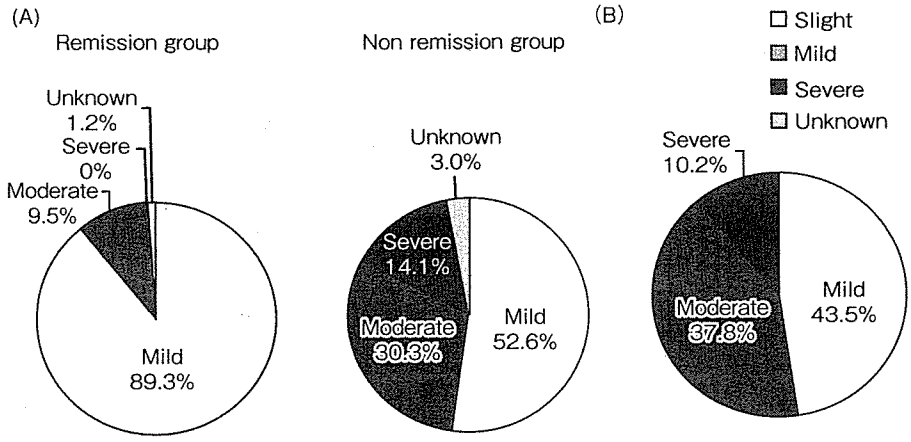

Fig. 7. (A) The severity of bronchial asthma on the first hospital visit. (B) The distribution of the severity of bronchial asthma obtained from the survey by the joint research group of national hospital and sanatorium.

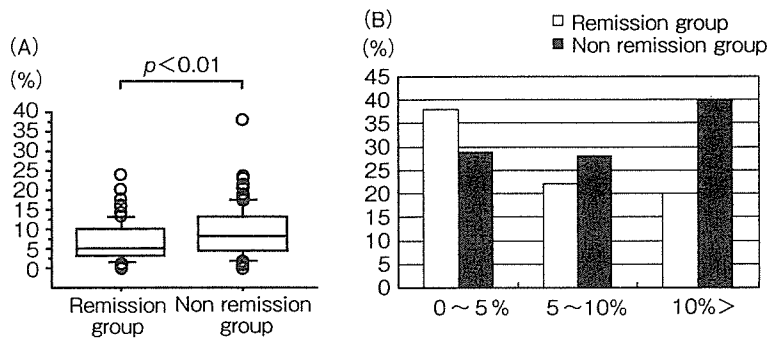

Fig. 8. (A) The comparison of the ratio of eosinophils in whole blood on the first visit in remission group and non remission group. (B) The distribution of the ratio of eosinophils in whole blood.

の末梢血好酸球数は寛解群 6.5%，非寛解群 9.4% と非寛解群で有意に多かった ($p<0.01$) (Fig. 8 (A))．またヒストグラムでは非寛解群では 10% 以上の好酸球数を呈した症例が約 40% であった (Fig. 8 (B))．初診時の血清中総 IgE 値は寛解群，非寛解群ともに有意差はないが (Fig. 9 (A))，ヒストグラムで検討すると IgE 値≧1000 IU/ml を越えるアトピー素因の強い症例は非寛解群で多かった (Fig. 9 (B))．初診時の喀痰（自然痰）中の好酸球数は Hansel の基準を用いて半定量したが，寛解群，非寛解群ともに Score 4 と中等度以上の好酸球数を認めた症例が多かった (Fig. 10)．初診時の FEV1% は寛解群 73.2%，非寛解群 66.9% と寛解群で有意に高値であった ($p<0.01$, Fig. 11 (A))．またヒストグラムでは寛解群は FEV1%≧70% が 60% 以上存在するのに対し，非寛解群では FEV1%≦50% の低肺機能症例が 10% 以上存在していた (Fig. 11 (B))．初診時アセチルコリン気道過敏性 ($AchPC_{20}$) では寛解群では $AchPC_{20}$ = 3565 μg/mL，非寛解群では 1455 μg/mL であり寛解群では有意に気道過敏性が軽度であった ($p<0.01$, Fig. 12 (A))．またヒストグラムでは初診時 Ach 閾値＞10000 μg/mL である症例は寛解群で約 30% 存在した．ここで初診時 Ach 閾値＞10000 μg/mL の症例では気道過敏性は正常域であるが可逆性および喀痰中の好酸球を認め，臨床的にも喘息であると診断し得た症例を対象としている．逆に初診時 Ach 閾値＜313 μg/mL 以下の症例からは寛解症例は

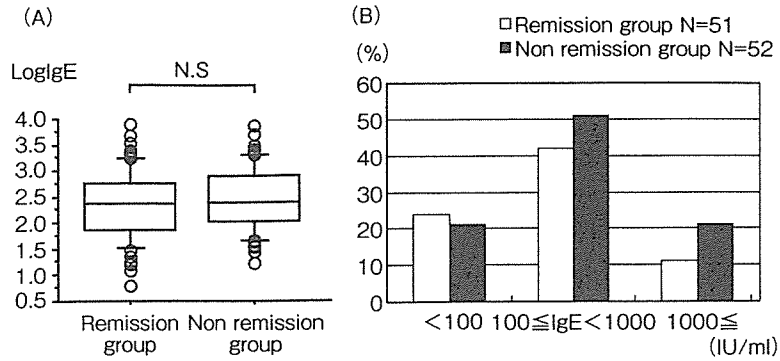

Fig. 9. (A) The comparison of the total IgE level in serum on the first hospital visit in remission group and non remission group. (B) The distribution of the total IgE level in serum.

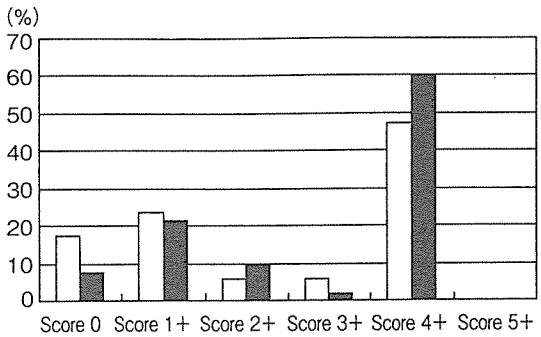

Fig. 10. The distribution of score of the number of eosinophils in sputum in remission group and non remission group. □ Remission group : N=51 ■ Non remission group : N=52

出ておらず，初診時 Ach 気道過敏性が軽度であるか高度であるかは成人喘息患者の予後をある程度規定する可能性があると考えられる．

また治療内容については初診時，徐放性テオフィリン製剤を使用した症例は寛解群で 71.3%，非寛解群で 82.7% であり，終診時にはそれぞれ 56.3%，78.8% と統計学的有意差は認めないものの寛解群では徐放性テオフィリン製剤使用が少ない傾向が認められた．このことは通院中の症状軽減によるテオフィリン製剤の使用の中止，また regular use が rescue use へ変化したことなどが推定される．非寛解群と比較して臨床症状が軽度であることを示唆する所見である．また本調査は平成 2〜4 年度までに受診中の患者対象のため吸入ステロイド併用症例の頻度は全体的にみても少なく，寛解群では初診時 1.3%，終診時 7.5% であり，非寛解群では初診時 3.8%，終診時 24.0% であった．非寛解群では初診時より吸入ステロイド使用が多く，終診時も使用症例が多かった．また対症療法を必要としない，無治療であった症例は寛解群において初診時 8.8%，終診時 23.4% であった．一方，非寛解群では初診時 1.9%，終診時 8.7% が無治療であった．以上のことから治療内容については寛解群，非寛解群ともに差は認めなかった．

考　案

成人喘息の予後に関する疫学調査は少ない．小児喘息と比較し，発症年齢が幅広く，患者個々の background が多彩であり，一定条件での追跡調査が難しいためであろうと思われる．従って成人喘息に治癒が期待できるのかどうかについていまだ明確な解答は得られていない．また治癒とはいかなくとも臨床的な長期寛解状態が可能かどうかについても明らかではない．本研究はいわゆる成人喘息の寛解率調査ではなく，そもそもの研究の動機はかつて当院に成人喘息患者として通院していたが，その後通院しなくなった患者がその後無症状でいるのか，あるいは通院治療を続けているのかを調査することが目的であった．アンケート調査はあくまでも患者自身の症状把握であるため，「症状がない」の中に軽度の咳嗽や本人が発作と自覚しない呼吸困難の症状が含まれている可能性は十分にある．従ってくすりを必要とせず症状がないという判断はあくまでも患者の主観による臨床的寛解としなければならない．しかし，くすりを必要とせず 3 年あるいは 5

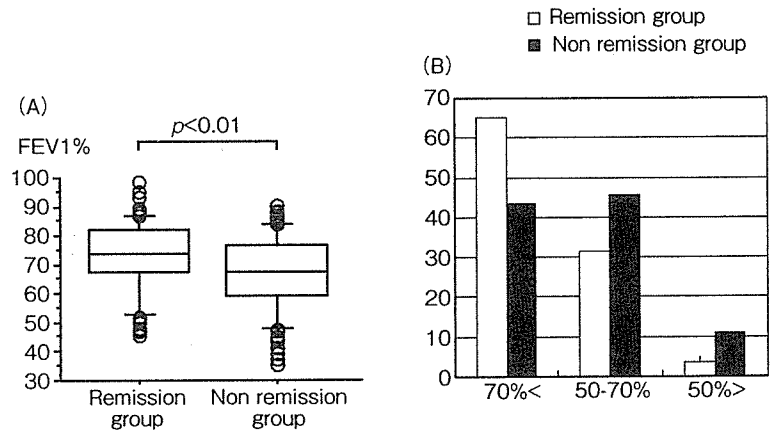

Fig. 11. (A) The comparison of FEV1% on the first hospital visit in remission group and non remission group. (B) The distribution of FEV1%.

