Kraft

Eisenbarth SC and colleagues speculated that it worked somehow through a new pathway. Although they haven't worked out all the pathways of the mechanism yet, it is important enough to shed light on the concept that it's more of a dose as well as timing.

Kaminogawa

Do you already have evidence or literature on this mechanism?

Kraft

No, this is Eisenbarth's paper. The TLR4-deficient mice didn't manifest this response. The TLR4 receptor was critical for this whole process and it worked through an MyD88-independent pathway. So, that is also new information.

Kondo

Thank you. Many scientists are interested in the balance of Th1/Th2, and we also investigated the factors that affect this balance. Let me introduce some here.

First, we investigated the roles of IL-10 in allergy. IL-10 was identified by Mosman et al. in 1989 as a factor that suppresses the production of cytokines in Th1 cells. Further studies revealed that it is also produced by various other cell types and has various effects. However, at that time, the involvement of the cytokine network in allergy had not been sufficiently investigated.

So, we investigated these points. IL-10 dose dependently suppressed the production of Th1 cytokines such as IL-12 and IFN-γ that were strongly expressed in the peripheral blood mononuclear cell fraction *in vitro* (Fig. 6). In contrast, in the T-cell clones, IL-10 dose dependently suppressed the production of Th2 cytokines (IL-4). These results show that IL-10 suppresses the production of Th1 cytokines (IL-12 and IFN-γ) strongly expressed *in vitro*, while it also suppresses Th2 cytokines. Therefore, IL-10 probably regulates the balance of Th1 and Th2.

We also investigated the production of these cytokines in the control group and the allergy group. In the allergy group, the production of IL-10 was significantly reduced. Furthermore, in a comparison of children younger than 2 years old and children older than 2 years, although it was not statistically significant, the production of IL-10 was lower in children with allergy younger than 2 years old.

Kraft

I believe that IL-10 is a critical mediator in this Th1/Th2 balance, and its roll begins early in life more studies evaluating the genetics of regulating IL-10 function should be performed.

PREVENTIVE THERAPY AND ITS DEVELOPMENT BASED ON HYGIENE HYPOTHESIS -POTENTIAL OF PROBIOTICS-

Kondo

Okay, as the next topic, I would like to discuss how we can make use of hygiene hypothesis in the treatment/prevention of allergy.

In the discussion we have had so far, it has been suggested that allergy can be prevented by enhancing the Th1 immune response and suppressing the excessive Th2 immune response. In fact, studies that apply these hypotheses to treatment have already been undertaken. One of them is the application of "probiotics." Professor Kaminogawa is a specialist in this field. Professor Kaminogawa, could you briefly tell us about "probiotics?"

Kaminogawa

Bacterial infections enhance the Th1 response and suppress allergy that is affected by the Th2 response; this is the concept of hygiene hypothesis. And for example, bifidobacteria and lactobacilli that inhabit the intestine have the ability to induce non-allergy-type lymphocytes, namely Th1. Indeed, these bacteria are fewer in children with allergy and abundant in control children without allergy, suggesting that they also suppress allergy *in vivo*. The concept of the application of "probiotics" is to use these bifidobacteria and lactobacilli to prevent allergy.

Kondo

Then "probiotics" means bacteria.

Nihon University College of Bioresource
Science Department of Food Science and Technology
Professor Shuichi Kaminogawa

