不十分と考えている８)。

スギ花粉症に対する手術療法のコントロールスタディーとしては我々が炭酸ガスレーザーに関して重症度に関して行ったものが唯一のものであり薬物投与群と比較してレーザー手術群が有効であった事を報告している７)。花粉症に対する効果の判定は花粉の飛散量に大きく左右されるため今回の検討ではシーズンを通しての生活への支障度、QOL を重視した判定基準を用いた。

今回の重症度評価では少量飛散年であった 2004 年の著効率は 67%であり、大量飛散年であった 2005 年では 29%、やや少なかった 2006 年ではでは 62%であった。レーザー治療の治療成績には花粉飛散量が大きく影響した。大量飛散年には薬物の併用が必要になると思われる。我々の通年性アレルギー症例における検討でも炭酸ガスレーザー治療は薬物の有効な中等症以下の重症度症例において薬物を中止できる可能性が高い、薬物無効例では効果は不十分であると述べてきた。今回の検討はスギ花粉症症例においても同様の傾向を示したが、関西地方の平年程度の飛散ではでは半数の患者で点眼薬を除く薬物の投与を中止できるといえる。JRQLQ による QOL 評価では少量飛散年でも大量飛散年でも点鼻ステロイドとほぼ同程度であった。

スギ花粉症に対する炭酸ガスレーザーの越年効果を評価するために行った抗原誘発検査の結果からは越年効果は証明できなかった。しかし、症例数が少ない上、年度間の花粉飛散量の変動が大きく、2005 年の大量飛散の影響が off season にも影響している可能性があり、今後改めて検討する必要がある。しかし、昨年度のＡＰＣ治療の越年効果に関するアンケート調査では、花粉量が平年並みであれば越年効果は困難と考えられた事と、通年性アレルギー性鼻炎に対する APC の効果が１年を過ぎると落ちてくる事も考え合わせて、薬物抵抗性の花粉症の患者には花粉量が平年並みより少ない年以外は毎年レーザーを受ける事が望ましいと考えた。

カモガヤ花粉症合併例は単独例と比較して重症度、満足度ともほぼ同じであった。カモガヤ花粉症合併例でカモガヤ飛散終了まで効果が持続した事から、越年効果はなくても、少なくともスギ、ヒノキ、カモガヤの飛散が終了する６月までは持続すると考えられた。

スギ花粉飛散中のレーザーの効果もしばしば議論になるが、今回は検討していないが、強い患者希望で季節中照射をおこなった自験例のなかに照射後に著しい鼻閉を来す症例があり、原則的に我々はおこなっていない。薬物と併用すれば季節中照射も可能かもしれないが、花粉症に対する作用機序が扁平上皮化成だとすれば、効果発現に数週間を要するわけで、ヒノキやイネ科花粉への効果は期待できてもスギ花粉飛散への効果は限られると思われる。

E. 結論

スギ花粉症に対する炭酸ガスレーザーの有効性と越年効果を重症度評価とともに、ＱＯＬおよび抗原誘発試験によって評価した。レーザー治療の効果はスギ花粉飛散量に大きく影響された。しかし、関西を含む西日本の平均的な飛散量であれば、半数の患者で点眼薬以外の薬物療法を終止できると思われた。越年効果に関しては今後さらに検討が必要だが平年なみの花粉飛散量の季節では認められなかった。スギ・ヒノキ花粉症は致死的でなく、一種の自然災害ともいえる疾患だが、国民の 20%が罹患し、未発症抗体陽性患者数から考えると、今後国民の半数が発症する可能性がある。このような疾患の対策に対する国民の要求は根治性よりも費用対効果にあると思われる。根治性を求めて遺伝子治療などの高度先進的な医療を開発するとともに、既存の抗アレルギー薬やマスク、メガネ、レーザー治療などの対症的治療の安全性や有効性や経済性を改善することや、甜茶や乳酸菌食品などの民間治療の有効性の検証も重要である。

F. 健康危険情報

照射当日の鼻閉と数日間の粘膜表面の scab の形成を除けば季節前照射に関して、問題となる副作用はなかった。

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

厚生労働科学研究費補助金（免疫アレルギー疾患予防・治療研究事業）
研究報告書

リアルタイムモニター飛散数と現状の治療によるQOLの関連性の評価と花粉症根治療法の開発
マウススギ花粉症モデル作製とCRTH2の関与に関する研究

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研究要旨
今回我々は、スギ花粉症の病態をより詳細に解析することを目的に、本疾患の感作・発症動態をより反映するマウスモデルの作製を試みた。SPF環境下にBALB/cマウス（雌：６－１０週齢）に対してスギアレルゲンCryj1をアジュバント非存在下に繰り返し点鼻感作した。反復点鼻投与により、マウスはくしゃみおよび鼻かき回数の有意な増加を示した。さらにCry j 1-特異的なIgEおよびIgG1抗体の産生、鼻粘膜内好酸球浸潤がみられ、ヒトのスギ花粉症を反映するモデルであることが示された。また顎下リンパ節細胞において、Cry j 1刺激によりIL-4およびIL-5産生が誘導された。さらに本モデルにおけるCRTH2の役割を解析する目的で、CRTH2ノックアウトマウス（CRTH2-/-マウス）における病態の変化を観察した。野生型マウスと比較して、CRTH2-/-マウスでは点鼻チャレンジ後のくしゃみ回数および鼻かき回数が有意に減少した。CRTH2-/-マウスでは抗原特異的なIgEおよびIgG1抗体の産生も抑制され、さらに鼻粘膜内好酸球浸潤も減弱した。以上よりCRTH2はスギ花粉症においてpro-inflammatoryに働くことが示され、CRTH2を制御することがスギ花粉症の治療として有用である可能性が示唆された。

A. 研究目的
花粉症・アレルギー性鼻炎の病態理解の一助となるように、我々は以前よりマンソン住血吸虫卵抗原などを繰り返し点鼻感作することにより誘導されるアレルギー性鼻炎モデルの作製を行ってきた。今回は、マウススギ花粉症モデルの作製を試み、スギ花粉アレルゲンをアジュバント非存在下に点鼻投与することによりアレルギー動態が誘導されるのか検討した。さらにCRTH2ノックアウトマウスを用いて、CRTH2の本モデルにおける役割を解析した。

B. 方法
BALB/cマウス（雌7週齢）に対してday 0、7、14にスギ花粉アレルゲンCry j 1を点鼻感作した。さらにday 21から7日間Cryj1を連日点鼻チャレンジした。これらの投与はPBSのみ、低用量（1 g）Cryj1、高用量（5 g）Cry j 1の3群に分けて行った。最終点鼻チャレンジ時のくしゃみ・鼻かき回数からCry j 1に対する過敏性の変化を検討した。チャレンジ後に採血を行い、血清中のCry j 1特異的IgEおよびIgG1/IgG2a抗体価をELISAにて測定した。さらに顎下リンパ節細胞を単離して、Cryj1刺激下で培養し上清中のサイトカインを測定した。また、鼻中隔粘膜内の好酸球浸潤の程度を観察した。さらに本モデルにおけるCRTH2の関与を解析する目的で、CRTH2ノックアウトマウスを用い同様の検討を行った。またCRTH2/TPアンタゴニストであるラマトロバン(30mg/kg)を内服させた場合の病態変化も観察した。

C. 結果
１．マウススギ花粉症モデルの作製
高用量のCryj1点鼻投与に対して、PBS投与に比較してBALB/cマウスは有意に亢進したくしゃみおよび鼻かき回数を示した。Cry j 1点鼻投与にて血清中のCryj1特異的IgEおよびIgG1抗体価はともに上昇した。さらにCry j 1感作群では鼻粘膜内好酸球浸潤が認められた。また、顎下部リンパ節細胞におけるCryj1刺激に対するIL-4およびIL-5の産生は高用量Cryj1を点鼻投与した群で増加した。
２．マウススギ花粉症モデルにおけるCRTH2の役割
CRTH2ノックアウトマウスでは、野生型マウスに比較して有意な症状の抑制、Cryj1特異的IgE/IgG1抗体産生の抑制、鼻粘膜浸潤好酸球数の低下、顎下部リンパ節細胞によるIL-4およびIL-5産生の有意な抑制がみられた。ラマトロバンを内服したマウスでも同様の傾向を示した。

D. 考察
これまでもスギ花粉症の動物モデルとしていくつかの報告がある（Murasugi T, et al. Eur. J. Pharmacol. 510: 143, 2005など）。しかしながらこれまでのモデルは全身感作あるいはアジュバントを用いるものが多く、臨床的な感作動態を反映しているとは言い難い。今回のモデルの特徴はスギ花粉アレルゲンをアジュバントなしに点鼻のみにて投与することにより感作および発症を誘導できる点であり、ヒトのスギ花粉症により近いモデルと言える。

アレルギー性炎症におけるCRTH2の位置づけについては相反する報告がなされている。最近、マウス喘息モデルではCRTH2はanti-inflammatoryに作用するとの報告が示された (Chevalier E, et al. J. Immunol. 175: 2056, 2005)。今回の結果はこの報告とは一致せず、マウススギ花粉症モデルにおいてはCRTH2はpro-inflammatoryに作用することが示された。マウスの系統差や用いた抗原を含む感作方法の違い、あるいは上気道と下気道による役割の差異などが影響したものと思われた。

E. 結論

ヒトの感作・発症の病態により近いマウススギ花粉症モデルを作製した。本モデルを用いた検討より、CRTH2を抑制的に制御することがスギ花粉症の予防・治療として有効である可能性が示唆された。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

Signals through CD40 play a critical role in the pathophysiology of *Schistosoma mansoni* egg antigen–induced allergic rhinitis in mice

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ABSTRACT

Background: *Interaction between CD40 and CD40L is thought to regulate immune responses in several allergic diseases. However, little is known about its in vivo role in the pathophysiology of allergic rhinitis. We sought to determine whether the lack of signals through CD40 affects the pathophysiology of allergic rhinitis using a murine model.*

Methods: *Wild type (WT) and CD40-deficient BALB/c ($CD40^{-/-}$) mice were sensitized intranasally to Schistosoma mansoni egg antigen (SEA). After repeated sensitization, histamine responsiveness, serum antibody titer including immunoglobulin E (IgE), nasal eosinophilia, and cytokine production by nasal mononuclear cells were determined in each group.*

Results: *Intranasal sensitization with SEA in WT mice elicited a strong Th2 response including SEA-specific IgE production, nasal eosinophilia, and interleukin (IL)-4, and IL-5 production by nasal mononuclear cells after antigen challenge. Production of SEA-specific IgE and IgG1 was abolished in SEA-sensitized $CD40^{-/-}$ mice. These mice showed impaired nasal eosinophilia and displayed markedly reduced histamine-induced nasal hyperresponsiveness as compared with WT mice. Furthermore, reduced production of IL-4 and IL-5 by nasal mononuclear cells was seen in $CD40^{-/-}$ mice.*