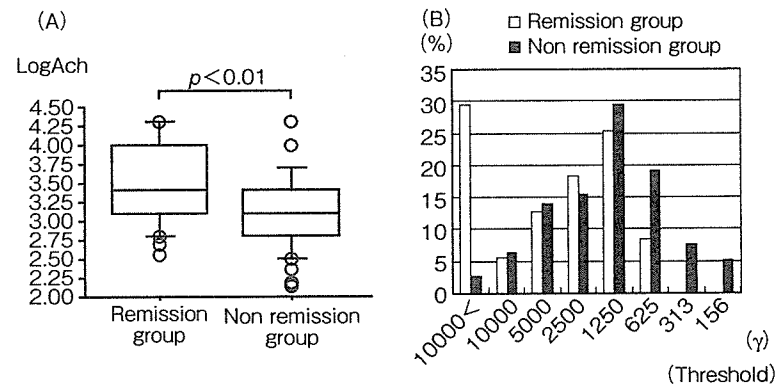

Fig. 12. (A) The comparison of bronchial reactivity to Ach (AchPC$_{20}$) on the first hospital visit in remission group and non remission group. (B) The distribution of bronchial reactivity to Ach (threshold amount of Ach).

年症状のない症例が少なからず存在することが明らかであり，この症例の中に成人喘息でも治癒し得る症例が存在する可能性があると考える．当院では過去に平成7年厚生科学研究疫学研究班調査において同様の調査を行った．それは昭和63年度から平成1年度まで当院受診歴があるがその後受診していない成人喘息患者を対象とし，アンケート調査を今回と同様に行ったものである．結果，954名中，462名より回答を得た(回答率48.4%)．くすりを服用しなくとも過去3年以上発作がないと答えた症例は462名中74名（16.0%）であり，今回の調査と類似していた[5]．これらの結果から成人喘息患者の中には臨床的長期寛解症例が存在する可能性が示唆され，その中に将来の治癒患者が含まれる可能性がある．

成人喘息では寛解の頻度や寛解導入の予測因子に関する報告は少ないが，Vonk[7]らによると0～39歳発症（中央値4歳）の喘息患者の26年後を追跡調査し，閉塞性障害が消失した症例では閉塞性障害が残存している症例と比較し，臨床症状が軽く，その内30.1%の症例ではヒスタミン気道過敏性が消失（HistPC$_{20}$>32 mg/mL）していると報告している．

一方，小児に関する予後の報告は多く，小児喘息119症例の30年後を追跡した調査では吸入ステロイドを使用せず臨床症状が消失しているいわゆる臨床的寛解症例は51.7%存在し，その中でもさらに%FEV1が90%以上，ヒスタミン気道過敏性が正常域である寛解

症例は22%であるという報告[8]，101症例の小児喘息を対象とした調査では20年後の臨床的寛解率は57%であり，その中で93.1%の症例がヒスタミン気道過敏性が消失していたという報告[9]，budesonide治療後臨床症状が消失した40症例を対象とし，治療を中止後も悪化を認めない症例が52%存在するという報告[10]などがある．

小児喘息の再発，非寛解に関する因子は1・HD，miteに対する感作，2・気道過敏性，3・女性，4・current smoking，5・early age at onsetが報告されている[11][12]．アウトグローに関する予測因子として，気道過敏性の残存が関与する[13][14]，閉塞性障害の程度が関与する[8]と双方があり未だに結論は出ていない．

また末梢血[15]，および気道[16]の好酸球性炎症の程度は小児喘息の寛解に関与しないという報告がある一方で，今回の我々の成人を対象とした調査では喀痰中の好酸球数は有意差を認めないものの，末梢血では非寛解群で多いという結果であった．この結果から成人と小児では気道過敏性に関わる好酸球性炎症の意味が異なることを示唆する可能性を有するものと考える．

小児では抗原感作，アトピー素因を乳幼児期に獲得し，成長とともにアトピー素因が減少する症例がアウトグローに関与する[8][14][17]と報告されているが今回の結果では病型や総IgE値，アレルギー疾患についての家族歴について有意差を認めなかったことから成人喘息においてはアトピー素因の有無は予後に影響しない可能性が示唆された．さらに喫煙歴についてはnonsmokerでは寛解群59.5%，非寛解群55.9%，exsmokerは寛解群20.3%，非寛解群35.7%，currentsmokerは寛解群20.3%，非寛解群8.3%であり統計学的有意差($p=0.024$)を認めた．喫煙が喘息発作，予後に影響を与えることはすでに知られていることであり，寛解群にnonsmokerが多く，一方，非寛解群でexsmokerが多いことは予想された結果であった．一方，寛解群にcurrent smokerが20.3%も存在することは驚くべき結果であった．しかし，寛解群にcurrent smokerが多いのは，成人喘息患者調査で小児発症成人喘息群にcurrent smokerが多いのと意味合いは似ているのではないかと思われる．すなわち，小児発症は成人発症に比べて軽症が多いことが患者調査で知られているが，current smokerが多いことも小児発症に軽症が多いことの裏付けなのかもしれない．故に，寛解群で初診時にcurrent smokerが多いのは，やはり，もともと軽症だからという理解も可能であろう．あるいは，寛解群の一部は喫煙に影響されないという可能性も示唆される結果である．

成人喘息は発症年齢により，小児喘息が寛解せずに成人まで継続して続いている「小児発症喘息」，成人になって初めて発症した「成人発症喘息」，小児喘息が一度寛解し，成人になってから再発した「成人再発喘息」，さらに思春期に発症した「思春期発症喘息」に分類される[18]-[20]．1992年の厚生省成人喘息実態調査研究班の秋山[19]らの報告では発症年齢別の割合は小児発症成人喘息11.1%，成人再発喘息3.7%，成人発症喘息77.7%であった．今回の調査では全国調査と比較して寛解群，非寛解群ともに小児発症喘息と成人再発喘息が多く，特に非寛解群では小児発症喘息が多かった．この事実は小児発症喘息の長い病歴が予後を左右するとも考えられる．しかし，発症年齢，通院期間，最終受診年齢にも差は認めず，服薬内容にも寛解群と非寛解群では差を認めないことから治療内容や通院期間が必ずしも予後を規定するわけではないという可能性も考えられる．これらのことから初診時より臨床症状や気道過敏性が軽度である症例が治療内容に影響されず寛解しうる可能性を有する群ともいえるかもしれない．また過去に秋山[21]は初診時気道過敏性が非常に亢進している，すなわち閾値が低い（Ach閾値156 μg/mL以下）患者群16例につき，その背景因子，初診時および現在の重症度，入院・挿管歴，最近の治療状況について検討した．初診時の重症度は軽症，中等症，重症とさまざまであるが，これらの症例のなかでは寛解に導入された例は一例もみられなかった．この結果から初診時アセチルコリン気道過敏性が高度亢進症例では寛解する可能性は低く，軽度亢進例が寛解予備群であることが示唆された．

また寛解に治療内容が関与するかどうかについてはKoh[22]らの思春期喘息を対象とし，臨床症状が消失したが気道過敏性が残存する喘息群と臨床症状および気道過敏性の残存する喘息群にbudesonide（800 μg）を6カ月間使用し，その結果，有症状の喘息症例では有意に気道過敏性が改善したが，臨床的寛解症例では気道過敏性は改善しなかった．このことは小児喘息における気道過敏性が好酸球性炎症のみを反映しないこと，また臨床的寛解に治療内容が影響しないことなどが推測される．我々の結果でも寛解群では吸入ステロイド導入率が少なく，徐放性テオフィリン製剤の使用も外来通院中に減少したことなどから，臨床的寛解に治療内容は影響しない可能性も示唆される．

以上より，今回のアンケート調査で対象となった無治療無症状喘息症例はいわゆる臨床的治癒，あるいは，機能的治癒か否かは明らかではない．しかし，成人喘息においても治療を必要とせず，臨床症状を認めない症例がわずかながらにも存在することはアンケート調査から伺えると考えられる．そして無治療無症状継続群は将来の寛解予備群の可能性を有すると推定される．寛解予備群の予測因子としては初診時重症度が軽症であること，発症より早期に受診していること，初診時呼吸機能が良好，特にアセチルコリン気道過敏性が軽度である，ことなどが考えられる．アトピー素因，治療内容などは寛解の可能性の有無には直接影響を与えないと考えられた．この事実からは軽症喘息の一部は治療によらない，すなわち自然寛解する要素を持ち得ている可能性が示唆される．

成人喘息の予後に関する報告は少ない．今後はこれらの無治療無症状群が客観的所見からも真の寛解状態にあるか否かを検討していく必要があると思われる．

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PROGNOSIS OF BRONCHIAL ASTHMA IN ADULTS

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Background : The prognosis of adult asthma, whether a long-term remission is available, is still unknown. In this paper we investigated the prognosis of adult asthma patients.
Methods : We sent a questionnaire by mail to 1168 patients who had been taken care in our clinic until 1990–1992, but disappeared afterward. Those patients were asked their present status of clinical condition of asthma including symptoms, medication, etc.
Results : Delivery of mail was failed in 370 patients because of changed address. 430 of 798 patients replied the mail and 86 patients out of 430 patients were in remission state with no symptom without any medications. The characterictis of these patients in remission are early hospital visit after developing asthma, mild in severity, mild in obstructive lung function and mild in bronchial hypersensitivity to acetylcholine at the first visit hospital.
Conclusion : We concluded that some of adult asthmatic patients might become in clinical remission.

