) were radioimmunoassayed. Values are the means ± S.E. from 7 to 8 rats. Statistical significance: *** $P < 0.001$ vs. corresponding control. $^{\#\#}$ $P < 0.01$ vs. the antigen-free control.

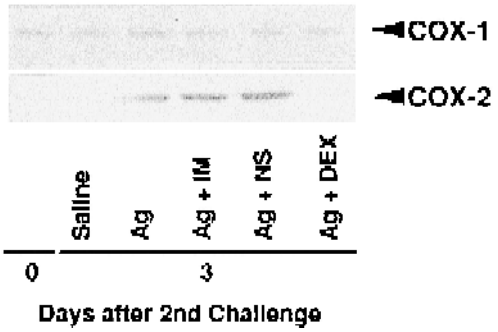

Fig. 7. Effects of indomethacin, NS-398, and dexamethasone on the levels of cyclooxygenase-1 and cyclooxygenase-2 in the granulation tissue 3 days after the second-time antigen challenge. Indomethacin (IM, 100 μg), NS-398 (NS, 100 μg), or dexamethasone (DEX, 10 μg) in 0.5 ml saline was injected into the pouch just after the second-time antigen challenge, and 1 and 2 days after the second-time antigen challenge. Control rats received the same amount of saline. Three days after the second-time antigen challenge, the rats were killed and the granulation tissue was dissected. The levels of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in the granulation tissue were detected by Western blot analysis as described in the legend to Fig. 3. Similar results were obtained in five separate experiments with different rats.

4. Discussion

The present investigation clearly demonstrated that antigen challenge into the capsule of the granulation tissue of immunized rats induces the formation of cyclooxygenase-2, but not that of cyclooxygenase-1, in the granulation tissue and in the leukocytes infiltrated into the pouch fluid. Furthermore, the selective cyclooxygenase-2 inhibitor, NS-398, suppressed the recurrence of allergic inflammation induced by the immunologic mechanism. These findings strongly suggest that cyclooxygenase-2 plays a role in the recurrence of allergic inflammation provoked where granulation tissue has been formed. There are several reports showing that cyclooxygenase-2 is induced at the site of inflammation provoked by injection of carrageenan into the air pouch of rats (Masferrer et al., 1994), injection of carrageenan (Seibert et al., 1994) or adjuvant (Anderson et al., 1996) into the footpad of rats, and injection of croton oil in Freund's complete adjuvant into the air pouch of mice (Appleton et al., 1995). Together, these findings and the present findings indicate that cyclooxygenase-2 is commonly induced at the site of inflammation provoked by either a non-immunologic mechanism or an immunologic mechanism.

The types of cells in the granulation tissue and infiltrated leukocytes in which cyclooxygenase-2 is induced remains to be clarified. Describing the chronic granulomatous tissue of the air pouch-type model in mice, Appleton

et al. (1995) reported that no cells within the granulomatous tissue in which the predominant cell types are polymorphonuclear leukocytes and macrophages showed immunoreactivity for cyclooxygenase-2 at day 3, and a small number of macrophage- and fibroblast-like cells positively labeled for cyclooxygenase-2 was first seen at day 5, the number increasing and reaching a peak at 14 to 21 days. Because these authors induced inflammation by injecting 0.1% croton oil in Freund's complete adjuvant directly into the air pouch made on the dorsum of the mice, the mechanism of the induction of inflammation was different from that of the recurrence model of allergic inflammation employed in the present investigation. Although they examined the distribution of the cells that were labeled with cyclooxygenase-2 antibody, Appleton et al. (1995) did not examine the effect of the selective inhibitor of cyclooxygenase-2. Masferrer et al. (1994) examined the effect of NS-398, a selective cyclooxygenase-2 inhibitor, on the carrageenan-induced air pouch-type inflammation model in rats, and showed that NS-398 is a potent anti-inflammatory agent and does not produce the typical side-effects associated with the non-selective, cyclooxygenase-1-directed anti-inflammatory drugs. The carrageenan-induced inflammation model is also different from the allergic inflammation model in the mechanism of the induction of inflammation. Anderson et al. (1996) also reported that the selective inhibitor of cyclooxygenase-2, SC-58125, suppressed the adjuvant-induced paw edema in rats.