Conclusion: *These results show that signals through CD40 play a critical role in not only IgE production but also pathophysiology of allergic rhinitis such as nasal hyperresponsiveness and nasal eosinophilia.* (Am J Rhinol 20:165–169, 2006)

A member of the tumor necrosis factor receptor superfamily, CD40 is expressed on thymic epithelial cells, B cells, and a number of professional antigen-presenting cells. Many studies have indicated that the interaction of CD40 with its ligand CD40L, which is expressed on activated T cells, plays a critical role in induction of T-cell–dependent B-cell responses. Thus, CD40/CD40L interaction regulates B-cell proliferation, immunoglobulin (Ig) production, isotype switching, and induction of B-cell memory.[1,2] Studies using both $CD40^{-/-}$ and $CD40L^{-/-}$ mice as well as blocking antibody (Ab) have shown that CD40/CD40L interaction also modulates inflammatory responses by up-regulating production of proinflammatory cytokines and nitric oxide from monocytes.[3,4]

Allergic rhinitis is associated with the expansion of Th2-type immune responses characterized by the production of allergen-specific IgE, nasal eosinophilia, and nasal hyperresponsiveness.[5,6] Several studies using CD40- or CD40L-deficient mice have shown a role for CD40/CD40L interaction in the pathophysiology of asthma, one of the most typical allergic diseases in the lower airway.[7–10] However, it remains unclear whether CD40/CD40L interaction also is involved in pathogenesis of allergic rhinitis, and to date, no reports have established the *in vivo* role of CD40 or CD40L in the initiation of allergic rhinitis. We have developed a murine model of allergic rhinitis, which received repeated intranasal administration of *Schistosoma mansoni* egg antigen (SEA) in the absence of an adjuvant and consequently showed Th2 response and nasal mucosa inflammation with eosinophil infiltration.[11] To determine whether CD40/CD40L interaction plays a role in pathogenesis of allergic rhinitis *in vivo*, we compared nasal hyperresponsiveness, Ab production, nasal eosinophilia, and nasal mononuclear cell cytokine production in SEA-sensitized $CD40^{-/-}$ mice with that in similarly sensitized wild-type (WT) mice. We believe that this is the first study showing the *in vivo* role of CD40 in nasal pathophysiology such as nasal eosinophilia and nasal hyperresponsiveness in a murine model of rhinitis.

MATERIALS AND METHODS

Animals

Homozygous CD40-deficient BALB/c ($CD40^{-/-}$) mice and WT BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Six- to 7-week-old female mice were used in all of the experiments. These mice were maintained in the specific pathogen-free condition in accordance with instituted guidelines.

Sensitization of Mice

We sensitized WT and $CD40^{-/-}$ mice as previously described.[11] In brief, 5 μg of SEA was instilled into the anterior nose once a week for 3 weeks (on days 1, 8, and 15). One week later, mice were challenged intranasally with 1 μg of SEA for 7 consecutive days (on days 22–28; nasal challenge). Mice were bled by tail snipping on day 6 after the third sensitiza-

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tion and 18 hours after the final challenge. After 18 hours following the final challenge, the mice were killed and nasal mononuclear cells were obtained for further analysis as described before.[11]

Measurement of Nasal Hyperreactivity

To assess nasal hyperreactivity in SEA-sensitized mice, responsiveness to intranasally administered histamine was measured by determining the concentration of histamine that causes nasal rubbing and sneezing. Briefly, 10 μL of histamine (Sigma, St. Louis, MO) in different doses was administered into the nostrils of SEA-sensitized $CD40^{-/-}$ and WT mice. Nasal symptoms (nasal rubbing and sneezing) were evaluated by counting the number of nasal rubbings and sneezing that were caused for 5 minutes after nasal administration of histamine. The concentration of histamine at which mice rubbed >40 times and also sneezed >5 times was identified as the threshold concentration. This point was expressed to the limiting concentration of histamine (log10 M). The nasal responsiveness was measured 1 day before both the first sensitization and the death (day 0 and day 29, respectively). The nose symptom assay was examined blindly.

Ab Enzyme-Linked Immunosorbent Assay

The levels of SEA-specific IgG_1 and IgG_{2a} in the serum were measured by enzyme-linked immunosorbent assay (ELISA) as previously described.[11] The serum total IgE and SEA-specific IgE was determined also by ELISA as previously described.[11]

Histological Examination

The heads were removed, fixed, and decalcified. Coronal nasal sections were stained with Luna solution and the number of eosinophils in the posterior part of the nasal septum was counted microscopically under a high-power field (10 × 40).[12] This count was done blindly.

In vitro Culture of Nasal Mononuclear Cells and Cytokine Determination

Nasal mononuclear cells from WT and $CD40^{-/-}$ mice were isolated by enzyme extraction, as previously described, with slight modification.[12] Cells were cultured in 96-well flat-bottom plates in 5% CO_2 at 37°C with and without plate-bound purified hamster anti-mouse CD3e monoclonal Ab (1μg/mL; Biosciences Pharmingen, San Jose, CA). After 72 hours of culture, supernatants were harvested. Interleukin (IL)-4, IL-5, and interferon (IFN) γ production in culture supernatant were measured by captured ELISA as previously described.[13] The detection limit for IL-4, IL-5, and IFN-γ in this system was 10, 200, and 100 pg/mL, respectively.

Statistics

Nonparametric Mann–Whitney *U* test was used to evaluate the differences in the values obtained. A value of $p < 0.05$ was considered significant. Values were given as mean ± SEM.

RESULTS

Impaired Histamine-Induced Nasal Responsiveness in SEA-Sensitized $CD40^{-/-}$ Mice

Nasal allergic symptoms such as sneezing and nasal rubbing were determined before the sensitization (day 0) and after the final nasal challenge (day 28). Before sensitization, histamine responsiveness was not different between WT and $CD40^{-/-}$ mice. In WT mice, histamine responsiveness was remarkably enhanced after the final challenge ($p < 0.01$). However, histamine responsiveness was not changed in CD $40^{-/-}$ mice after the final challenge, and the responsiveness was significantly lower as compared with that of control mice (Fig. 1).

SEA-Sensitized $CD40^{-/-}$ Mice Show Reduced Recruitment of Eosinophils into Nasal Mucosa

After intranasal sensitization with SEA, WT mice displayed significant eosinophil infiltration into nasal mucosa (Fig. 2*A*). In contrast, only a few eosinophils were present in nasal mucosa from SEA-sensitized $CD40^{-/-}$ mice ($p = 0.003$, Fig. 2*B*). The number of eosinophils infiltrating into nasal septum per field (10 × 40) was 26.5 ± 6.0 and 8.0 ± 5.5 (median value with range) in WT ($n = 6$) and $CD40^{-/-}$ mice ($n = 8$), respectively. There were no significant differences in the numbers of infiltrating lymphocytes between the groups.

Analysis of Ab Response in SEA-Sensitized $CD40^{-/-}$ and WT Mice

After the nasal sensitization, WT mice produced significant amounts of Th2-associated total IgE and SEA-specific IgE and IgG_1 but no Th1-associated IgG_{2a} (Figs. 3 and 4). On the other hand, similarly sensitized $CD40^{-/-}$ mice abolished the production of both total IgE and SEA-specific IgE during the

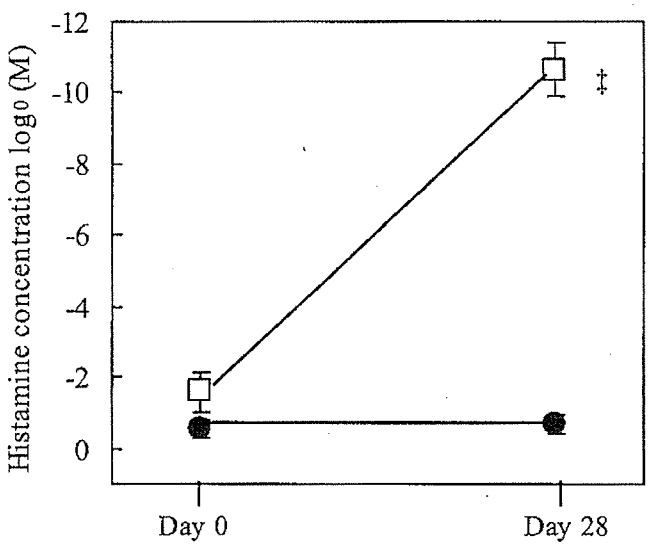

Figure 1. Histamine-induced nasal responsiveness. WT mice and $CD40^{-/-}$ mice were intranasally sensitized with SEA once a week for 3 weeks. Then, 1 week later, all mice were challenged with SEA for 7 consecutive days. The nasal hyperresponsiveness for histamine was determined before sensitization (day 0) and after nasal challenge (day 28). Histamine responsiveness of WT (open square) and $CD40^{-/-}$ mice (closed circle) was expressed by the strongest histamine concentration that caused mice both >5 times sneezing and >40 times nasal rubbing. Data shown are mean ± SEM of five mice per group. Data are representative of two separate experiments (‡$p < 0.01$ compared with the WT group).

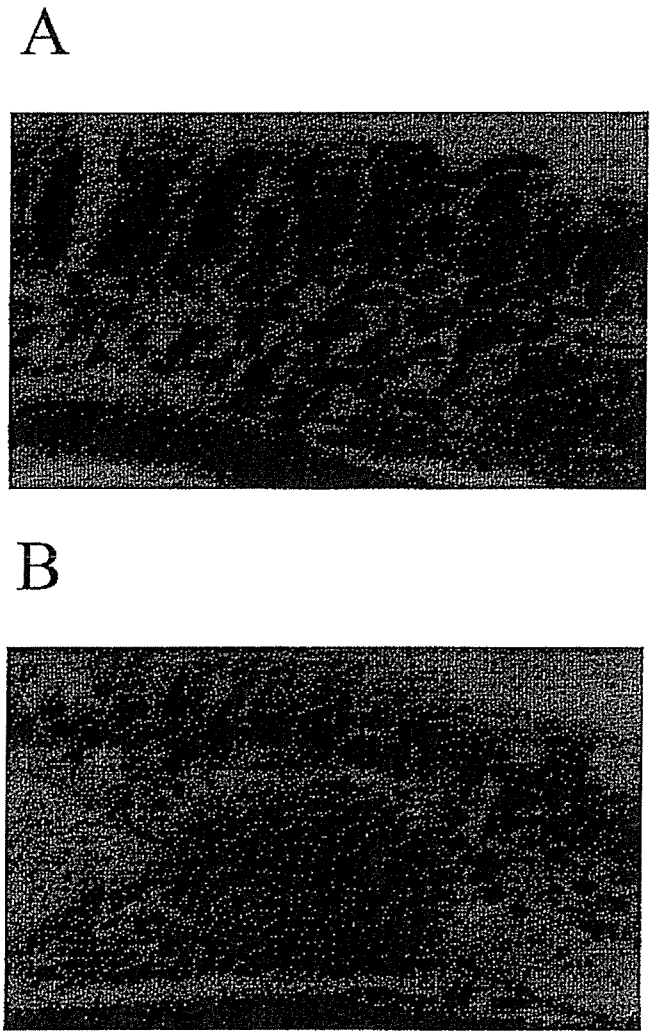

Figure 2. Nasal eosinophilia of mice intranasally sensitized with SEA. WT and CD40$^{-/-}$ mice were intranasally sensitized with SEA. After nasal challenge, mice were killed, and coronal nasal sections were fixed, decalcified, and stained with Luna solution. Typical nasal sections are shown: (A) WT mouse and (B) CD40$^{-/-}$ mouse.