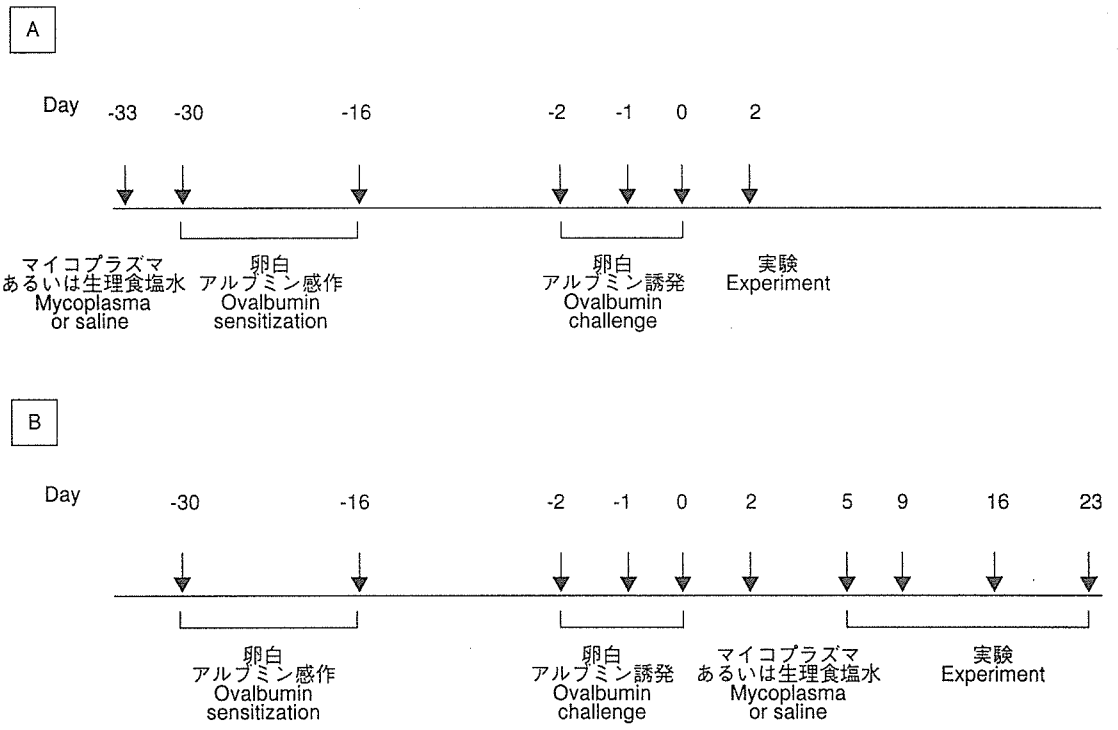


図3　喘息モデルマウスにおける感染の影響実験(タイムライン)
(A) *M. pneumoniae* 感染後の OVA 感作・誘発のタイムライン．(B) OVA 感作・誘発後の *M. pneumoniae* 感染のタイムライン．
Fig. 3　The effects of infection in mouse asthma model (time line)
(A) Time line for *M. pneumoniae* infection followed by OVA sensitization and challenges. (B) Time line for OVA sensitizations and challenges followed by *M. pneumoniae* infection.

(文献4より引用)

ギー反応が予防できるのではないかという方向性がみえてまいりました．実際に，こうした理論を治療に応用する試みも始められております．そのひとつが「プロバイオティクス」の応用です．上野川先生はこの分野のご専門家でいらっしゃいます．上野川先生，「プロバイオティクス」について簡単にご説明いただけますでしょうか．

上野川

微生物の感染が Th1免疫応答を促進し，Th2反応に影響を受けるアレルギーを抑制するというのが衛生仮説の概念ですが，例えば，腸内に生息しているビフィズス菌やラクトバチルス菌も，非アレルギー型のリンパ球，つまり Th1を誘導する能力をもっているわけです．実際に，アレルギー小児にはこれらの細菌が少なく，対照のアレルギーではない小児の腸内には多いことから，生体でもアレルギー抑制の働きをしていると考えられています．これらビフィズス菌やラクトバチルス菌をアレルギー発症予防に役立てようというのが「プロバイオティクス」の応用です．

近藤

「プロバイオティクス」は細菌のことを指しているわけですね．

上野川

いわゆる「善玉菌」のことです．大腸内のマイクロフローラ(図7)は，ヒトの健康と病気の双方に重要な役割を有しています．例えば，有害な作用をもつものとして病原体の *Ps.aeriginosa*，*Proteus*，*Staphylococci* など，健康を促進するものとして *Lactobacilli*，*Eubacteria*，*Blfidobacterium* などがあります．特にヒトの共生生物としてのバクテリアは免疫系における恒常性の維持に役立っています．腸内細菌によって防御されている免疫システムは，IgA を介して病原菌を破壊する働きを有しています．