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Association of Transforming Growth Factor-β1 Single Nucleotide Polymorphism C-509T with Allergy and Immunological Activities

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Key Words
Immunological activities · Single nucleotide polymorphisms · T-cell proliferation · TGFβ1

Abstract

Background: A single nucleotide polymorphism (SNP) C-509T within the tumor growth factor β1 (TGFβ1) gene has been associated with atopic asthma and asthma severity. To further understand the mechanisms involved, the association of C-509T with allergy, T-lymphocyte proliferation and plasma TGFβ1 concentration has been explored in a case-control study with allergic and nonallergic subjects. ***Methods:*** The recruited subjects including allergic (n = 38) and nonallergic (n = 25) participants have been genotyped for C-509T using allele discrimination assay. Association of C-509T with allergy status was examined using logistic regression analysis in both dominant and recessive models. Association of C-509T with T-cell proliferation in control and antigen-stimulated peripheral blood mononuclear cells (PBMCs), plasma TGFβ1 and total IgE level were tested by multiple regression analysis. ***Results:*** Individuals with homozygous mutant TT genotype showed a higher risk of allergy (TT: odds ratio = 5.099, 95% confidence limit: 1.355–19.190, p = 0.016) after covariates were adjusted. A trend to increased plasma TGFβ1 in subjects with T allele has been discovered. In the meantime, the T allele is associated with lower T cell proliferation in controls and maximum response to above antigens. A low T-cell proliferation is correlated with higher plasma TGFβ1 concentration ($p < 0.01$). The in vitro studies confirmed the suppressing effect of TGFβ1 on T-cell proliferation at physiological range. A significant inhibitory effect on IL-4 production was also observed. ***Conclusions:*** A C to T base change in TGFβ1 SNP C-509T has been associated with a higher risk of allergy. The mechanisms are not clear. Elevated TGFβ1 levels associated with the C-509T polymorphism might suppress immune activation as well as Th2 cytokine production.

Introduction

Allergic diseases such as asthma, allergic rhinitis and atopic dermatitis have been considered as complex polygenic disorders resulting from a combination of genetic and environmental factors. In past years, a lot of efforts have been pursued to look for possible genetic components contributing to allergies and asthma [1–4]. Studies from our group and others have indicated that tumor

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growth factor β1 (TGFβ1) is a promising candidate gene whose genetic polymorphisms may play a key role in allergic diseases [5–10]. Among reported genetic polymorphisms in the TGFβ gene, a C to T base exchange at –509 position in the promoter region was associated with elevated levels of total IgE in allergic asthma patients in our previous study [8]. Two subsequent independent case-control studies in relatively large populations reported the association of variant T allele with asthma, and even with asthma severity [5, 6]. The exact role and possible mechanisms involved are still not clear.

TGFβ1 is a 25-kDa disulfide-linked homodimeric multifunctional cytokine. With respect to its diverse activities in normal cell growth and development, TGFβ1 might be the most important growth factor in immunomodulatory effects because knockout mice died of massive inflammatory lesions [7]. Both anti-inflammatory and inflammatory properties have been reported [8]. In the present case-control study including allergic and non-allergic subjects, we have been able to further study the significances of the TGFβ1 C-509T genetic polymorphism in allergic diseases, its association with TGFβ1 production, immune modulation activity reflected from antigen-stimulated T-cell proliferation in peripheral blood mononuclear cells (PBMCs), and even regulatory effects on cytokine production.

Patients and Methods

Subjects

Adult volunteers either with a history of atopy or allergic diseases, e.g. allergic rhinitis, atopic dermatitis, and asthma, or no allergy (healthy individuals) have been recruited. Atopy or allergy status was determined based on identified allergen through positive skin test or allergen-specific IgE test, and allergic symptoms. To avoid possible confounding effects for T-cell proliferation to antigen, patients with other diseases especially an immune deficiency, allergic patients with a history of immunotherapy, and patients who have taken steroids or other immunosuppressive agents at least 3 days before blood drawing met the exclusion criteria. All subjects signed informed consent with National Jewish Institutional Review Board approval before being enrolled and donating blood.

Genotyping

Peripheral venous blood was obtained from each subject. Genomic DNA was extracted from whole blood using the QIAamp blood kit (QIAGEN, Santa Clarita, Calif., USA). Briefly, blood cells were first lysed, and supernatant containing genomic DNA was mixed with ethanol and applied to QIAamp spin column. After washing several times, purified genomic DNA attached in column was eluted finally in TE buffer.

The allelic discrimination assay using the ABI PRISM 7700 Sequence Detection System (AB Applied Biosystems, Foster City, Calif., USA) has been developed for genotyping of TGFβ1 C-509T (HUgn0007040). Primers used for amplification of DNA fragments are: forward primer at position –572~–551 (-CATGGGAGGT-GCTCAGTAAAGG-), reverse primer at position –437~–454 (-GGTAGGAGAAGAGGGTCTGTCAAC-). Probe 1 containing allele C at position –519~–494 was linked with reporter dye FAM (6-carboxyfluorescein) at the 5′ end and black hole quencher (Biosearch Technologies) at the 3′ end: -CCCTTCCATCCCT-CAGGTGTCCTGTT-. Probe 2 containing allele T instead of C was labeled with reporter dye 6-JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein). Each reaction contains 25 μl of Taqman Universal Master Mix (AB Applied Biosystems), 900 n*M* of forward and reverse primers in concentration, 100 n*M* of probe 1 and probe 2 in concentration, and 200 ng of genomic DNA in 50 μl total volume. Thermal cycling conditions for allelic discrimination assay are: 2 min at 50°C, 10 min at 95°C for AmpliTaq Gold Activation, and 40 cycles of 15 s at 95°C for denaturing and 1 min at 60°C for annealing/extension. Rn for a given reaction tube, defined as the ratio of the emission intensity of the reporter dye over the emission intensity of the passive reference, was obtained. The relative Rn value of FAR to JOE reporter dye for each reaction was utilized to separate CC (1.1–1.43), CT (0.74–1.1) and TT (0.30–0.74) after adjustment with non-template control based on the 95% confidence limits in each group. Duplicate experiments have been performed confirming reproducibility. Three distinct groups for CC, CT and TT have been recognized based on our setup thresholds.

Cell Culture and Cell Proliferation

PBMCs were isolated from human heparinized venous blood by density gradient centrifugation on Ficoll-Paque (Amersham, Uppsala, Sweden), and washed three times in HBSS (Gibco, Grand Island, N.Y., USA). Purified PBMCs were suspended in complete RPMI 1640 medium (Gibco), which was supplemented with 5% heat-inactivated fetal calf serum (Gemini Bio-Products, Calabasas, Calif., USA), 200 U/ml penicillin, 200 μg/ml streptomycin, 50 μg/ml gentamicin, 4 m*M* *L*-glutamine (Gibco), 10 m*M* HEPES (Sigma), 1 m*M* sodium pyruvate (Gibco), 0.1 m*M* nonessential amino acid (Gibco), and 50 μ*M* 2-mercaptoethanol.

To assess lymphocyte proliferation response, PBMCs were cultured at a concentration of 10^5 cells/well in 96-well flat-bottom plates in a volume of 200 μl. PBMCs were incubated with medium control; tetanus toxoid (Aventis Pasteur, Swiftwater, Pa., USA) at concentrations of 0.02, 0.07, and 0.2 lac-forming units (LFU)/ml; timothy (pollen) *Phleum pratense* allergic extract (Bayer, Elkhart, Ind., USA) at concentrations of 4, 40, and 400 PNU/ml, and standardized cat hair allergic extract (Fel d1; ALK, Horsholm, Denmark) of 1, 10, and 100 BAU/ml. On day 6, 1.0 μCi/well [^{3}H] thymidine (Amersham, Arlington Heights, Ill., USA) was added to 100 μl of cell culture and incubated for 6 additional hours. Cells were finally harvested onto filter paper disks via a multiwell harvester and radioactivity (counts/min) counted with a 1450 Microbeta Plus Liquid Scintillation Counter (Wallace, Orange, Calif., USA). All samples were run in triplicate.

To assess the effect of TGFβ1 on cell proliferation, PBMCs were also cultured at a concentration of 10^5 cells/well in 96-well flat-bottom plates in a volume of 200 μl for 6 days. Human recombinant TGFβ1 (R&D Systems, Minneapolis, Minn., USA) was added as indicated in the Results. Radioactivity was counted as described above.

To assess the effect of TGFβ1 on cytokine production, PBMC were cultured at a concentration of 10^6 cells/well and treated as described in the Results. Supernatant was collected at 48 h, and IL-2, IFNγ, IL-4 were measured using ELISA assays.

Plasma TGFβ1 Measurement

Plasma was first isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Paque and further centrifuged at 3,000 *g* for 10 min to eliminate platelets, and then frozen at –70°C. Plasma TGFβ1 was measured using the TGFβ1 ELISA Kit (Biosource, Nivelles, Belgium). Based on the manufacturer's instruction, latent TGFβ1 was first extracted from platelet-depleted plasma, and incubated with biotinylated anti-TGFβ1 in TGFβ1-antibody-coated wells for 3 h as well as TGFβ1 standard and diluent control. Streptavidin-HRP working solution was added for 30 min after thorough washing. Stabilized chromogen solution was finally added to each well for 30 min and terminated by stopping solution before absorbance (optical density, OD) was detected at a wavelength of 450 nm. The concentration of plasma TGFβ1 was calculated after deriving from a standard curve. The minimum detectable dose of TGFβ1 is 15.6 pg/ml. Duplicate experiments have been performed.