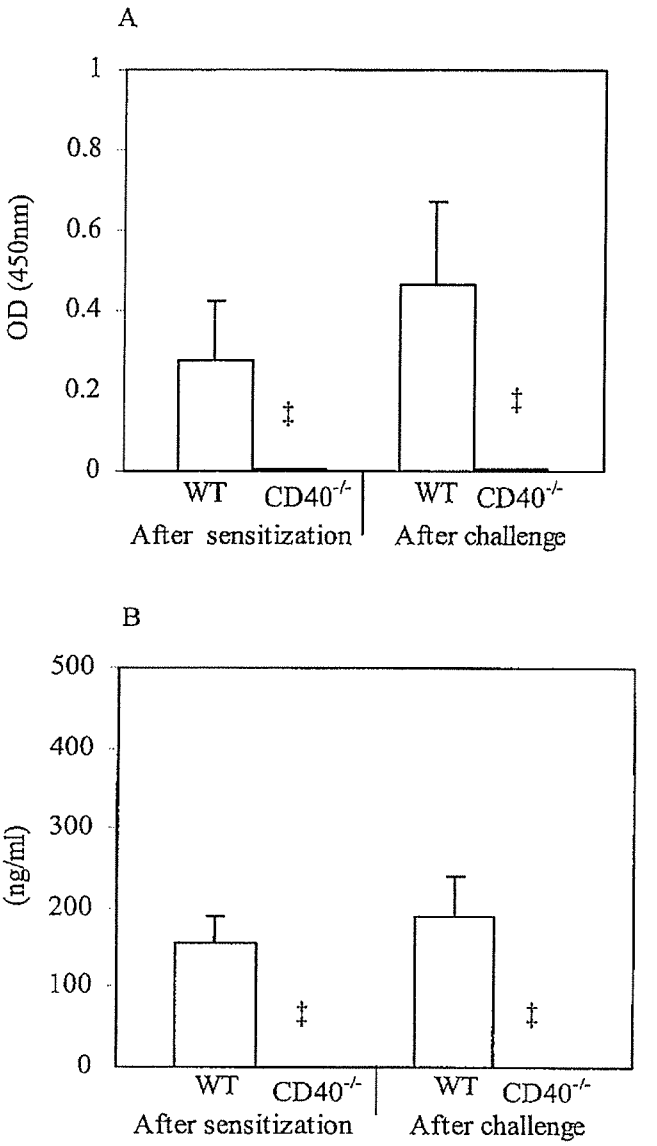

Figure 3. (A) Titers of SEA-specific IgE and (B) amount of total IgE. WT mice and CD40$^{-/-}$ mice were intranasally sensitized with SEA once a week for 3 weeks. Then, 1 week later, all mice were challenged with SEA for 7 consecutive days. WT (open column) and CD40$^{-/-}$ mice (closed column) were bled by the tail snipping on day 6 after the third sensitization and 18 hours after the final challenge. SEA-specific IgE and serum total IgE were detected with sandwich ELISA. Results show (A) the mean optical density at 450 nm ± SEM at 1:4 dilution and (B) the mean nanograms per milliliter ± SEM of five serum samples from each group. Data are representative of two separate experiments (‡$p < 0.01$ compared with the WT group).

period observed (Fig. 3). CD40$^{-/-}$ mice also failed to produce significant levels of SEA-specific IgG_1 and IgG_{2a} (Fig. 4).

Analysis of Cytokine Production by Nasal Mononuclear Cells in SEA-Sensitized CD40$^{-/-}$ and WT Mice

After *in vitro* stimulation with anti-CD3, nasal mononuclear cells from WT mice produced significant levels of IL-4 and IL-5. In contrast, nasal lymphocytes from CD40$^{-/-}$ mice produced only basal levels of IL-4 or IL-5 (Fig. 5, *A* and *B*). As regards IFN-γ, there were no significant differences between WT and CD40$^{-/-}$ mice (Fig. 5C).

DISCUSSION

In this study, we showed that CD 40$^{-/-}$ mice sensitized intranasally with SEA mount poor Th2 response, produce little or no IL-4 and IL-5 as well as SEA-specific IgG_1 and IgE. Furthermore, CD40$^{-/-}$ mice display markedly reduced nasal eosinophilia and nasal hyperresponsiveness in response to histamine. These findings indicate that CD40 plays a critical role in the pathophysiology of SEA-induced murine allergic rhinitis.

An engagement of CD40 with CD40L provides an essential

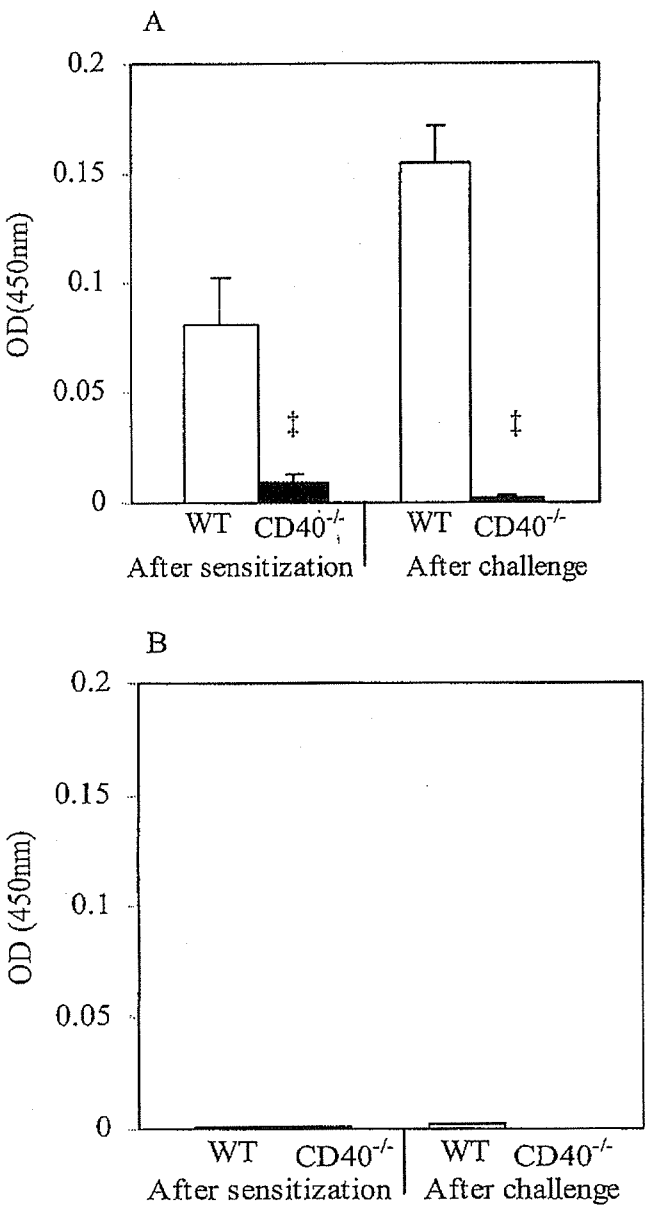

Figure 4. SEA-specific (A) IgG_1 and (B) IgG_{2a} isotypes. After boosting sensitization with SEA, WT mice and $CD40^{-/-}$ mice were intranasally challenged with SEA for 7 consecutive days. Sera were taken from WT (open column) and $CD40^{-/-}$ mice (closed column) 6 days after last boosting sensitization and 18 hours after last nasal challenge. They were assayed for SEA-specific IgG_1 and IgG_{2a} in indirect ELISA. Results show the mean optical density at 450 nm ± SEM of five serum samples from each group at 1:100 dilutions. Data are representative of two separate experiments (‡$p < 0.01$ compared with the WT group).

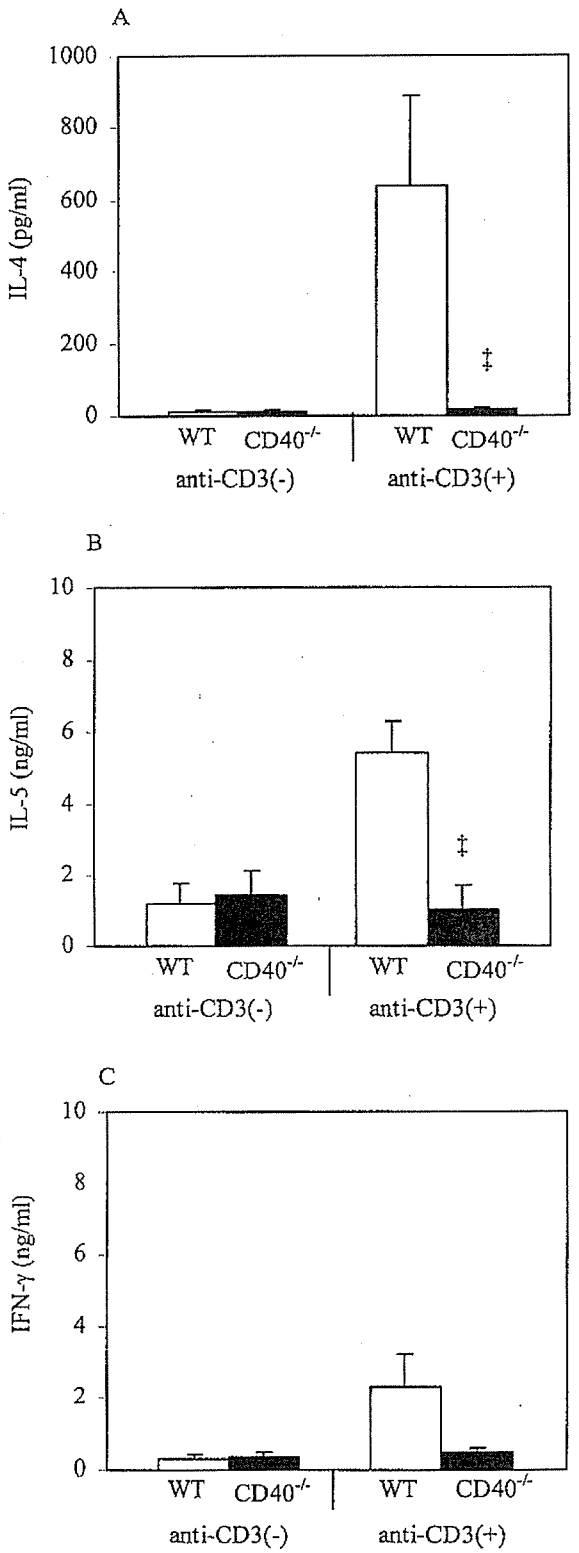

Figure 5. Production of (A) IL-4, (B) IL-5, and (C) IFN-γ by nasal mononuclear cells from WT (open column) and $CD40^{-/-}$ (closed column) mice sensitized intranasally with SEA. Nasal mononuclear cells were isolated and cultured in vitro *for 72 hours without or with plate-bound antimouse CD3e monoclonal Ab. Cytokines were measured by ELISA. Results were expressed the mean ± SEM. Data are representative of two separate experiments (†$p < 0.05$, ‡$p < 0.01$).*

costimulatory signal to the B cell that is required for T-cell–dependent class switching for all Ig's except IgM.[1] A previous study has shown that CD40/CD40L interaction is required for initiation of the immune response to antigens that inherently induce a very strong Th2 response and production of IL-4, which facilitates isotype switching to IgE and IgG_1.[7,14] On the other hand, Ferlin *et al.* found that CD40 signaling causes