もともとプロバイオティクスというのはギリシア語で，「生命の益になるもの」という意味で，「生命の害になるもの」というアン

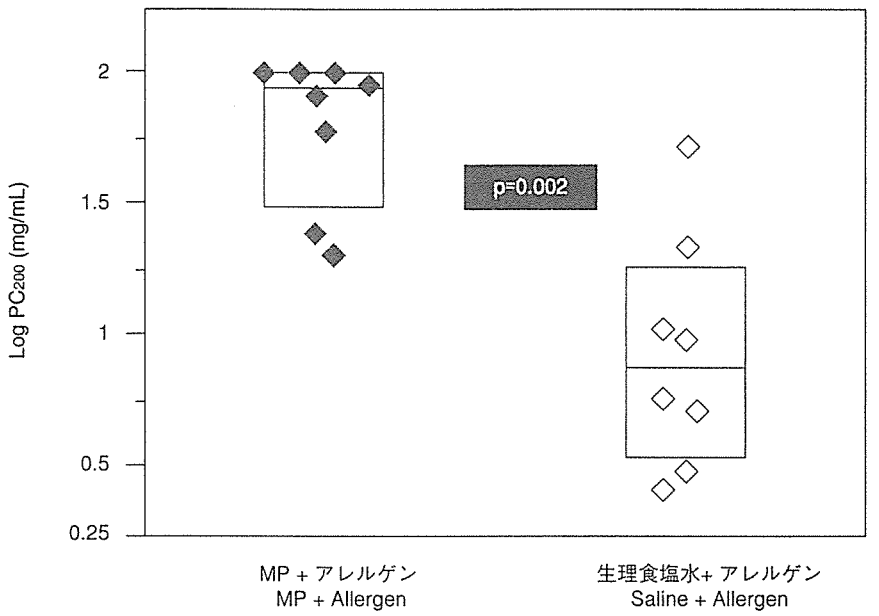


図 4　BHR 測定値におよぼす *M. pneumoniae* 感染の影響

M. pneumoniae (MP) 感染，あるいは食塩処理後にアレルゲン (OVA) 感作・誘発を行ったマウスにおけるエアロゾル化メタコリン誘発試験の BHR 測定値．PC_{200}が低いほど BHR が高いことを意味する．図内の長方形は，25 - 75の百分位を意味し，ボックス内の水平線は中央値を示す．MP ＋アレルゲンおよび生理食塩水＋アレルゲンは，それぞれ n=8である．

Fig. 4　The effects of *M. pneumoniae* infection on BHR measurement

Measurement of BHR to aerosolized-methacholine challenge in mice which were infected with *M. pneumoniae* (MP) or treated with saline followed by allergen (OVA) sensitizations and challenges. Lower values of log PC_{200} represent greater BHR. The vertical boxes indicate 25th to 75th percentiles, and the horizontal lines in the boxes represent median values. n=8 for MP ＋ Allergen and Saline ＋ Allergen groups.

（文献4より引用）

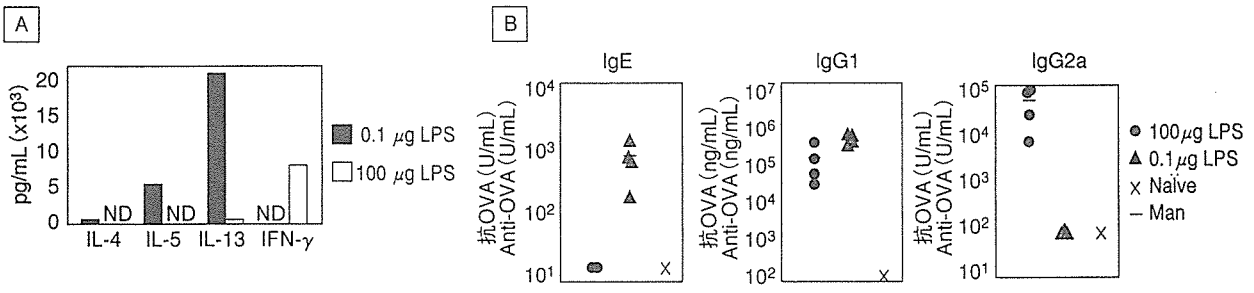


図 5　サイトカインおよび抗体産生におよぼす LPS の影響

(A) 低用量 (色付き棒) と高用量 (中抜き棒) の LPS グループにおける肺排出 LN からのサイトカイン生産．4つのうち代表的な実験を1つ示す．ND は検出限界以下を示す．(B) 低用量 (△) 高用量 (○) LPS グループの血清抗体と，未処理の BALB/c マウス (×) からのプールされた血清との比較．線は平均を示す．IgG1, IgE, IgG2a 反応に関して，$p < 0.05$ (LPS 高用量 vs. 低用量)．

Fig. 5　The effects of LPS on the production of cytokine and antibody

(A) Cytokine production from lung draining LNs in low (solid bars) and high (open bars) dose LPS gropes. One representative experiment of four is shown. ND, not detectable. (B) Serum antibodies of low (△) and high (○) dose LPS groups are compared with pooled sera from naive BALB/c mice (×). Line depicts the mean. $p < 0.05$ (LPS high vs. low dose) for IgG1, IgE, and IgG2a responses.