Plasma Total IgE Measurement

Plasma total IgE was measured using the ELISA kit from ALerCHEK (Portland, Maine, USA). According to the manufacturer's instructions, 20 μl of standard IgE and plasma sample were diluted with 80 μl of specimen diluent and incubated in anti-human IgE-coated microwells for 60 min at room temperature. After thorough washing with washing buffer, 100 μl of HRP-conjugated goat anti-human IgE was added for another 60 min. Finally, 100 μl of TMB/peroxide substrate were incubated for 30 min after another thorough washing and terminated by 0.5 *N* sulfuric acid before OD at 450 nm was determined. Total IgE concentration (IU/ml) in plasma was derived from the constructed standard curve. Duplicate experiments have been performed.

Cytokine Assays

IL-2, IFNγ and IL-4 production in cell culture supernatants was measured using ELISA kits (Biosource) with a sensitivity of <5 pg/ml, 0.03 IU/ml, and 2 pg/ml, respectively. According to the instructions, standard, control and samples from supernatant were incubated in specific-antibody-coated microwells together with antibody conjugate for 2 h at room temperature on a horizontal shaker set. After thorough washing, 200 μl of freshly prepared chromogenic solution were pipetted into each well and incubated for 15 min at room temperature on a horizontal shaker set before 50 μl of stop solution were added. The OD value at 450 nm was read, and plasma concentrations of cytokines were calculated based on standard curves finally.

Statistical Methods

Statistical analyses were performed using SAS software version 9.1.3. Balance of categorical variables regarding gender, race, ethnicity, genotype and allele frequency between allergy and non-allergy groups was tested using the χ^2 test or Fisher's exact test in PROC FREQ. The Hardy-Weinberg equilibrium for TGFβ1 C-509T allele frequency was calculated according to Thompson et al. [9]. Logistic regression was utilized to find the association between the TGFβ1 C-509T genotype and allergy status, while gender (male or female), race (White, Asian or Black) and TGFβ1 C-509T genotype (CC and CT, or TT only) has been considered in model building. Backward selection with slentry = 0.2, slstay = 0.2 options was used to remove low-risk factors. To adjust for possible confounding influences in the association of the C-509T genotype with numerical variables such as T-cell proliferation, stepwise-wise selection with slentry = 0.2 and slstay = 0.2 has been run with race, gender, allergy status, and TGFβ1 C-509T genotype entered in multiple regression models. Regression diagnostics have been performed to make sure that no violation of assumption occurs. Pearson's correlation coefficient has been used to estimate correlations between two numerical variables. Nonparametric approaches (the Kruskal-Wallis test and Wilcoxon's two-sample test) have been applied for multiple comparison among groups. p values less than 0.05 are considered as statistically significant.

Results

We recruited 63 volunteers including 38 with a history of allergic or atopic diseases and 25 normal subjects. All of them are nonsmokers. The properties of the recruited population are summarized in table 1. There is no significant difference between allergy and non-allergy groups regarding the composition of gender, race, ethnicity (χ^2 test or Fisher's exact test, $p > 0.05$) and age (Wilcoxon's rank sum test, $p > 0.05$). The allele frequency of TGFβ1 C-509T is consistent with the Hardy-Weinberg equilibrium ($\chi^2 = 3.93$, $p > 0.1$). Except for medium and phytohemagglutinin (PHA) control, T-cell proliferation stimulated by tetanus toxoid and timothy allergy is significantly higher in the allergy group than in the non-allergy group. There is a trend towards an increase in T-cell proliferation for cat hair allergen in the allergy group although it is not statistically significant ($p = 0.0710$).

Association of the TGFβ1 C-509T Genotype with Allergy

The association of the TGFβ1 C-509T genotype with allergy was examined using logistic regression. The possible covariates such as gender (male or female) and race (White, Asian or Black) have been considered and were removed according to the selection criterion (see Statistical Analysis). In the recessive model, the allergy status was associated with the TT genotype (odds ratio = 5.099, 95% confidence limits 1.355–19.190, $p = 0.016$). However, there is no significant association between allergy status and CC in the dominant model (odds ratio = 0.823, 95% confidence limits 0.26–2.383, $p = 0.7199$). This suggests that the TT variant of the TGFβ1 gene at promoter –509 is associated with increased risk of allergy in humans.

Table 1. Subject characteristics (mean and SD)

	Allergy	No allergy	Total	p value
Subjects	38	25	63	
Age, years	36 (10)	38 (12)	36 (11)	0.5737
Sex, %				
Female	58	52	56	0.6450
Race, %				
White	79	68	75	
Asian	16	32	22	
Black	5	0	3	0.2725
Ethnicity, %				
Non-hispanic	89	100	94	
Hispanic	11	0	6	0.0937
TGFβ1 C-509T genotype, %				
CC	37	44	40	
CT	53	20	40	
TT	11	36	20	0.0158
TGFβ1 C-509T allele frequency, %				
C	63	54	60	
T	37	46	40	0.9114
Timothy allergy, %				
Yes	84	0	51	<0.0001
Cat allergy, %				
Yes	58	0	35	<0.0001
CPM for control	8.49 (1.64)	8.12 (1.34)	8.34 (1.52)	0.2243
CPM for tetanus toxoid	9.70 (1.13)	9.04 (1.12)	9.44 (1.16)	0.0216
CPM for cat hair allergen	9.19 (1.36)	8.59 (1.13)	8.95 (1.30)	0.0710
CPM for timothy allergen	9.36 (1.15)	8.79 (1.08)	9.13 (1.15)	0.0409
CPM for PHA	11.45 (0.66)	11.45 (0.51)	11.45 (0.60)	0.8088
Total IgE	1.58 (0.75)	0.84 (0.72)	1.32 (0.82)	0.0009
Plasma TGFβ1, ng/ml	3.10 (0.47)	3.16 (0.41)	3.12 (0.45)	0.6014

Racial and ethnic classification is according to the standard used in Census 2000. For age and other numerical data, means (SD) are shown. Values for CPM and total plasma IgE are natural logarithmic transformed. Wilcoxon's rank sum score test was used for comparisons between allergy and non-allergy groups.

Association of TGFβ1 C-509T with Plasma TGFβ1 Level

To understand the possible mechanisms associated with the above finding, the association of C-509T with plasma TGFβ1 levels has also been estimated using multiple regression. The results indicated that there is a trend to higher plasma TGFβ1 concentration in the presence of T at the C-509T allele (CC: 3,008.34 ± 369.96, CT: 3,143.76 ± 529.17, and TT: 3,312.66 ± 386.55 pg/ml). However, statistically significant differences could not be shown in either the dominant model (CC only: $p = 0.1198$) or the recessive model (TT only: $p = 0.1346$) while possible covariates such as allergy, gender, age and race have been adjusted for. The hypothesis that a higher concentration of plasma TGFβ1 in the TT genotype accounts for the association of C-509T with allergy may need to be verified in larger studies in the future.

Association of the TGFβ1 C-509T Genotype with T-Cell Proliferation in Human PBMCs

With respect to the importance of TGFβ1 in the immunomodulation of allergen-induced cell proliferation, the association of the TGFβ1 C-509T genotype with antigen-stimulated cell proliferation in human PBMCs has also been examined using multiple regression analysis. Based on selection rules, the adjusted variables are listed in table 2 as indicated. Outcomes including unstimulated cell proliferation (medium control) and cell proliferation induced by maximum tetanus toxin, cat hair allergen and timothy allergen extracts showed a negative relationship

Table 2. Association of the TGFβ1 C-509T genotype with T-cell proliferation and IgE (n = 63)

Outcomes (ln value)	Adjusted variables	Regression coefficients for TT or CC (mean ± SE)	t value	p value
TT (recessive)				
CPM for medium control	White	−1.26 ± 0.48	−2.64	0.0127
CPM for tetanus toxoid	Black	−1.04 ± 0.34	−3.08	0.0031
CPM for cat hair allergen	cat allergy, White	−1.02 ± 0.41	−2.50	0.0152
CPM for timothy allergen	grass allergy, White	−0.93 ± 0.36	−2.55	0.0134
CPM for PHA	age, White	−0.07 ± 0.192	−0.37	0.7132
Plasma IgE	allergy	0.003 ± 0.26	0.01	0.9902
CC (dominant)				
CPM for medium control	age, White, Asian	1.20 ± 0.37	3.25	0.0019
CPM for tetanus toxoid	age, allergy	0.70 ± 0.28	2.48	0.0159
CPM for cat hair allergen	White, cat allergy	0.67 ± 0.32	2.07	0.0429
CPM for timothy allergen	White, grass allergy	0.84 ± 0.29	2.95	0.0047
CPM for PHA	age, Asian	0.03 ± 0.16	0.19	0.8464
Plasma IgE	allergy	0.07 ± 0.20	0.34	0.7376

with TT ($p < 0.05$) in the recessive model except for PHA. Conversely, the T-cell proliferation had a positive relationship with CC in the dominant model ($p < 0.05$) except for PHA. These results indicate that the C-T mutation is associated with reduced T-cell proliferation in general. We have used the stimulation index (ratio of antigen-stimulated cell proliferation over control) as response variable for tetanus toxin, cat allergen and timothy allergen. Neither the TT nor the CC genotype was correlated with antigen-specific cell proliferation (data not shown), suggesting that suppression might not be antigen specific.

The analysis for the association of the TGFβ1 C-509T genotype with plasma total IgE indicates that IgE is apparently not associated with the TGF C-509T genotype ($p = 0.9902$ for TT and $p = 0.7376$ for CC). The C-509T genotype does not exhibit an allergy-status-dependent difference ($p = 0.5676 > 0.05$ for TT and $p = 0.6718 > 0.05$ for CC) in IgE level.