IL-4–independent IgE switching *in vivo*.[15] We have previously reported that SEA-sensitized IL-4–deficient mice fail to produce significant levels of IgE, suggesting that IL-4 is critical for IgE production in SEA-induced rhinitis.[16] Therefore, it is perhaps not surprising that SEA-sensitized $CD40^{-/-}$ mice, which produced significantly less IL-4, displayed low titers of SEA-specific IgE, IgG_1, and total IgE. The lack of SEA-specific IgE and IgG_1 production in $CD40^{-/-}$ mice observed in this study is also consistent with the similar observation reported by others using these mice.[1,2,7,9]

IL-5 is a Th2-derived cytokine that and plays a central role in the differentiation, proliferation, accumulation, and migration of eosinophils to the site of inflammation.[17] In this study, reduced production of IL-5 by nasal mononuclear cells was seen in $CD40^{-/-}$ mice that could contribute to reduced levels of eosinophilia. The impaired infiltration of eosinophils in this study is not simply caused by the lack of IL-4 or IgE production because we have previously shown that IL-4–deficient mice, which are unable to produce SEA-specific IgE, show significant numbers of eosinophils in their nasal mucosa after intranasal sensitization with SEA.[16] The production of other cytokines, such as granulocyte-macrophage colony-stimulating factor and IL-13 or chemokines, such as eotaxin, may influence the inflammatory process.[18] The present results together with our previous findings suggest that CD40-dependent but IL-4/IgE–independent mechanism regulates eosinophil trafficking into nasal mucosa after SEA sensitization.

In this study, we found that histamine-induced nasal hyperresponsiveness was markedly reduced in SEA-sensitized $CD40^{-/-}$ mice. Although there are several reports investigating the role of CD40 in bronchial hyperresponsiveness,[7,9,10] this is the first report regarding the role of CD40 in the hyperresponsiveness in the upper airway. In fact, our result is not consistent with the previous reports focusing on bronchial hypersensitivity.[7,9,10] Takahashi *et al.* showed that enhanced bronchial hyperresponsiveness accompanied the increase of lung eosinophilia in $CD40^{-/-}$ mice.[10] On the other hand, Mehlhop *et al.* have suggested that CD40L but not CD40 is central to bronchial hyperresponsiveness, although both $CD40^{-/-}$ mice and $CD40L^{-/-}$ mice sensitized with *Aspergillus* showed the same level of lung eosinophilia as WT mice and produced no serum IgE.[9] These conflicting observations may be caused by several factors such as the type of antigen (SEA, ovalbumin, or *Aspergillus*), the route for sensitization (intranasal versus intraperitoneal), phase examined (induction or effector phase), or usage of adjuvants (*e.g.*, alum).

In conclusion, $CD40^{-/-}$ BALB/c mice fail to display reduced nasal hyperresponsiveness after repeated intranasal challenge with SEA. These findings show that CD40 plays a central role in pathophysiology allergic rhinitis. These results have implications for future investigations on understanding the underlying mechanism of allergic rhinitis and may have therapeutical implication for this disease.

ACKNOWLEDGMENTS

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Presence and characterization of prostaglandin D2–related molecules in nasal mucosa of patients with allergic rhinitis

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ABSTRACT

Background: *Prostaglandin D2 (PGD2) is the major prostanoid produced in the acute phase of allergic reactions. However, its pathophysiological role in addition to the pathway of production in allergic rhinitis remains unclear. We sought to determine the expression of synthases and receptors for PGD2 in human nasal mucosa. These expressions were compared between allergic and nonallergic patients.*

Methods: *The expression and localization of hematopoietic-type (h)-PGD2 synthase (PGDS) and lipocalin-type (l)-PGDS were detected by immunohistochemistry. The expression of D prostanoid (DP) receptor and chemoattractant receptor–homologous molecule expressed on Th2 cells (CRTH2) was determined by quantitative real-time PCR.*

Results: *The h-PGDS but not l-PGDS was clearly expressed in nasal mucosa. The expression of h-PGDS in allergic patients was significantly higher than in control patients without mucosal hypertrophy. A variety of infiltrating cells including mast cells, eosinophils, macrophages, and lymphocytes as well as constitutive cells such as epithelial cells and fibroblasts expressed h-PGDS. The expression of both DP and CRTH2 was confirmed also. Although either the amount of DP or the amount of CRTH2 was not correlated with serum levels of IgE, the amount of CRTH2 but not DP was highly and significantly correlated with the number of eosinophils infiltrating into nasal musosa.*

Conclusion: *These results suggest that PGD2 is released via the action of h-PGDS from various cells, and the expression of h-PGDS may be associated with the hypertrophic inflammation in the nose. In addition, ligation of PGD2 to CRTH2 appears to be selectively involved in eosinophil recruitment into the nose regardless of atopic status.*

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Prostaglandins are known to regulate immune responses.[1] Among these, prostaglandin D2 (PGD2) is thought to be involved in allergic reactions.[2] Levels of PGD2 increase after allergen provocation in lavage fluids from both upper and lower airways,[3,4] and it shows a variety of biological activities such as vasodilation.[5,6] Indeed, nasal challenge with PGD2 induces a sustained nasal obstruction.[7] In several experimental models, PGD2 induces eosinophil infiltration and Th2-type cytokine release into airways.[8,9]

Two ligands for PGD2, D prostanoid receptor (DP) and chemoattractant receptor–homologous molecule expressed on Th2 cells (CRTH2) have been isolated and characterized.[10,11] The expression of DP is observed in not only the central nervous system (CNS) but also inflammatory cells such as eosinophils.[12,13] Deletion of DP leads to a reduction in eosinophil inflammation and Th2 cytokine production in mice.[14] On the other hand, CRTH2 is originally found in Th2 cells, eosinophils and basophils,[11] and recent investigations revealed that the expression is also observed in organs such as lung.[15] Ligation of PGD2 to CRTH2 promotes chemotaxis, and also leads to a dose- and time-dependent increase of the number of blood leukocytes.[11,16]

PGD2 is formed from arachidonic acid by successive enzyme reactions: oxidation of arachidonic acid to PGH2, catalyzed by cyclooxygenase, and isomerization to PGD2 by PGD2 synthases (PGDSs). To date, two distinct types of PGDSs have been cloned: one is lipocalin-type PGDS (l-PGDS), and the other is hemopoietic-type PGDS (h-PGDS).[17,18] The l-PGDS is found mainly in the CNS and also is localized in other organs such as the cochlea and male epididymis.[17,19,20] On the other hand, h-PGDS is widely distributed in the peripheral tissues such as the lungs and also is localized in antigen-presenting cells, mast cells, and Th2 cells.[21–23] However, the expression and localization of these two PGDSs in airways remains unclear.

In this study, we sought to determine the expression and localization of PGDS and receptors for PGD2 in human nasal mucosa and compared these expressions between allergic and nonallergic subjects. In addition, we analyzed the correlation of the amount of PGDS and these receptors with pathophysiological parameters. We believe that the results presented here may provide insights into the role of local interaction between PGD2 and its receptors in the pathophysiology of allergic rhinitis.

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MATERIALS AND METHODS

Patients and Samples

Eleven Japanese patients (20–52 years old; mean, 30.5 ± 13.1 years; eight men and three women) with perennial allergic rhinitis were studied. All of them had severe nasal obstruction graded according to the criteria outlined by Okuda *et al.*[24], and showed high levels of nasal resistance on inspiration determined by active anterior rhinomanometry (MPR-2100; 0.410–2.014; mean, 0.793 ± 0.512 Pa/s per cm^3 at 100 Pa; Nihon Kohden, Tokyo, Japan). All the patients exhibited positive titers of IgE to *Dermatophagoides farinae* ranging from 2.13 to 99.26 unit allergin (UA)/mL when assayed by the capsulated hydrolic carrier polymer (Pharmacia, Tokyo, Japan). Eleven patients (19–62 years old; mean, 36.7 ± 15.6 years; seven men and four women) with nonallergic hypertrophic rhinitis and seven patients (33–62 years old; mean, 43.3 ± 9.5 years; four men and three women) with mucocele of the paranasal sinus were selected as controls. None of the control subjects had a history of allergic diseases, and no subjects exhibited positive titers of IgE to *D. farinae* (<0.35 UA/mL). Patients with nonallergic hypertrophic rhinitis showed high levels of nasal resistance (0.402–1.000; mean, 0.587 ± 0.176 Pa/s per cm^3). On the other hand, none of the patients with mucocele had hypertrophy of the inferior turbinates, and all exhibited normal levels of nasal resistance (0.088–0.208; mean, 0.148 ± 0.039 Pa/s per cm^3). Informed consent for participation in the study was obtained from each subject, and the study was approved by the Human Research Committee of Okayama University Graduate School of Medicine and Dentistry. None of the patients received immunotherapy or used immunosuppressive drugs during this study.

Primary Antibodies

Rabbit polyclonal antibodies against human h-PGDS and l-PGDS were generated as described previously.[25,26] Purified antihuman c-kit (104D2; mouse IgG1), lymphocyte common antigen (LCA; 2B11 + PD7/26; mouse IgG1), CD68 (PG-M1; mouse IgG3), and vimentin (Vim3B4; mouse IgG2a) mAbs were purchased from Dako (Kyoto, Japan). Purified anti-human eosinophil cationic protein/eosinophil protein X (ECP/EPX; EG2; mouse IgG1) mAb was purchased from Pharmacia. Rabbit and mouse IgG (Sigma, St. Louis, MO) were used as a negative control for each specific primary antibody.

Immunohistochemistry

Immunohistochemical staining for PGDSs was described previously.[20,25,26] The specificity of staining was checked by incubating sections with the polyclonal antibody that had been preabsorbed with excess amounts of purified recombinant human PGDS. The numbers of positive cells were counted in four fields of high power (10 × 40), and the average was determined.