（文献 5 より引用）

Duke University Medical Center
Division of Pulmonary, Allergy and Critical Care Medicine
Associate Professor of Medicine **Monica Kraft**

チバイオティクスと対峙されて用いられる言葉なのです．しかし，プロバイオティクスもアンチバイオティクスも宿主を疾病から防御する点では同じですが，前者が共生的で，後者が抗生的であることころが違います(図 8)．

Fuller の定義によると，プロバイオティクスとは，「腸内フローラバランスを改善することにより動物に有益な効果をもたらす生菌添加物」を指します．しかし，現在ではプロバイオティクスの定義はより幅広くなり，「十分量が摂食または局所的に塗布されたときに，その消費者に有益な利益を与える"生きた"微生物」とされています．

日本で使用されているプロバイオティクスには，乳酸菌，ビフィズス菌などがあります．最近，これらのプロバイオティクスが，腸管において病原菌感染を防ぐ働きをしたり，免疫系を改善したり，がん予防をするのではないか，といわれていますよね．

近藤

なるほど．どの程度までデータは示されているのでしょうか．疫学的データはございますか．

上野川

アレルギー罹患率の高いスウェーデンと比較的低いエストニアの子どもを対象として行われた興味深い疫学調査の結果があります． Björksten 教授らスウェーデンの研究者が行った調査[6]ですが，それによるとアレルギーの小児の腸内フローラにはビフィズス菌や乳酸桿菌が少ないのです(図 9, 10)．

近藤

Kraft 先生は，このプロバイオティクスについてはご存知ですか．

Kraft

ええ，知っています．まだ研究途上の分野だとは思いますが，私を含め，米国の研究者も非常に興味をもっています．プロバイオティクスを利用した食事療法によって，ヒト腸内のフローラ環境が変わるというデータを目にしたことがあります．喘息に関する小規模研究において，喘息に対するプロバイオティクスの良好な効果を示唆する結果が得られています．遠くない将来，喘息に関しても大規模な研究が行われるのではないでしょうか．とても楽しみですね．

近藤

「プロバイオティクス」がアレルギー予防に働くという考えは，さきほどから話題にしている衛生仮説を考える上でも非常に重要だと思いますが，アレルギー疾患においては実際にどのような報告がされているのでしょうか．

上野川

まず，1998年にわれわれのグループが食物アレルギーモデルマウスを用いて，乳酸菌が血清中の IgE， IgG を抑制し，全身性のアナフィラキシー症状を抑制したという報告を行いました(図 11)[7]．

その後，2002年に Kalliomaki 博士らフィンランドのグループが，妊娠中または授乳中の母親とその子供(生後6ヵ月まで)に乳酸菌を与えると，生後2年間にわたって湿疹症状が減少することを示しました(図 12)[8]．

Kraft

メカニズムとしてはどのようなものが考えられますか．

上野川

これはあくまで仮説ですが，乳酸菌やビフィズス菌といったグラム陽性菌の細胞壁にあるリポタイコ酸，ペプチドグリカンを介しての作用だと思います．これらの菌が免疫細胞と接触した場合に，まずこれらの物質が抗原提示細胞上の TLR2を介してシグナルを伝達して Th1細胞を誘導し，アレルギー反応を抑制するのではないでしょうか(図 13)．