Effect of TGFβ1 on T-Cell Proliferation

We also tested whether higher concentration of plasma TGFβ1 is correlated with lower T-cell proliferation capacity (fig. 1). From the graphs, negative associations of the plasma TGFβ1 concentration with natural log-transformed value of control, tetanus toxoid, cat allergen, timothy allergen, and even PHA-stimulated cell proliferation were found. Total IgE seems not to be associated with plasma TGFβ1 amounts. After adjusting for possible covariates such as gender, race and age in multiple regression models, the linear correlation still exists (table 3). These results support our hypothesis that human subjects with the T allele in the TGFβ1 C-509T genetic polymorphism suppressed immunological activity resulting probably from higher TGFβ1 production.

Table 3. Regression analysis of T-cell proliferation and IgE with plasma TGFβ1 concentration adjusted for gender, age, and race (n = 63)

Outcomes (ln value)	Regression coefficients for TGFβ1 (mean ± SE), $\times 10^{-3}$	t value	p value
CPM for medium control	−1.573 ± 0.4251	−3.70	0.0005
CPM for tetanus toxoid	−0.808 ± 0.3354	−2.41	0.0196
CPM for cat hair allergen	−1.239 ± 0.3711	−3.34	0.0016
CPM for timothy allergen	−0.970 ± 0.3317	−2.92	0.0051
CPM for PHA	−0.356 ± 0.1924	−1.85	0.0705
Plasma IgE	−0.138 ± 0.2291	−0.6	0.5485

Furthermore, recombinant human TGFβ1 was added at three different concentrations (32 and 3.2 ng/ml and 32 pg/ml) simultaneously in in vitro studies while PBMCs were cultured in the presence of tetanus toxoid (0.02 LFU/ml) as a stimulator. TGFβ1 significantly inhibited cell proliferation at 32 ng/ml ($p = 0.012$) with a dose-response trend (fig. 2) consistent with a suppressing effect of TGFβ1.

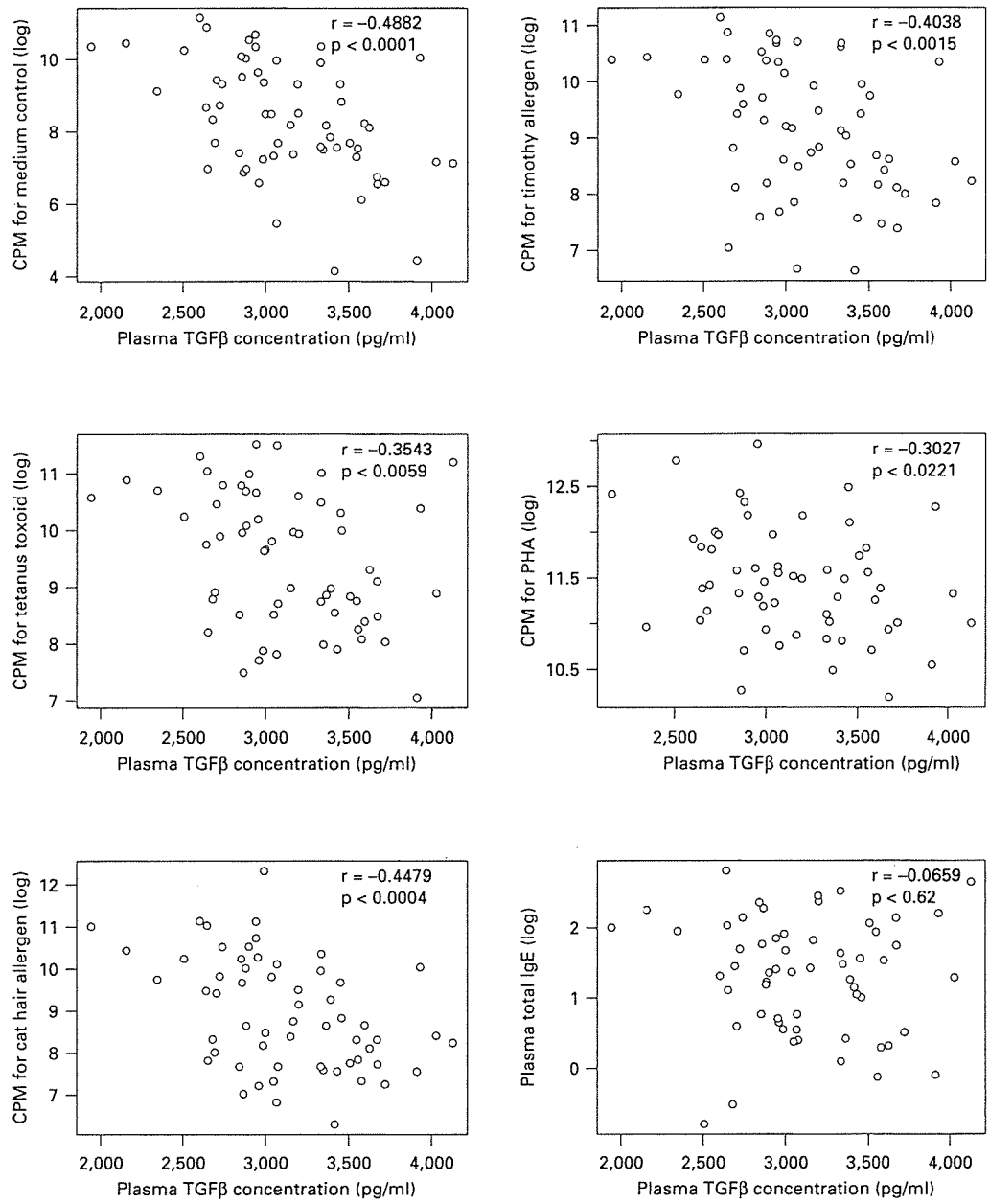

Fig. 1. Scatter plot of plasma TGFβ1 with plasma total IgE and T-cell proliferation in response to antigens in 63 subjects. Counts per minute (CPM) and total IgE are the natural log-transformed value of maximum T-cell proliferation in medium control, tetanus toxoid, cat allergen, timothy allergens, PHA-stimulated PBMCs and plasma IgE level. r = Correlation coefficient.

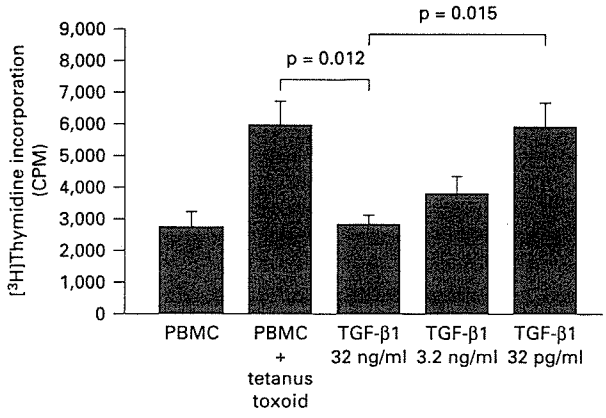

Fig. 2. Inhibition of T-cell proliferation by TGFβ1. [^{3}H]thymidine uptake (counts/min, CPM) of T-cells stimulated with 0.02 LFU/ml tetanus toxoid in the presence of TGFβ1 for 6 days (n = 8).

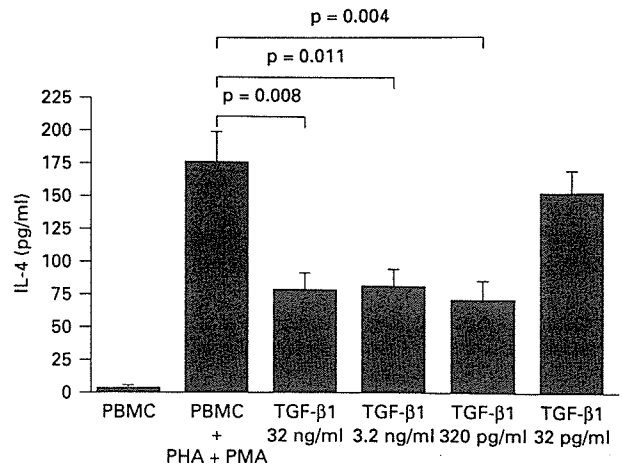

Fig. 3. Inhibition of IL-4 synthesis by TGFβ1. PBMCs were stimulated with PHA 10 μg/ml and 10 ng/ml phorbol 12-myristate 13-acetate (PMA) in the presence of TGFβ1 (n = 5).

Inhibiting Effect of TGFβ1 on Cytokine Production

Cytokines have been considered to be very important in regulating T-cell proliferation. The inhibitory effect of TGFβ1 on cytokine production was further studied in vitro. PBMCs were incubated in the presence of 10 μg/ml PHA and 10 ng/ml phorbol 12-myristate 13-acetate with TGFβ1 at four different concentrations (32 and 3.2 ng/ml, and 320 and 32 pg/ml) for 48 h. Supernatant was collected and measured for IL-4, IL-2 and IFNγ using ELISA. Figure 3 showed that TGFβ1 significantly inhibited IL-4 synthesis even at 320 pg/ml ($p = 0.004$). However, the effects of TGFβ1 on IL-2 and IFNγ production were not statistically significant (data not shown). The suppressing effect of TGFβ1 on cytokine production, especially of the Th2 type, may be involved in the association of the TGFβ1 genotype with reduced immunological activities.