To determine the cells expressing h-PGDS, double-staining immunohistochemistry was performed. The nasal sections were incubated with rabbit anti-human h-PGDS antibody or control rabbit IgG. After washing, the sections were incubated with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled amino acid polymer (Histofine Simple Stain MAX-PO [R]; Nichirei Co., Tokyo, Japan) followed by reaction with AEC (Simple Stain 3-amino-9-ethylcarbazole solution; Nichirei Co.). In this condition, cells expressing h-PGDS are stained as red. The sections were further incubated with mouse mAbs against human c-kit, LCA, CD68, vimentin, and EG2 or control mouse IgG overnight at 4°C. After washing, the sections were incubated with goat anti-mouse immunoglobulins conjugated to alkaline phosphatase–labeled amino acid polymer (Histofine Simple Stain MAX-PO [M]; Nichirei Co.) followed by reaction with fast blue (Fast Blue Substrate Kit; Nichirei). In this condition, cells bearing c-kit, LCA, vimentin, or EG2 are stained as blue. Percentages of cells coexpressing h-PGDS and surface markers are calculated as follows: cells stained with both h-PGDS (red) and surface markers (blue)/cells stained with surface markers (blue) × 100 (%). Preliminary experiment revealed that goat anti-mouse immunoglobulins conjugated to alkaline phosphatase–labeled amino acid polymer did not react with rabbit anti-human h-PGDS antibody, and *vice versa*, goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled amino acid polymer did not react with mouse mAbs against human c-kit, LCA, CD68, vimentin, and EG2.

RT-PCR

Surgically excised inferior turbinates were immediately soaked into RNAlater RNA stabilization reagent (Qiagen, Tokyo, Japan) and stored at −30°C until used. Total cellular RNA was extracted by RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The extracted material was treated with amplification grade deoxyribonuclease I (Sigma) for 15 minutes at room temperature. Reverse transcription of the samples to cDNA was done using a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions.

Real-time quantitative PCR assay was performed as described elsewhere.[27] In brief, the assay was performed on GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) using QuantiTect SYBR Green PCR (Qiagen). The primers used for PCR had the following sequences and product size: CRTH2, forward 5′-CTACAATGTGCTGCTCCTGAA -3′ and reverse 5′-CAGGTGAGCACGTAGAGC-3′ (375 bp); DP, forward 5′-GCAACCTCTATGCGATGCA-3′ and reverse 5′-CAAGGCTCGGAGGTCTTCT-3′ (260 bp); and GAPDH, forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′ (452 bp [556 bp in genomic DNA]).[11] GAPDH from serially diluted human genomic DNA was amplified as template, and the standard curve was plotted. The amount of DP and CRTH2 was estimated by dividing the signals into that of GAPDH.

Statistical Analysis

Dunn's procedure as a multiple comparison procedure or Student's *t* test was used to determine the significance of the values obtained. Pearson's correlation coefficient was analyzed using StatView software (Abacus Concepts Inc., Berkeley, CA). A level of $p < .05$ was considered statistically significant. Values were given as means ± SD.

RESULTS

Expression of h-PGDS But Not l-PGDS in Nasal Mucosa

Using anti-human h-PGDS polyclonal antibody, expression of h-PGDS was clearly detected in nasal mucosa of patients with perennial allergic rhinitis (Fig. 1 *A*). This signal was completely lost when the antibody had been preabsorbed with excess amounts of purified recombinant human PGDS (Fig. 1 *B*). Cells expressing h-PGDS were widely located in both the layers of the epithelium and the lamina propria. On the other hand, expression of l-PGDS was merely detected in the mucosa (data not shown). Negligible reaction with irrelevant rabbit IgG was seen in this study.

The expression of h-PGDS in nasal mucosa was similar in patients with allergic and nonallergic hypertrophic rhinitis. However, the expression in allergic patients was significantly higher than in patients with mucoceles ($p = 0.023$; Fig. 2 *A*). The mean numbers ± SD of cells expressing h-PGDS per field (10 × 40) were 140.1 ± 78.2, 98.1 ± 88.4, and 51.1 ± 41.3 in patients with allergic rhinitis, nonallergic hypertrophic rhinitis, and mucoceles, respectively.

Phenotype Analysis of Cells Expressing h-PGDS

We analyzed the phenotypes of nasal cells expressing h-PGDS by double staining immunohistochemistry. Majority of c-kit$^+$, CD68$^+$, and ECP/EPX$^+$ cells expressed h-PGDS (Fig. 3, *H–J*). In addition, several LCA$^+$ and vimentin+ cells also expressed h-PGDS (Fig. 3, *K* and *L*). Among the c-kit$^+$, ECP/EPX$^+$, CD68$^+$, LCA$^+$, and vimentin+ cells, 86.9 ± 6.9, 77.5 ± 9.7, 83.3 ± 10.6, 17.1 ± 8.6, and 28.4 ± 13.7% coimmunostained for h-PGDS, respectively. There is no cross-reactivity of secondary antibodies when we used rabbit and mouse IgG as negative controls for each specific primary antibody.

Expression of Messengers for DP and CRTH2 in Nasal Mucosa

The expression of CP and CRTH2 mRNA in nasal mucosa was analyzed from 11 patients with allergic rhinitis and 11 patients with nonallergic hypertrophic rhinitis. The expression of DP was clearly detected in all the nasal mucosa, whereas the expression of CRTH2 varied among the subjects (Fig. 4). In general, the expression of DP was significantly higher than that of CRTH2 ($p = 0.003$, Student's paired *t*-test). The mean amounts of DP/GAPDH and CRTH2/GAPDH ± SD in nasal mucosa were 0.0042 ± 0.0050 and 0.0011 ± 0.002, respectively. There is no difference in expression of DP/GAPDH or CRTH2/GAPDH comparing the two rhinitic groups. However, the expression of CRTH2 but not DP was significantly higher in patients with allergic rhinitis as compared with control subjects (Fig. 5).

The amount of DP in nasal musosa was not correlated with either the level of serum total IgE or the number of eosinophil in nasal mucosa (Fig. 6, *A* and *B*). In addition, the amount of the CRTH2 did not correlate with the level of serum total IgE (Fig. 6 *C*). However, analysis with Pearson's correlation coefficient revealed that the amount of CRTH2 highly ($r = 0.824$) and significantly ($p < 0.001$) correlated with the number of eosinophils infiltrating into nasal musosa (Fig. 6 *D*). In addition, the positive correlation was found in both allergic ($r = 0.551$; $p = 0.079$) and nonallergic ($r = 0.949$; $p < 0.001$) patients but not controls ($r = 0.552$; $p = 0.214$).

DISCUSSION

Our results revealed that h-PGDS but not l-PGDS was detected in the human nose. There have been a few reports indicating the expression of PGDS in human airway.[28–31] Seymour *et al.* examined the expression of PGDS in bronchial mucosa but did not describe whether they analyzed the expression of h-PGDS or l-PGDS.[28] Miwa *et al.* reported that nasal mast cells were positive for h-PGDS in rats, but it is known that h-PGDS genes are expressed in a highly species-specific manner,[29,30] and, more recently, Nantel *et al.* showed that h-PGDS was only detected in few resident mast cells in normal human mucosa, but they did not describe the expression of l-PGDS.[31] Thus, we believe that this is the first report establishing selective expression of h-PGDS in the human upper airway.

A variety of cells in the human nose express h-PGDS. More

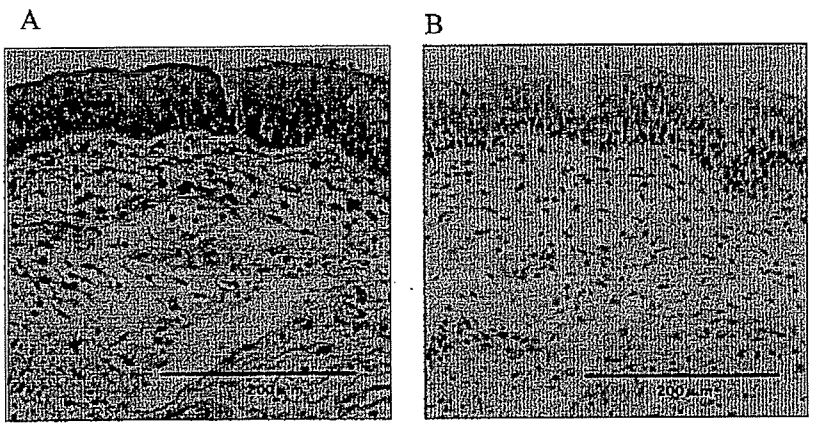

Figure 1. Immunohistochemical staining of h-PGDS in human nasal mucosa. Sections of inferior turbinates were reacted with rabbit (A) polyclonal Ab against h-PGDS or (B) the Ab preabsorbed with excess amounts of purified recombinant human PGDS, and then stained using Vectastain Elite avidin-biotin-peroxidase kit with diaminobenzidine substrate, as described in the Methods section (bar = 200 μm).

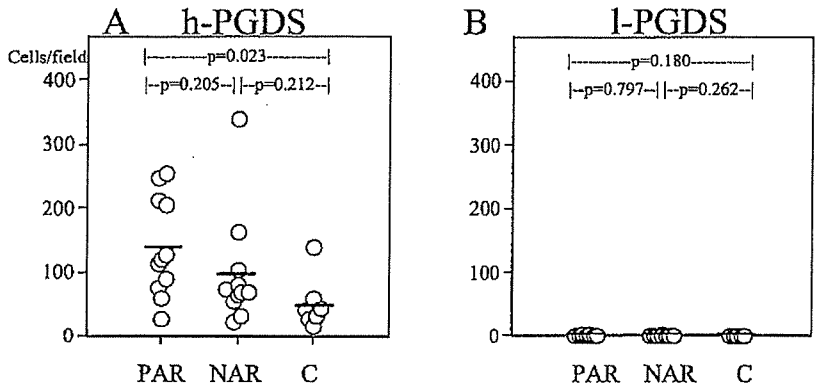

Figure 2. Number of cells expressing (A) h-PGDS and (B) l-PGDS in nasal mucosa of patients with perennial allergic rhinitis (PAR), nonallergic hypertrophic rhinitis (NAR), and mucocele of paranasal sinuses as a control (C). Bar represents mean number of positive cells. Values of p were obtained by Dunn's procedure as a multiple comparison procedure.