It is also necessary to clarify the mechanism for the induction of cyclooxygenase-2 in the granulation tissue and in the infiltrated leukocytes after antigen challenge in the present model. Akarasereenont et al. (1994) reported that tyrosine kinase activity is a crucial step in the induction of cyclooxygenase-2 protein in murine macrophages. Therefore, the activation of tyrosine kinase might be a key event in the signal transduction pathways for the induction of cyclooxygenase-2 at the site of allergic inflammation. Because tyrosine kinase is activated by several cytokines, antigen challenge may induce the production of cytokines, which induce cyclooxygenase-2 protein. After the antigen challenge, the amount of prostaglandin E_2 in the pouch fluid increased with time (Fig. 1). In this model, prostaglandin E_2 production was the greatest, followed by 6-keto-prostaglandin $F_{1\alpha}$ > prostaglandin $F_{2\alpha}$ > thromboxane B_2 (Ohuchi et al., 1984). The antigen-induced production of prostaglandins might be due to the induction of cyclooxygenase-2 in the granulation tissue and in the leukocytes infiltrated into the pouch fluid. A portion of the increase of cyclooxygenase-2 levels in the granulation tissue might be attributable to the extravascular leukocytes remaining in the granulation tissue. However, when the relative amount of cyclooxygenase-2 is much less than that of cyclooxygenase-1, prostaglandin production in the inflammatory tissue may depend on the release of arachidonic acid from membrane phospholipids by phospholipase A_2, and not on the induction of cyclooxygenase-2. At present, it is difficult to determine quantitatively by Western blot analysis the amount of cyclooxygenase-2 relative to that of cyclooxygenase-1. When certain cytokines, such as interleukin-1, are produced at the inflammatory site, phospholipase A_2 might be expressed in high levels (Angel et al., 1994; Denis, 1994), resulting in elevated levels of arachidonic acid. Together with the induction of cyclooxygenase-2, highly expressed phospholipase A_2 also may participate in the production of prostaglandin E_2 at the inflammatory site.

Finally, as to the effect of the steroidal anti-inflammatory drug, dexamethasone, cyclooxygenase-2 induction by antigen challenge in the granulation tissue was inhibited by treatment with dexamethasone, and cyclooxygenase-1 levels in the granulation tissue were not altered by treatment with dexamethasone (Fig. 7). The same effects of dexamethasone were observed in the leukocytes infiltrated into the pouch fluid (data not shown). Inhibition by dexamethasone of the induction of cyclooxygenase-2 in the cell culture system has also been reported (O'Banion et al., 1992; DuBois et al., 1994; Mitchell et al., 1994). The antigen-induced increase in prostaglandin E_2 contents in the pouch fluid was almost completely suppressed by dexamethasone treatment (Fig. 6). This might be due to the inhibition of the induction of cyclooxygenase-2. The induction of phospholipase A_2 may also be inhibited by dexamethasone (Hoeck et al., 1993; Schalkwijk et al., 1993), thereby decreasing the substrate arachidonic acid for cyclooxygenase-1 and cyclooxygenase-2. However, the anti-inflammatory activity of dexamethasone cannot be attributed solely to the inhibition of the induction of cyclooxygenase-2 and phospholipase A_2, because glucocorticoids have various anti-inflammatory activities independent of the inhibition of arachidonic acid metabolism (Tsurufuji et al., 1980; Tsurufuji and Ohuchi, 1989).

In conclusion, on antigen challenge, cyclooxygenase-2 was induced in the granulation tissue and in the leukocytes infiltrated into the pouch fluid of immunized rats, while antigen challenge did not alter the cyclooxygenase-1 level. Furthermore, NS-398, a selective cyclooxygenase-2 inhibitor, showed potent anti-inflammatory activity as did indomethacin, a non-selective cyclooxygenase-1/cyclooxygenase-2 inhibitor. These findings suggest that cyclooxygenase-2 plays a role in the recurrence of allergic inflammation. This is the first demonstration of the role of cyclooxygenase-2 in the antigen-induced recurrence of inflammation, and the effectiveness of the selective cyclooxygenase-2 inhibitor for the suppression of allergic inflammation.

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