Discussion

TGFβ belong to a super family of about 40 structurally related polypeptide growth factors [10]. Perturbation of the TGFβ superfamily has been implicated in various human diseases, e.g. cancer, angiogenesis, fibrotic diseases, and atherosclerosis, due to their mediation of many key events in normal growth and development including cellular differentiation, proliferation, motility, organization and death [11]. TGFβ1 is one of the best-studied factors in mammalian tissues. The role of TGFβ1 in asthma and allergic diseases has also been well documented in the literature [12]. Increased TGFβ1 expression in asthma has been repeatedly reported. Overexpression of TGFβ1 mRNA and proteins in bronchial biopsies from subjects with severe and moderate asthma has been observed compared with controls without asthma [13–15]. In asthmatic airways, TGFβ1 mRNA expression mainly produced from eosinophils was related to the degree of disease severity, subepithelial fibrosis and airway remodeling [13, 14, 16].

The genetics of atopy and asthma has been a very interesting research area in past years. Deciphering secrets in genetic variations related to allergy and asthma development may greatly benefit our efforts in the prevention and treatment of asthma and allergic diseases. The candidate gene approach, in which genes of relevance to the physiological changes of the disease process are tested for direct involvement with the disease, has been successfully applied to link genetic variation in the promoter region of a number of cytokine genes to asthma and allergic diseases. The promoter region of the TGFβ1 gene has been characterized showing regulatory impacts on transcriptional processes [17]. Our previous work demonstrated that a TGFβ1 promoter polymorphism at C-509T with the TT homozygote genotype is linked to allergic and asthmatic patients with elevated serum IgE [18]. Two case-control independent studies in relatively large populations of white individuals also found the association of

variant T allele with asthma, even with severity of asthma [5, 6, 19]. In our present study, the T variant of the genetic polymorphism TGFβ1 C-509T is significantly associated with allergy status. These results consistently support our hypothesis that the TGFβ1 T allele genotype is an important risk factor in allergy development.

C to T base change is located in the region of the promoter associated with Yin Yang-1 activator consensus sequence. It has been reported to increase promoter function using luciferase constructs [20]. The T allele has been demonstrated to enhance the YY1 binding site on the TGFβ1 promoter region and may account for the increased transcription of TGFβ [6]. The C-509T polymorphism was significantly associated with increased concentration of latent TGFβ1 in serum in a human study [21]. Our present results also showed an increased trend of plasma TGFβ1 to the T allele. Limited sample sizes and differences in population properties may explain the lack of statistical significance in this study. It is reasonable to deduce that subjects with the T allele may display a higher level of TGFβ1 in other tissues such as respiratory airways or skin. The observed overexpression of TGFβ1, e.g. in bronchial biopsies of asthmatic patients as discussed before, may at least partly result from their enhanced capacity to produce TGFβ1 determined by their genetic components such as the T allele mutation in C-509T, although a feedback mechanism from the inflammatory reaction in asthmatic patients cannot be excluded.

It has been well known that immunological activity plays a crucial role in initiating and developing allergic reactions in response to allergens. In mice, knockout studies with TGFβ1 indicated that TGFβ1 might be one of the most important growth and regulatory factors in the immune system because the knockout mice died of massive inflammatory lesions [22]. However, the evidences about its effects in the immune system seem confusing and contradictory. Its anti-inflammatory effect has been demonstrated by inhibiting the proliferation of T and B lymphocytes [23, 24]. On the other hand, TGFβ1 has been found to be a growth factor by inducing proliferation of macrophages, dendritic cells, and even T cells [25, 26]. Our present study indicates that a C to T mutation at the TGFβ1 genetic polymorphism C-509T is correlated with suppressed T-cell proliferation in vivo. This suggests that subjects with the T allele in the absence or presence of allergy demonstrated reduced lymphocyte response after exposure to allergen, even at the basic level. Increased production of TGFβ1 seems to be one of the reasons, since the plasma TGFβ1 concentration is negatively correlated with the degree of T-cell proliferation, and TGFβ1 suppresses T-cell proliferation in vitro at physiological concentrations. Human subjects with the T allele may own a relatively lower immune activity due to more TGFβ1 inside their body.

Allergic diseases are aberrant immune responses to antigen mainly mediated by IgE. Our previous study indicated that the C to T base exchange is associated with elevated IgE in atopic asthmatic patients. However, other studies did not confirm our findings [6]. Our present study failed to show a significant association with the total IgE level while other possible covariates have been considered. Lack of adjustment of covariates, e.g. the allergy status, in our previous report might be one reason for the elevated IgE in atopic asthma patients. These results implied a complicated relationship between TGFβ1 and total IgE. As we know, TGFβ1 is believed to inhibit IgE predominately in immunity. It might be related to its downregulatory effect on various cell surface molecules including CD23 and MHC class II antigen in human cells, which may be very important in promoting IgE synthesis [27, 28]. TGFβ1 could also inhibit e-germline transcription [29] directly through transcription factors or through inhibiting Th2 cytokines such as IL-4. Elevated TGFβ1 in TT allele subjects is expected to reduce the IgE level. However, as IgE synthesis is a very complicated process involving lots of cytokines (e.g. IL-13 and IL-10), even suppression of IgE is environment directed. It has been reported that at a later stage of differentiation, IgE B cells are refractory to the inhibitory effect of TGFβ, as shown by the slight but significant increase in the spontaneous secretion of IgE by PBMC of atopic patients [30]. Finally, TGFβ1 downregulation of IgE may be a mechanism where Th2 inflammation may still be prominent, with no increase in IgE, a situation that might be seen in intrinsic asthma for example. Therefore, we may not be surprised to see the lack of association of this genetic polymorphism with the IgE level in plasma in our present study.

Both T-cell proliferation and production of IgE and cytokines, including Th2 and Th1, are believed to play an important role in allergic inflammation. In our study, the Th2 cytokine IL-4 was depressed rather than IL-2 and IFNγ, implying that Th2 cytokines are crucial in explaining the observed suppressing effect of TGFβ1 on T-cell proliferation. However, the suppressing effect of TGFβ1 on the Th1-type reaction or initiation of T-cell reactions may still exist. This is because the TGFβ1-induced downregulation of the IL-2 receptor at both protein and mRNA level has been reported [29]. TGFβ1 has been shown to inhibit IL-2-induced tyrosine phosphorylation, activation of Jak-1 and Stat 5, and IL-2-induced expression of

α and β chains of IL-2R [30]. Interaction of IL-4 and IFNγ has been reported. Reduction of IL-4 by TGFβ1 may reduce the forces to inhibit IFNγ production. These may be some reasons that we failed to observe the significant effect on IFNγ. With respect to the cytokine IL-4, T-cell proliferation in the allergic reaction, an increased TGFβ1 concentration in T-allele subjects may play a role in the suppression of the allergic reaction or inflammatory effect rather than an increase in the risk of allergic status. The mechanisms involved are still not clear. More studies in larger patient cohorts and on the mechanisms regarding its role in allergic development may be worth of much more attention in the future. Other roles than cell proliferation for TGFβ1 may be hidden behind the association of TGFβ1 C-509T with allergy. As an example, TGFβ1 is an important stimulant of fibrosis, inducing formation of the extracellular matrix, and promoting wound healing and scar formation. Therefore, the profibrotic effect of TGFβ1 may play a more important role in asthma development. Another important function for TGFβ1 is their regulatory activity in cell differentiation. IL-3 and M-CSF in cooperation with TGFβ induces granulocyte macrophage colony-stimulating factor-independent differentiation of human CD34+ hematopoietic progenitor cells into dendritic cells with features of Langerhans' cells. TGFβ1 may play an important role in the differentiation and maturation of dendritic cells and monocyte differentiation. TGFβ1 may play an important role in regulating some cell cycle arrest rather than others, as indicated in CD34+ CD38-c-kit(high) vs. c-kit(low/–) cells. Due to the complexity in the development of allergic diseases, the T allele may be combined with other genetic variations, which produce a different environment leading to the allergic status.

In summary, our study demonstrated the modulatory effects of TGFβ1 and its polymorphism on cell proliferation and cytokine production. Further investigations with larger sample sizes could further solidify our findings. Clarification of the association of the TGFβ1 C-509T polymorphism with allergic diseases may raise new and better methods for diagnosis, and more importantly, the potential for new pharmacological interventions related to the products of the candidate genes.

Acknowledgments

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Original article

Role of CCL21 and CCL19 in allergic inflammation in the ovalbumin-specific murine asthmatic model

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Background: Dendritic cells are the most powerful of the antigen-presenting cells and are known to play important roles in sensitization and inflammation in allergen-specific asthma. Various cytokines and chemokines are involved in the maturation and activation of dendritic cells. Among them is CC chemokine ligand (CCL)21, a key chemokine in the entry of naive T cells and antigen-stimulated dendritic cells into the T-cell zones of secondary lymphoid organs, which is a critical process in antigen-specific T-cell activation.

Objective: We studied the role of CCL21 in airway inflammation in asthma by using BALB/c-plt/plt (plt) mice, which possess genetic defects in expression of both CCL21 and CCL19.

Methods: Plt and control BALB/c mice were immunized with ovalbumin and alum 4 times and thereafter were subjected to a 2-week regimen of ovalbumin inhalation.

Results: In plt mice, ovalbumin-specific IgE response was delayed compared with control BALB/c mice, but they had the same level of response after final immunization. Although airway inflammation and response to acetylcholine were significantly reduced compared with BALB/c mice, significant eosinophilic inflammation and hyperresponsiveness were also observed in plt mice after 2 weeks of inhalation. Four weeks after cessation of inhalation, airway inflammation and hyperresponsiveness in plt mice were greater than in BALB/c mice. At the time of resolution of airway inflammation, IL-10 production was enhanced in BALB/c mice but not in plt mice.