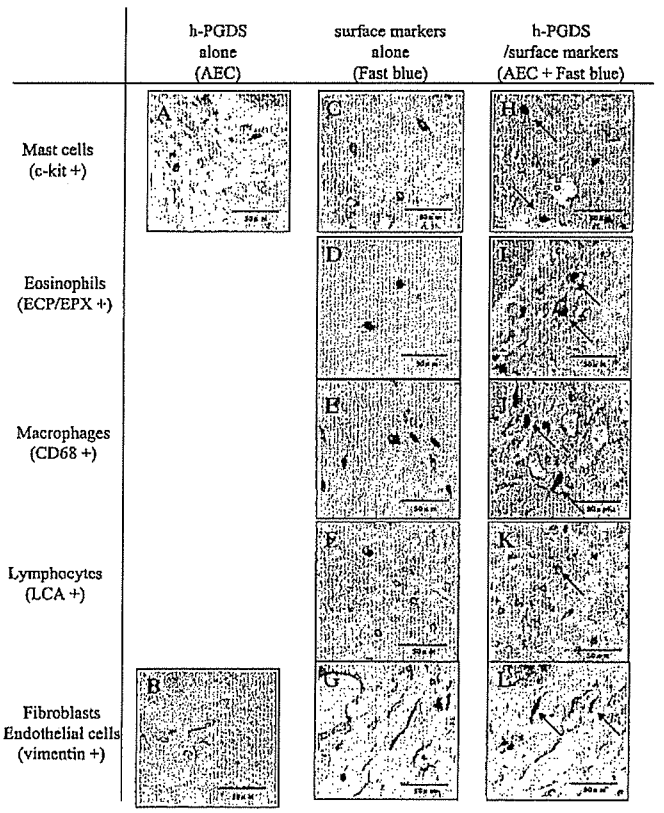

Figure 3. Expression of h-PGDS in infiltrating c-kit$^+$ (H), ECP/EPX (EG2)$^+$ (I), CD68$^+$ (J), LCA$^+$ (K), and vimentin+ (L) cells in nasal mucosa. As controls, sections were stained with anti-h-PGDS polyclonal Ab alone (A, B) or anti-c-kit (C), ECP/EPX (EG2) (D), CD68 (E), LCA (F), and vimentin (G) mAb alone. Arrows indicate double immunostained cells (bar = 50 μm).

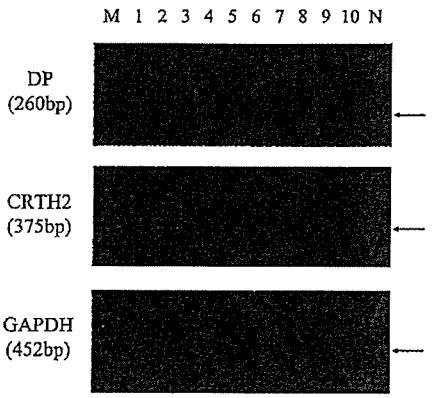

Figure 4. Expression of DP, CRTH2, and GAPDH in nasal mucosa of patients with perennial allergic rhinitis (lanes 1–5) and nonallergic hypertrophic rhinitis (lanes 6–10). mRNA was extracted from the inferior turbinates, and then the levels of DP, CRTH2, and GAPDH were detected by RT-PCR as described in the Methods section (M, molecular marker; N, distilled water).

than 80% of mast cells (c-kit$^+$) and macrophages (CD68$^+$) expressed h-PGDS, and this result may be consistent with previous reports that these cells are major sources of PGD2 production in various organs.[21,22] In addition, 17.1 ± 8.6% of LCA$^+$ lymphocytes expressed h-PGDS. H-PGDS is preferentially expressed in human Th2 but not Th1 cells.[23] Thus, our result may undertake the possibility that a subset of lymphocytes bearing h-PGDS in the nose were Th2 cells. Interestingly, most ECP/EPX$^+$ eosinophils also expressed h-PGDS. Although it is known that eosinophils can produce PGE2, little is known whether eosinophils can produce PGD2.[32] Our result suggests that eosinophils have the potential to produce PGD2.

In addition to the infiltrating cells, constitutive cells in the nose, particularly epithelial cells, also expressed h-PGDS (Fig. 1). The fact that airway epithelial cells can produce PGD2 supports our finding.[33] Vimentin+ cells also express h-PGDS. A recent investigation revealed that bronchial fibroblasts spontaneously release PGD2, and the production increases dramatically in response to the mixture of LPS, IL-1β, and TNF-α.[34]

The expression of h-PGDS was similar in patients with allergic and nonallergic rhinitis, both showing hypertrophy in nasal mucosa, as determined by rhinomanometry. However, the expression was lower in patients with mucoceles, those without mucosal hypertrophy. These results suggest that PGD2 produced *via* the action of h-PGDS may be involved in the nasal hypertrophic inflammation regardless of allergic status.

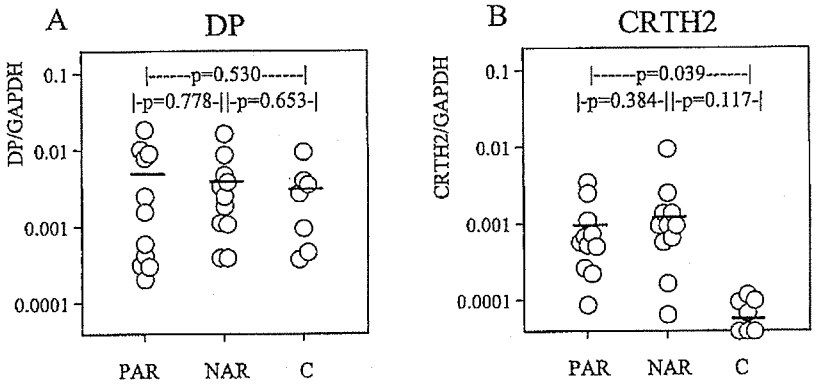

Figure 5. Comparison of the amount of (A) DP and (B) CRTH2 in nasal mucosa of patients with perennial allergic rhinitis (PAR), nonallergic hypertrophic rhinitis (NAR), and control subjects (C). The amount of DP and CRTH2 were estimated using real-time RT-PCR. Bar represents mean amounts of the messengers. Values of p were obtained by Student's unpaired t-test.

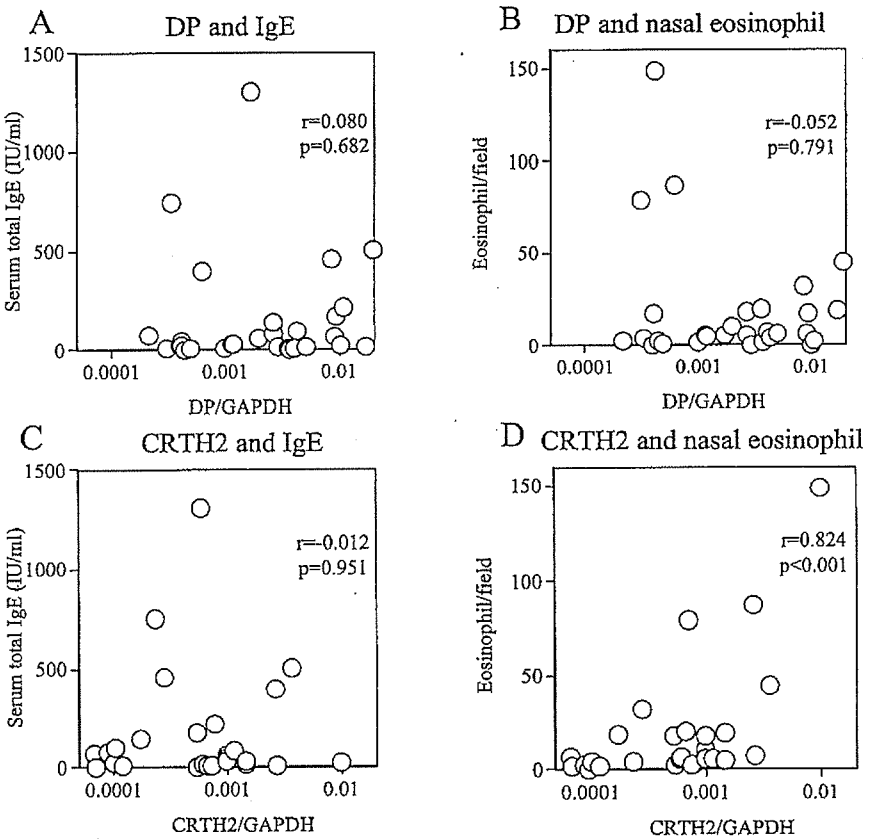

Figure 6. Correlation of the amount of (A and B) DP or (C and D) CRTH2 in the nose with (A and C) serum IgE levels or (B and D) the number of eosinophils infiltrating into nasal mucosa. Values were determined by Pearson's correlation coefficient.

The expression of DP was found to be similar in all nasal samples tested regardless of the presence of allergic rhinitis. Our results suggest that nasal mucosa may constitutionally express DP and support the report that the ligation of PGD2 to DP can affect airway inflammation and function.[14] In fact, Nantel *et al.* recently showed that DP is expressed in epithelial goblet cells, serous glands, and vascular endothelium in nasal polyps as well as normal nasal mucosa.[31] On the other hand, the amount of CRTH2 was varied among the patients and significantly lower than that of DP as a whole. However, it may be possible that the expression of CRTH2 displays down-modulation in inflamed tissues.[9]

Interestingly, the amount of CRTH2, but not DP, positively and significantly correlated with the number of infiltrating eosinophils, suggesting that CRTH2 is selectively involved in eosinophil recruitment at inflammatory sites *in vivo*. Gervais *et al.* reported that eosinophil chemokinesis was induced by CRTH2-selective agonist but not DP-selective agonist *in vitro*,

although both molecules are detectable on circulating eosinophils.[13] On the contrary, the amount of CRTH2 did not correlate with the level of serum total IgE. Together with the finding that the amount of CRTH2 in the nose was similar in allergic and nonallergic patients, these results suggest that the nasal expression of CRTH2 may be independent of atopic status.

In conclusion, we showed the expression of synthases and receptors for PGD2 in nasal mucosa. In particular, we suggest an important role of CRTH2–PGD2 interaction in the eosinophilic inflammation in the nose that may be associated with the high expression of h-PGDS. These observations may provide a basis for future therapeutic approaches in the management of allergic rhinitis by inhibiting the interaction between PGD2 and its receptors.

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E prostanoid 2 (EP2)/EP4-mediated suppression of antigen-specific human T-cell responses by prostaglandin E_2

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Summary

Prostaglandin E_2 (PGE_2) is a lipid mediator that displays important immunomodulatory properties, such as polarization of cytokine production by T cells. Recent investigations have revealed that the effect of PGE_2 on cytokine production is greatly influenced by external stimuli; however, it is unclear whether PGE_2 plays a significant role in major histocompatibility complex-mediated antigen-specific T-cell responses via binding to one of four subtypes of E prostanoid (EP) receptor alone or in combination. In the present study, we sought to determine the effect of PGE_2 on antigen-specific $CD4^+$ T-cell responses in humans, especially in terms of receptor specificity. We used purified protein derivative (PPD) and Cry j 1 as T helper type 1 (Th1) and Th2-inducing antigens, respectively. We generated several different Cry j 1- and PPD-specific T-cell lines (TCLs). PGE_2 significantly and dose-dependently inhibited the proliferation and subsequent production of interleukin-4 by Cry j 1-specific TCLs and of interferon-γ by PPD-specific TCLs upon antigen stimulation. Administration of EP2 receptor agonist and EP4 receptor agonist suppressed these responses in an adenylate cyclase-dependent manner, while EP1 and EP3 receptor agonists did not. Messenger RNA for EP2, EP3 and EP4, but not EP1, receptors were detected in Cry j 1- and PPD-specific TCLs, and no differences in EP receptor expression were observed between them. Furthermore, PGE_2 and EP2 receptor agonist significantly inhibited interleukin-5 and interferon-γ production by peripheral blood mononuclear cells in response to Cry j 1 and PPD stimulation, respectively. These results suggest that PGE_2 suppresses both Th1- and Th2-polarized antigen-specific human T-cell responses via a cAMP-dependent EP2/EP4-mediated pathway.

Keywords: antigen; E prostanoid; human; prostaglandin E_2; T cells

Introduction

The induction and exacerbation of allergic diseases, such as allergic rhinitis, are mediated by allergen-specific $CD4^+$ T cells, particularly T helper type 2 (Th2) cells.[1,2] Interleukin-4 (IL-4) derived from Th2 cells up-regulates the production of allergen-specific immunoglobulin E (IgE), and is suppressed by interferon-γ (IFN-γ) derived from Th1 cells.[3,4] In addition, IL-5 derived from Th2 cells plays an important role in allergic inflammation because it selectively promotes the chemotaxis, activation and survival of eosinophils.[5] Antigen-specific T cells require two distinct signals for functional activation. The first signal results from interaction of the antigen/major

Abbreviations: APC, antigen-presenting cell; bp, base pair; cAMP, cyclic adenosine-3′5′-; c.p.m., counts per minute; EP, E prostanoid; IFN, interferon-γ; IgE, immunoglobulin E; IL-4, interleukin-4; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; PGE_2, prostaglandin E_2; PKA, protein kinase A; PPD, purified protein derivative; RT-PCR, reverse transcription-polymerase chain reaction; TCL, T-cell line; Th1, T helper type 1.

histocompatibility complex (MHC) complex with the T-cell receptor.[6] The second requires costimulatory signals.[7] In addition, the external and internal microenvironment, including various bacterial products and sex hormones, can affect T-cell function.[8,9]

PGE_2 is a major prostanoid released from a variety of immune cells, including macrophages, via activation of cyclooxygenase enzymes and PGE_2 synthase.[10] PGE_2 is known to affect the production of cytokines by $CD4^+$ T cells. In general, PGE_2 has either no effect or enhances the production of Th2 cytokines, such as IL-4 and IL-5, while dramatically inhibiting the production of Th1 cytokines, such as IFN-γ and IL-2.[10–14] However, recent investigations suggest that this effect of PGE_2 on cytokine production is highly subject to external influence.[15–18] Surprisingly, most information was based on nominal T-cell receptor signals including mitogens, antibodies to membrane receptors, or other surface proteins, phorbol esters and ionophores. It is currently not known whether PGE_2 plays a significant role in the MHC-mediated, antigen-specific proliferation and cytokine production of $CD4^+$ T cells in humans.

PGE_2 acts upon binding to one of four subtypes of receptor, E prostanoid 1 (EP1), EP2, EP3 or EP4, alone or in combination.[10] The EP1 receptor is coupled to a Gq/p protein, resulting in phosphatidylinositol production and increased intracellular concentrations of Ca^{2+}.[10,19] The EP3 receptor is mostly coupled to a Gi protein, by which it inhibits adenylate cyclase.[10,20] EP2 and EP4 receptors, on the other hand, are coupled to a Gs protein that stimulates adenylate cyclase.[21] Although the production of T-cell-derived cytokines is known to be regulated by a cyclic-adenosine monophosphate (cAMP)-dependent pathway[17], the specific role of each PGE_2 receptor subtype in antigen-specific human T-cell responses is not fully understood.

The present study was performed to determine whether PGE_2 affects antigen-specific $CD4^+$ human T-cell responses using purified protein derivative (PPD) and Cry j 1 as Th1- and Th2-inducing antigens, respectively. In addition, we examined the expression of four subtypes of EP receptors by antigen-specific T cells, and sought to determine which receptor subtypes are involved in the action of PGE_2. We believe that the findings presented in this study might provide new insight into the physiological role of PGE_2 in human antigen-specific T-cell responses and inspire novel approaches to the treatment of allergic diseases.

Materials and methods

Subjects

Twelve Japanese patients (five men and seven women; age 18–49 years, mean 32·5 years) with Japanese cedar pollinosis were examined. Written informed consent was obtained from each subject. The patients showed elevations of serum IgE specific for Japanese cedar pollen using a radioallergosorbent test (capsulated hydrolic carrier polymer (CAP-RAST); Pharmacia, Uppsala, Sweden), with sensitivities ranging from 1·81 to 50·90 UA/ml (mean 19·40 UA/ml). None of the patients used immunosuppressive drugs or underwent immunotherapy during this study. As a control, six healthy Japanese volunteers not sensitized with Japanese cedar pollen as determined by skin scratch test were enrolled (three men and three women; age 18–46 years, mean 30·7 years).

Antigens and reagents

Cry j 1 was purified from crude extracts of *Cryptomeria japonica* pollen using a well-established procedure.[22] Endotoxin contamination was considered to be negligible because an Endospec™ ES test was negative (Seikagaku Kogyo Corporation, Tokyo, Japan). PPD was purchased from Nihon BCG Seizo Co. (Tokyo, Japan). Ovalbumin was purchased from Sigma (St Louis, MO). Protein concentration was determined using a bicinchoninic acid assay, according to the manufacturer's instructions (Pierce, Rockford, IL). PGE_2 was purchased from Cayman (Ann Arbor, MI). The receptor-selective agonists for EP1 (ONO-DI-004), EP2 (ONO-AE1-259-01), EP3 (ONO-AE-248) and EP4 (ONO-AE1-329) were provided by Ono Pharmaceuticals (Osaka, Japan). PGE_2 and the agonists were dissolved to stock concentrations of 10^{-2} M in dimethylsulphoxide (DMSO; Sigma) and stored at −80° until use. SQ22536 and RP-8-Br-cAMPS were purchased from Sigma and BioLog Life Science (San Diego, CA), respectively.

Isolation and culture of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated and cultured as described previously.[23] In brief, 1×10^6/ml cells were incubated in the presence or absence of either 10 μg/ml Cry j 1, 10 μg/ml ovalbumin or 2 μg/ml PPD, along with PGE_2 and the receptor-selective agonists at 37° in a 5% CO_2/air mixture. Culture supernatant was collected after 72 hr and stored at −80° until the time of cytokine production assay.

Generation and culture of antigen-specific T-cell lines

The $CD4^+$ Cry j 1^- and PPD-specific T-cell lines (TCLs) used were generated using a procedure described previously.[24] In flat-bottomed microtitre plates (Corning Inc., Corning, NY), 2×10^4 cells from the TCLs were mixed with 1×10^5 irradiated autologous PBMCs (PBMCx) as antigen-presenting cells (APCs). Following this, the cells were cultured in the presence or absence of either 10 μg/ml

Cry j 1, 10 μg/ml ovalbumin or 2 μg/ml PPD in 0·2 ml of culture medium containing serial concentrations of PGE_2/receptor-selective agonists or control buffer (DMSO). Culture supernatant was collected to perform cytokine production assays and to harvest cells for proliferation assays, as previously described.[25]

To determine adenylate cyclase and protein kinase A type I activity, TCLs and/or APCs were incubated with SQ22536, an inhibitor of adenylate cyclase, or RP-8-Br-cAMPS, a protein kinase A (PKA) type I inhibitor, at 37° for 1 hr. Following this, the cells were washed with culture medium three times, after which they were mixed and cultured in the same manner described above.

Cytokine determination

Levels of IL-4, IL-5 and IFN-γ were measured in the culture supernatant by means of Opt EIA sets (BD Biosciences, San Jose, CA), according to the manufacturer's instructions.[24] The detection limit of these assays was 3 pg/ml for IL-4, 20 pg/ml for IL-5 and 20 pg/ml for IFN-γ.

Reverse transcription–polymerase chain reaction (RT-PCR)

Cry j 1- and PPD-specific TCLs were immediately soaked in RNAlater™ RNA stabilization reagent (Qiagen, Tokyo, Japan) and stored at −30° until use. Total cellular RNA was extracted using the Rneasy™ mini kit (Qiagen), according to the manufacturer's instructions. The extracted material was then treated with amplification grade deoxyribonuclease I (Sigma) for 15 min at room temperature. Reverse transcription of the samples to generate cDNA was performed using a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions.

Real-time quantitative PCR assays were performed as described elsewhere.[25] In brief, the assays were performed using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, CA, USA) with QuantiTect SYBR Green PCR (Qiagen). The PCR primer sequences and product sizes were as follows: EP1, forward 5′-CGCGC TGCCCATCTTCTCCAT-3′ and reverse 5′-CCCAGGCC GATGAAGCACCAC-3′ [(471 base pairs (bp)]; EP2, forward 5′-GCTGCTGCTTCTCATTGTCTCG-3′ and reverse 5′-TCCGACAACAGAGGACTGAACG-3′ (392 bp); EP3, forward 5′-GGACTAGCTCTTCGCATAACT-3′ and reverse 5′-GCAGTGCTCAACTGATGTCT-3′ (293 bp); EP4, forward 5′-ATCTTACTCATTGCCACC-3′ and reverse 5′-TCTATTGCTTTACTGAGCAC-3′ (212 bp); and glyceraldehyde 3-phosphate hydrogenase (GAPDH), forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′ (452 bp).[26] The expression level of EP1, EP2, EP3 and EP4 was estimated by dividing each signal into the signal for GAPDH.

Statistical analysis

Statistical comparisons were performed using the Bartlett test, followed by Wilcoxon's signed-rank test and Mann–Whitney's *U*-test. A level of $P < 0·05$ was considered statistically significant. Values were given as means ± standard deviation (SD).

Results

Effect of PGE_2 on Cry j 1- and PPD-specific cellular responses by TCLs

We generated a panel of both Cry j 1- and PPD-specific TCLs from three patients with Japanese cedar pollinosis. All six Cry j 1-specific TCLs proliferated in response to Cry j 1, and predominantly produced IL-4 (Fig. 1a–c). PGE_2 significantly inhibited the Cry j 1-induced proliferative response, as well as IL-4 production, in a dose-dependent manner (Fig. 1a,b). As a whole, IL-4 production was significantly inhibited by 47·01 ± 18·92% ($P = 0·028$ by Wilcoxon's signed-rank test), 87·96 ± 11·61% ($P = 0·028$) and 96·72 ± 0·87% ($P = 0·028$), upon exposure to 0·01 μM, 0·1 μM and 1 μM of PGE_2, respectively, compared to the buffer control (Fig. 2).