Conclusion: The chemokines CCL21 and CCL19 were critical for resolution of airway inflammation.

Clinical implications: The findings about the chemokines for induction and resolution of inflammation are key to establishing a new strategy for asthma immunotherapy.

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Key words: Asthma, airway hyperresponsiveness, CCL21, dendritic cells, airway inflammation

In allergic asthma, immune response to inhaled antigen is skewed to the T_H2-type response.[1-7] For the recognition of antigen, dendritic cells (DCs) work as professional antigen-presenting cells (APCs). Administration of antigen pulsed DCs induces allergic inflammation.[8,9] In addition, DC maturation is accelerated in the asthmatic airway.[10] Immature DCs possess the ability to take up and process antigens but have low antigen-presenting ability.[11] After taking up antigen, DCs become mature, expressing CD86, CD80, CD40, and CC chemokine receptor (CCR).[12,13] Mature DCs migrate to lymph nodes through the interaction of CCR7 and CC chemokine ligand (CCL)21 (secondary lymphoid tissue chemokine), where they effectively present antigen to naive T cells. CCL21 has potent activity in attracting naive and mature T cells, B cells, and DCs.[14,15] CCL21 was expressed in high endothelial venules in the peribronchial area and perivascular lymphatics in the lung. Another ligand of CCR7 is CCL19 (Epstein-Barr ligand chemokine), which also promotes efficient interaction of T cells and DCs at the site of lymph nodes. The process of antigen presentation has been clearly shown at the skin.[9] Antigen that penetrates the skin is taken up by DCs. Subsequently, these professional APCs enter afferent lymph vessels, a process promoted by CCL21. Antigen-laden APCs then induce the activation, expansion, and differentiation of antigen-specific T cells. These processes are efficiently performed only at skin-draining lymph nodes. Although CCL21 is also expressed in peribronchial areas and perivascular lymphatics in the lung,[16] the role of CCL21 and CCL19 in the asthmatic airway has not been clarified.

CCL21 is encoded by 2 genes, SCYA21a and SCYA21b, which encode CCL21-ser and CCL21-leu

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Abbreviations used
APC: Antigen-presenting cell
BALF: Bronchioalveolar lavage fluid
CCL: CC chemokine ligand
CCR: CC chemokine receptor
DC: Dendritic cell
PAS: Periodic acid-Schiff
Penh: Enhanced pause
plt: BALB/c-plt/plt

protein, respectively. CCL21-leu (secondary lymphoid tissue) is absent in mice with a paucity of lymph node T cells (BALB/c-plt/plt [plt] mice), which possess a defect in homing of naive T cells in lymph nodes, resulting in impaired secondary lymphoid organs.[17-19] Plt mice are also deficient in CCL19.[18] To test the role of CCL21 and CCL19 in antigen-specific asthma, we investigated the ability of plt mice to exhibit allergen-specific airway inflammation. We found that airway inflammation and airway response to acetylcholine were significantly reduced in plt mice compared with BALB/c mice. However, significant eosinophilic inflammation and hyperresponsiveness were also observed in plt mice after 2 weeks of inhalation. Interestingly, after cessation of inhalation for 4 weeks, airway inflammation and airway hyperresponsiveness of plt mice were greater than those of BALB/c, suggesting that in the resolution phase of airway inflammation, CCL21 and CCL19 play a critical role.

METHODS

Sensitization

BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). Plt mice were bred in the animal facilities of Musashino and Teikyo University School of Medicine under Specific Pathogen-Free conditions. Care and use of the animals followed the guidelines of the Principles of Laboratory Animal Care formulated by the National Society for Medical Research. The mice were initially immunized intraperitoneally 4 times with 10 μg ovalbumin + 2 mg aluminum hydroxide gel (on days 0, 28, 35, and 49) as previously reported with slight modifications.[20] After immunization, animals were subjected to inhalation of ovalbumin 3 mL/min every other day for 2 weeks (day 50 to day 63 from the first immunization) using a micromist nebulizer (Devilbiss, Somerset, Pa). Mean particle diameter was 3.0 μm. In some mice, recombinant macrophage inflammatory protein 3β/CCL19 (Epstein-Barr ligand chemokine; 5 μg/mouse), purchased from R&D Systems (Minneapolis, Minn), was administered by using an osmotic pump (Alzet Minipump; Durect Corp, Cupertino, Calif), which was implanted subcutaneously in the anterior back region. Four to 5 mice were used per treatment group in each experiment. Untreated mice were also used as controls.

Assessment of airway responsiveness

The assessment of airway responsiveness was undertaken 24 hours after the last ovalbumin inhalation, as previously reported.[20] We used barometric whole-body plethysmography (Buxco Electronics, Inc, Troy, NY) as described by Hamelmann et al.[21] Data are expressed as enhanced pause (Penh). Airway responsiveness was determined by calculating the ratio of the Penh to control Penh (%Penh). In some mice, invasive measurements were undertaken as previously reported.[22] Briefly, the anesthetized mice were tracheostomized and injected with pancuronium bromide. The animals were connected to a Harvard ventilator with 0.3 mL tidal volume and a respiratory frequency of 120/min. Next, they were placed in the whole-body plethysmograph (Buxco Electronics) to measure airway resistance.

Measurements of serum ovalbumin-specific IgE and IgG_{2a}

To measure ovalbumin-specific IgE, we coated the plate with ovalbumin using the AlaSTAT system (Diagnostics Products, Los Angels, Calif) with some modification as previously reported.[20] After incubation with samples diluted 10-fold, horseradish peroxidase–labeled rat antimurine IgE specific antibody, purchased from Morinaga Co, Ltd (Kanagawa, Japan), was added to the plate as the second antibody. Color was developed and measured at 450 OD.

For ovalbumin-specific IgG_{2a}, plates were coated with 50 μg/mL ovalbumin in 50 mmol/L carbonate buffer, pH 9.0. After blocking with SuperBlock (Pierce, Rockford, Ind), samples diluted 2000−fold were added in the plates. Antibodies were detected by peroxidase-labeled antimouse IgG_{2a} (1:500; Invitrogen, Carlsbad, Calif). The color was developed with TMB+ (Dako Corp, Glodtrup, Denmark) and stopped by 1N H_2SO_4. The antibody titers were calculated by comparison with internal standards.

Bronchoalveolar lavage fluid cell analysis and histologic examination

After measurement of pulmonary function, each mouse was exsanguinated. Bronchoalveolar lavage fluid (BALF) was obtained from selected mice by incubating and washing the lung with 1 mL saline until the recovered volume was 5 mL. BALF was centrifuged at 1500 rpm for 10 minutes at 4°C. Pellets were dissolved in 1 mL PBS, and the number of the cells was counted. Cytospin specimen was obtained by rotating at 640 rpm for 2 minutes. Then, the cells were stained with Diff Quik (International Reagents Corp, Osaka, Japan), and the cell fractions were examined on microscope.

The lungs were fully inflated using 10 cm H_2O pressure and fixed with 10% formaldehyde for hematoxylin-eosin and periodic acid-Schiff (PAS) staining.

RNA extraction and quantification of mRNAs

Lungs were frozen in liquid nitrogen immediately after isolation and were used for RNA extraction. Lung tissue was homogenized at 4°C, and total RNA was extracted by using ISOGEN, a modified acid guanidium-phenol-chloroform method (Nippon Gene Co, Ltd, Tokyo, Japan). RNA was treated with 10U DNase (Qiagen, Hiden, Germany) following the manufacturer's instructions. The purity of the RNA was established by spectrophotometer using a DNA purity calculating program (Hitachi Seisakusho, Tokyo, Japan). The absorbance OD 260/280 ratio was 1.8. After the amount of total RNA was measured, cDNA synthesis was performed with 0.125 μmol/L oligo-dT (Takara Biochemicals, Tokyo, Japan) as previously described.[20] The levels of mRNA were examined by real-time PCR using the Light Cycler-Fast Start DNA Master SYBR GreenI kit (Roche Diagnostics, Mannheim, Germany). In this system, double-stranded DNA is labeled with SYBR Green I and then detected. Quantification was performed on the basis of the standard curve obtained by using serial dilution of specific PCR products. Results are shown as ratios of the level of mRNAs standardized by the level of β-actin mRNA. The primers used were as follows: β-actin, 5′-CCTGTATGC-CTCTGGTCGTA-3′ 5′-CCATCTCCTGCTCGAAGTCT-3′ 260 bp; IL-13, 5′-GATATGAAGTTGAAGAGGAGAG-3′ 5′-GGCAAA-GAAGTAACAAAAGG-3′ 208 bp; IFN-γ, 5′-TCTTGGATAT-CTGGAGGAAC-3′ 5′-GACCTCAAACTTGGCAATAC-3′ 215 bp.

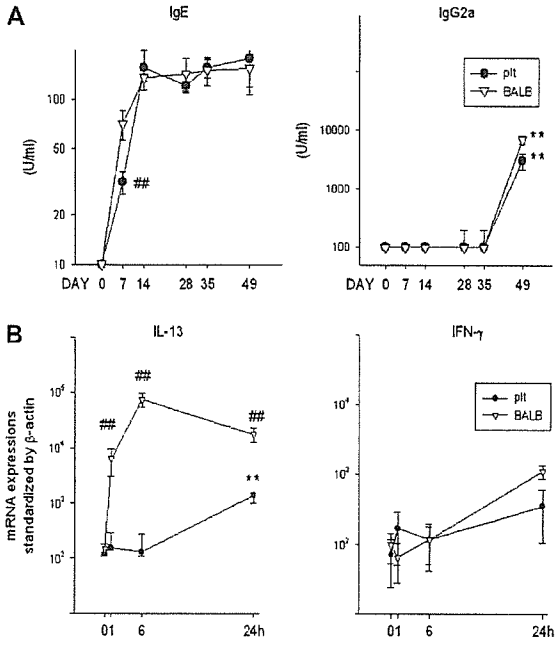

FIG 1. Time course of ovalbumin-specific IgE and IgG_{2a} response **(A)** and mRNA expressions of IL-13 and IFN-γ **(B)**. Each *bar* represents the mean ± SEM of values obtained from 5 different mice. Five mice per group were examined. Similar results were obtained from 4 other experiments. ##$P < .01$ BALB/c vs plt. **$P < .01$ indicated time vs baseline.

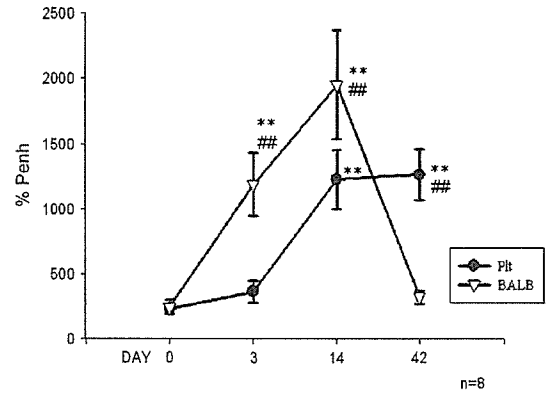

FIG 2. Time course of airway response to acetylcholine. Airway response to 100 mg/mL acetylcholine was expressed as % Penh. Each *bar* represents the mean ± SEM of values obtained from 6 different mice. Similar results were obtained from 3 other experiments. **$P < .01$ indicated day vs day 0. ##$P < .01$ BALB/c vs plt mice.

Quantification of IL-10 production

Lung cells were extracted from some of the mice in which airway responsiveness was measured and used for cell culture. Lungs were cut into small pieces and incubated at 37°C for 1 hour in 150 U/mL collagenase in RPMI. Then, lung pieces were homogenized in 1% penicillin/streptomycin (Gibco Invitrogen, Carlsbad, Calif)/RPMI 1640 using cell strainers with 70-μm pores (BD Falcon, Franklin Lakes, NJ) and centrifuged at 1500 rpm for 10 minutes at 4°C. Cells were then washed twice, and $CD3^+$ cells were separated by using the Dynabead mouse pan T-cell system following the manufacturer's instructions (Dynal Biotic ASA, Oslo, Norway). $CD3^+$ cells with about 5% adherent cells were then suspended in 1% penicillin/streptomycin/10% FCS in RPMI 1640 and cultured with or without ovalbumin (100 μg/mL) in 24-well plates (Corning, Inc, Corning, NY) at 1×10^6 cells/well at 37°C. After 2 days of culture, the supernatant was collected, and the levels of IL-10 were evaluated by the ELISA Quantising kit (R&D Systems).

Flow cytometry

Data were collected by using FACSAria (BD Biosciences, San Jose, Calif). A total of 1×10^6 cells from lung, spleen, blood, and lymph node were stained with indicated antibodies. Antibodies used were antimouse CD16/CD32 (Fc block), phycoerythrin-Cy7–conjugated rat antimouse CD25 antibody, CD4-Alexa 647 (BD Biosciences), and phycoerythrin-labeled CCL19-Fc chimera antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Cells were first treated with antimouse CD16/CD32 (Fc block) for 10 minutes. Then staining antibodies were added for 30 minutes at 4°C. All plots show log10 fluorescence.

Statistics

For comparisons of multiple parameters, we used ANOVA with Bonferroni correction. The level of statistical significance was set at $P < .05$. Data are expressed as means ± SEMs.

RESULTS

Ovalbumin-specific IgE and IgG_{2a} synthesis in plt mice

To elucidate the functional role of CCL21 and CCL19 in IgE synthesis, we determined the kinetics of IgE synthesis with immunization of ovalbumin and alum in plt mice and BALB/c mice. Examination of the ovalbumin-specific IgE level on day 7 after the first immunization revealed a significantly lower IgE level in plt mice compared with BALB/c mice (Fig 1, *A*; $P < .01$). On day 14, the difference was no longer evident, a tendency that was continued after the final immunization (Fig 1, *A*). These data suggested that induction of IgE was delayed but that the response was similar to the IgG response reported previously.[23] When we compared IgG_{2a} synthesis, there was significant synthesis on day 49 but no difference between plt and BALB/c mice (Fig 1, *A*).

Next, we examined IL-13 and INF-γ mRNA expression of the lung after ovalbumin inhalation. One hour after ovalbumin inhalation, significant IL-13 mRNA was detected in BALB/c mice but not plt mice (Fig 1, *B*). After 24 hours, IL-13 mRNA expression was detected in plt mice but significantly lower compared with BALB/c mice (Fig 1, *B*). IFN-γ expression was not different between BALB/c and plt mice. These data suggested that plt mice expressed significant T_H2 response but lower compared with BALB/c mice.

Airway hyperresponsiveness and inflammation in plt mice

To determine airway response, we first measured airway resistance after 100 mg/mL acetylcholine

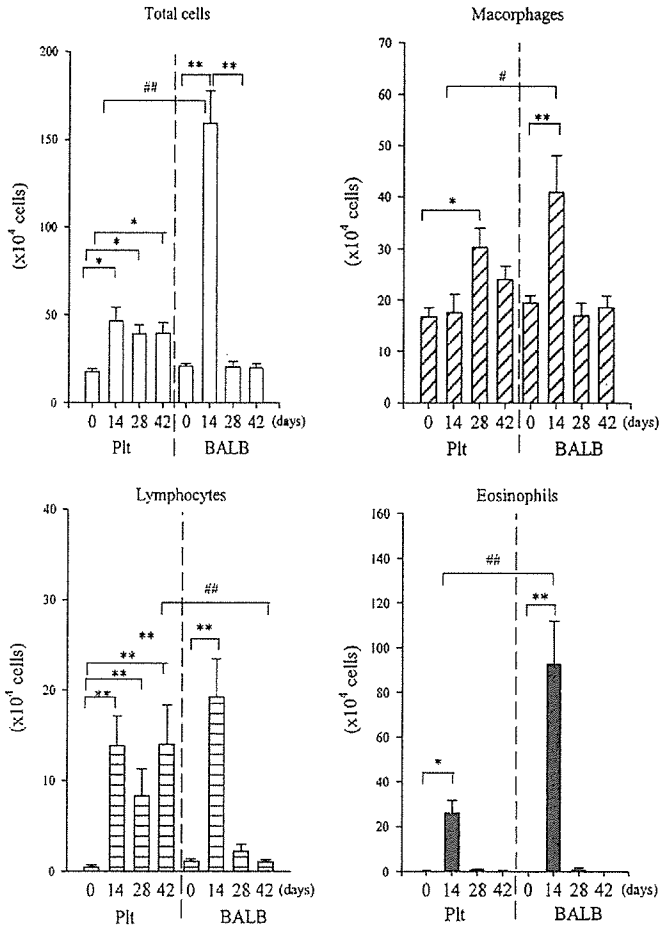

FIG 3. BALF cell analysis. Data shown are means ± SEMs of 8 mice per group. *$P < .05$; **$P < .01$ indicated day vs day 0. #$P < .05$; ##$P < .01$ BALB/c vs plt mice.

inhalation (Fig 2). We followed a sensitization system as previously reported.[20] Mice were analyzed 24 hours after the last ovalbumin inhalation at the times indicated in Fig 2. Before ovalbumin inhalation, no increase in airway resistance was observed after acetylcholine inhalation compared with saline inhalation. On day 3, a significant increase in airway response was observed in BALB/c mice but not in plt mice. On day 14, a further increase in airway response was observed in BALB/c mice. On day 14, plt mice also exhibited a significant increase in airway response compared with untreated mice; however, the response was significantly lower than in BALB/c mice. On day 42 (4 weeks after cessation of ovalbumin inhalation on day 14), airway response was decreased in BALB/c mice but not in plt mice. To confirm the results, airway resistance after inhalation of 2.5 mg/mL acetylcholine was also examined by using an invasive measurement on day 42. Airway resistance of plt mice and BALB/c mice was 4.33 ± 0.25 versus 2.94 ± 0.33 cm H_2O/L/s, respectively (n = 5 each group; $P < .05$).

To determine airway inflammation, BALF cells were also analyzed in BALB/c mice (Fig 3). On day 14, significant eosinophilia was observed in BALF. Increase in eosinophil and lymphocytes resolved on day 28 (2 weeks after cessation of exposure to ovalbumin). Although the level was lower, plt mice exhibited significant increase in the number of eosinophils on day 14 (Fig 3). In contrast to BALB/c, increases in lymphocytes remained present until day 42 (4 weeks after cessation of exposure to ovalbumin).

Histologic analysis revealed less airway inflammation at day 14 in plt mice than in BALB/c mice (Fig 4, *A*). PAS staining revealed that mucus-producing cells significantly appeared both in plt and BALB/c mice. However, the number of mucus-producing cells was small in plt mice as compared with BALB/c mice (Fig 4, *B*, on day 14). In BALB/c mice, airway inflammation resolved on day 28, but plt mice exhibited significant airway inflammation on day 28 (Fig 4, *A*). These data indicated that airway inflammation was delayed but that significant responses occurred in plt mice compared with BALB/c mice, as is the case with antibody response. It was also noted that resolution of airway inflammation and airway responsiveness was delayed in plt mice.