All six PPD-specific TCLs proliferated in response to PPD and predominantly produced IFN-γ (Fig. 1d–f). As observed in the Cry j 1-specific TCLs, PGE_2 significantly inhibited the PPD-induced proliferative response and IFN-γ production in a dose-dependent manner (Fig. 1d,f). As a whole, IFN-γ production was significantly inhibited by 25·93 ± 2·18% ($P = 0·028$), 53·10 ± 1·12% ($P = 0·028$) and 66·56 ± 1·48% ($P = 0·028$) upon exposure to 0·01 μM, 0·1 μM and 1 μM of PGE_2, respectively, compared to the buffer control (Fig. 3). The baseline production of IL-4 by Cry j 1- specific TCLs and IFN-γ by PPD-specific TCLs in the absence of antigen was 0 ± 0 and 203 ± 314 pg/ml, respectively, and no additional proliferation or cytokine production over background was observed with ovalbumin, the irrelevant antigen (data not shown). PGE_2 also significantly inhibited the PPD-induced proliferative response and IFN-γ production by PPD-specific TCLs from non-allergic healthy donors in a dose-dependent manner (data not shown).

Effect of EP receptor-selective agonists on antigen-specific cellular responses by TCLs

To determine which PGE_2 receptor subtypes might mediate the inhibitory effect of PGE_2 on antigen-specific cellular responses by TCLs, we used four EP receptor-selective agonists. Treatment with EP1 and EP3 receptor agonists did not affect the Cry j 1-specific proliferative response or IL-4 production. However, treatment with an EP2 receptor agonist strongly inhibited these responses especially

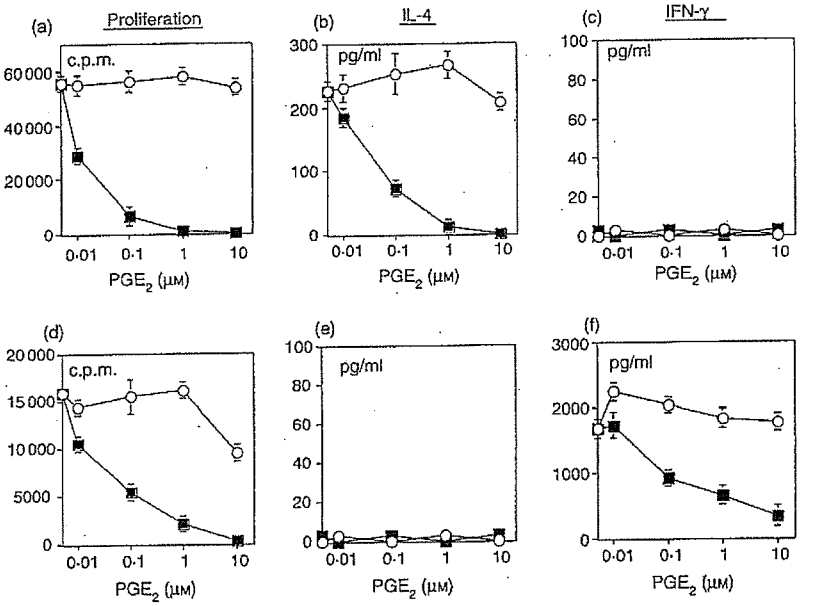

Figure 1. Inhibition of antigen-specific human T-cell responses by PGE_2. TCLs specific for Cry j 1 (a–c) and PPD (d–f) were cultured with APC and the respective antigen in the presence of serial concentrations of PGE_2 (■) or control buffer (DMSO: ○). Proliferation (a,d), IL-4 production (b,e), and IFN-γ production (c,f) were determined. The experiments were repeated at least six times using different TCLs. Typical results are shown in mean count per minutes (c.p.m.) ± SD from triplicate cultures for proliferation and mean concentration ± SD from triplicate cultures for cytokine production. Background proliferation and IL-4 production in the absence of Cry j 1 was 132 ± 25 c.p.m. and 0 pg/ml in YO-1, the Cry j 1-specific TCL (a–c), and the background proliferation and IFN-γ production in the absence of PPD was 155 ± 16 c.p.m. and 83 pg/ml in YO-P, the PPD-specific TCL (a–c).

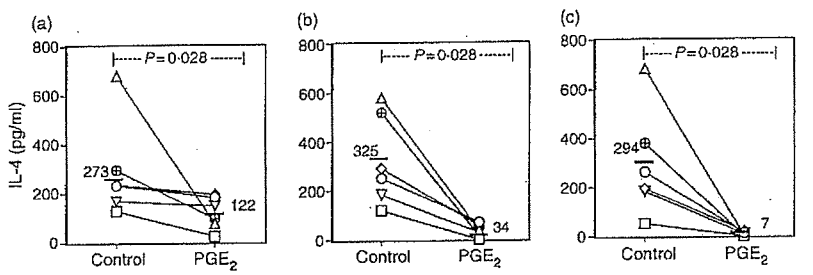

Figure 2. PGE_2-mediated inhibition of Cry j 1-induced IL-4 production by TCLs. Six Cry j 1-specific TCLs were mixed with APCs and cultured with 10 μg/ml of Cry j 1 for 65 hr in the presence of the following concentrations of PGE_2 or control buffer: 0·01 μM (a), 0·1 μM (b), or 1 μM (c). Following incubation, supernatant was collected and the IL-4 concentration of each sample was determined by ELISA. *P*-values were determined using Wilcoxon's signed-rank test. Data on each TCLs are representative of two separate experiments.

the proliferation. Treatment with an EP4 receptor agonist also inhibited these responses, although to a much lesser degree. Combined treatment with EP2 and EP4 receptor agonists had an additive effect. Treatment with EP1 or EP3 receptor agonists did not alter the inhibitory effects of the EP2 or EP4 receptor agonists (Fig. 4a,b). EP2 and EP4 receptor agonists had similar inhibitory effects on the PPD-specific proliferative response and IFN-γ production by PPD-specific TCLs although the inhibitory effect seemed to be modest compared with the effect on Cry j 1- specific responses (Fig. 4c,d).

Reversal of EP2/EP4-induced inhibition of antigen-specific T-cell responses by an inhibitor of adenylate cyclase that acts on APCs and T cells

EP2 and EP4 are coupled to a Gs protein that stimulates adenylate cyclase.[20] Thus, we sought to determine whether the inhibitory effect of PGE_2 mediated by EP2/EP4, is dependent on the activity of adenylate cyclase. Pretreatment of Cry j 1-specific TCLs alone with SQ22536, an inhibitor of adenylate cyclase, followed by the coculture with intact APC partially suppressed inhibition of the

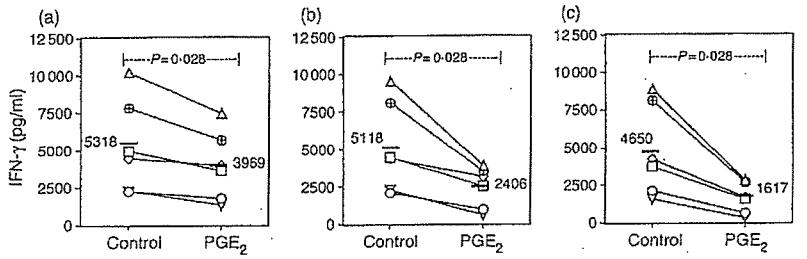

Figure 3. PGE_2-mediated inhibition of PPD-induced IFN-γ production by TCLs. Six PPD-specific TCLs were mixed with APCs and cultured with 2 μg/ml of PPD for 65 hr in the presence of the following concentrations of PGE_2 or control buffer: 0·01 μM (a), 0·1 μM (b), or 1 μM (c). Following incubation, supernatant was collected and the IFN-γ concentration of each sample was determined by ELISA. *P*-values were determined using Wilcoxon's signed-rank test. Data on each TCLs are representative of two separate experiments.

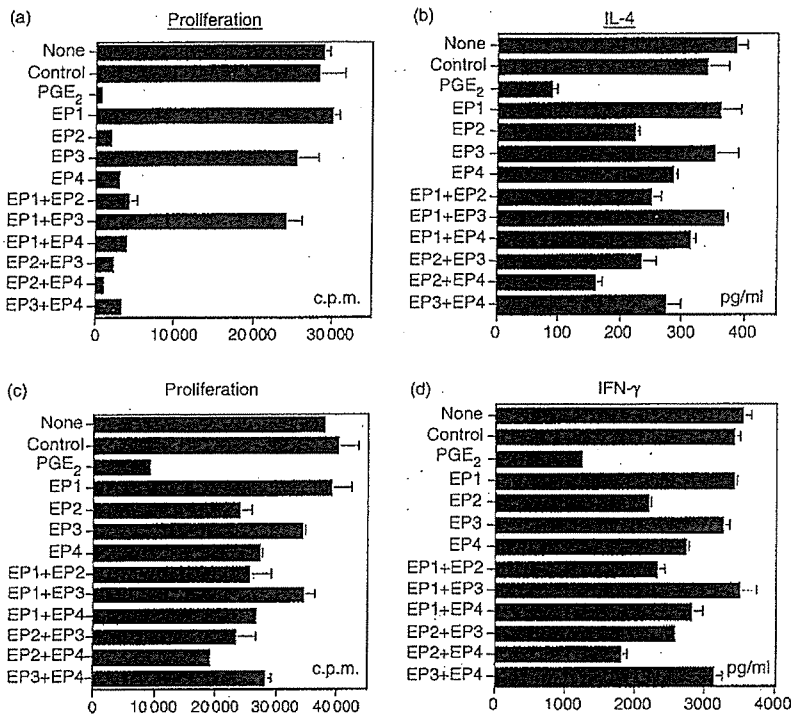

Figure 4. Effect of EP receptor-selective agonists on antigen-specific human T-cell responses. TCLs specific for Cry j 1 (a,b) and PPD (c,d) were cultured with APC and the respective antigen in the presence of PGE_2, an EP receptor-selective agonist or control buffer, each at a concentration of 0·2 μM. Experiments were also performed to examine the effects of 0·1 μM of each EP receptor agonist in combination. Proliferation (a,c), IL-4 production (b) and IFN-γ production (d) were determined. Typical results are shown in mean c.p.m. ± SD from triplicate cultures for proliferation and mean concentration ± SD from triplicate cultures for cytokine production. The baseline proliferation and IL-4 production by Cry j 1-specific TCLs in the absence of Cry j 1 were 83 ± 14 c.p.m. and 0 pg/ml, respectively and the baseline proliferation and IFN-γ production by PPD-specific TCLs in the absence of antigen were 1250 ± 96 c.p.m. and 17 pg/ml, respectively. Data are representative of at least three separate experiments. Similar results were seen when EP receptor agonists were added into the culture at 1 μM.

Cry j 1-specific proliferative response by EP2 and EP4 receptor agonists. Marked inhibition was observed when APCs alone were pretreated with SQ22536 followed by the coculture with intact TCL. In addition, pretreatment of both TCLs and APCs with SQ22536 completely reversed the inhibitory effects of EP2 and EP4 receptor agonists on the Cry j 1-specific response (Fig. 5a,b). Pretreatment with SQ22536 was also observed to inhibit EP2- and EP4-induced PPD-specific T-cell responses (Fig. 5c,d). In addition, pretreatment of TCLs and/or APCs with RP-8-Br-cAMPS, a PKA type I inhibitor, partially reversed the EP2 and EP4 receptor agonist-induced inhibition of both Cry j 1- and PPD-specific responses (data not shown).