近藤

大変興味深い報告ですね．プロバイオティクスのアレルギー抑制の機序として，周産期や幼少時のこれらの刺激やその成

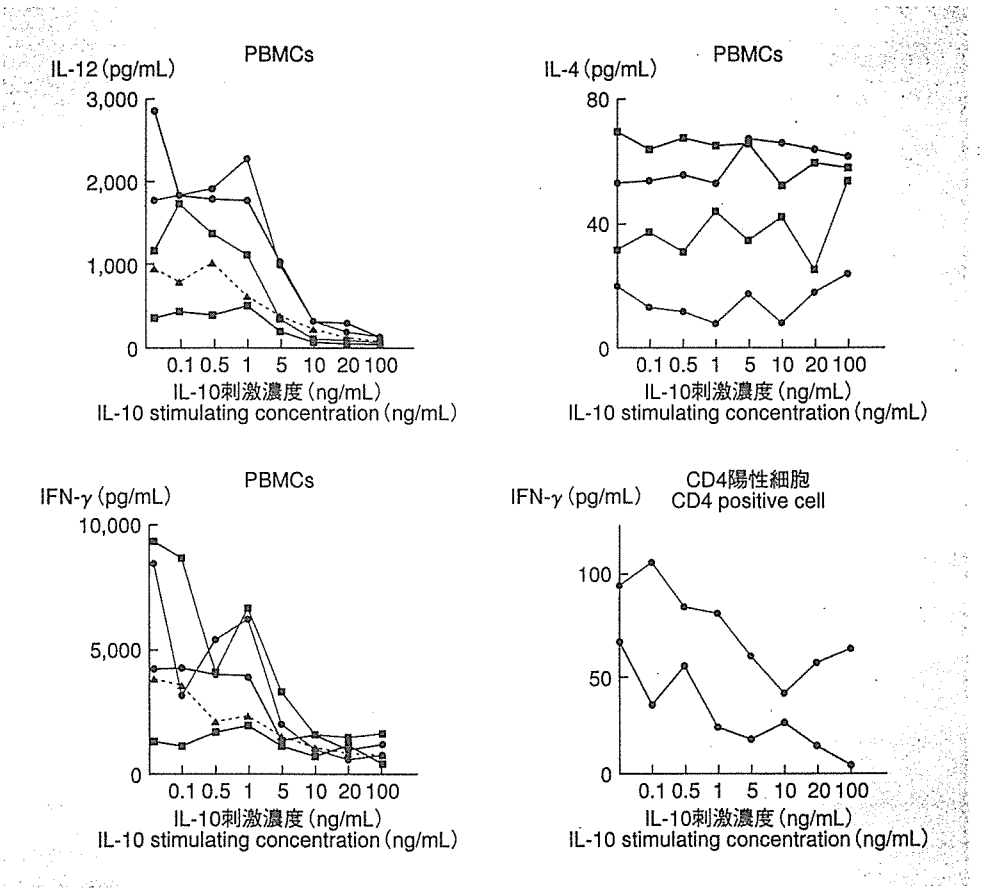


図6 IL-10の役割
Fig. 6 The roles of IL-10
IL-10は *in vitro* において強発現させたTh1系サイトカインであるIL-12, IFN-γに対して抑制的に働く.
IL-10 has suppressive effects on over expressed Th1 cytokines, IL-12 and IFN-γ *in vitro*.
(Asthma Frontier 2 : 21, 2003)

Kaminogawa

It means the so-called "goodies." The microflora in the colon (**Fig. 7**) play an important role in both human health and diseases. For example, *Ps. aeruginosa*, *Proteus*, and *Staphylococci* are pathogenic bacteria that have harmful effects and *Lactobacilli*, *Eubacteria*, and *Bifidobacteria* enhance human health. In particular, bacteria that live as symbionts to humans play a role in the maintenance of the homeostasis of the immune system. An immune system that is protected by enterobacteria can destroy pathogenic bacteria through IgA.

The word "probiotics" originates from a Greek word that means "beneficial for life," and it is the antonym of "antibiotics" (harmful to life). Both probiotics and antibiotics protect the host from diseases but the former is symbiotic and the latter is antibiotic (**Fig. 8**).

In Fuller's definition, probiotics means "live bacterial additives that bring beneficial effects to animals by improving the balance of intestinal flora." But now the definition of probiotics has been widened to "live bacteria that bring beneficial effects to the consumer when sufficient amounts are ingested or topically applied."

Probiotics used in Japan include lactobacilli and bifidobacteria. These probiotics are believed to prevent infection by pathogenic bacteria, improve the immune system, and prevent cancer.

Kondo

I see. What kind of evidence do we have? Is there epidemiological data?

Kaminogawa

There is an interesting study that compared children in Sweden where the prevalence of asthma is high and in Estonia where the prevalence of asthma is low. This study was conducted by Björksten B and colleagues[6]. In this study, the numbers of bifidobacteria and lactobacilli were lower in children with allergy (**Fig. 9, 10**).

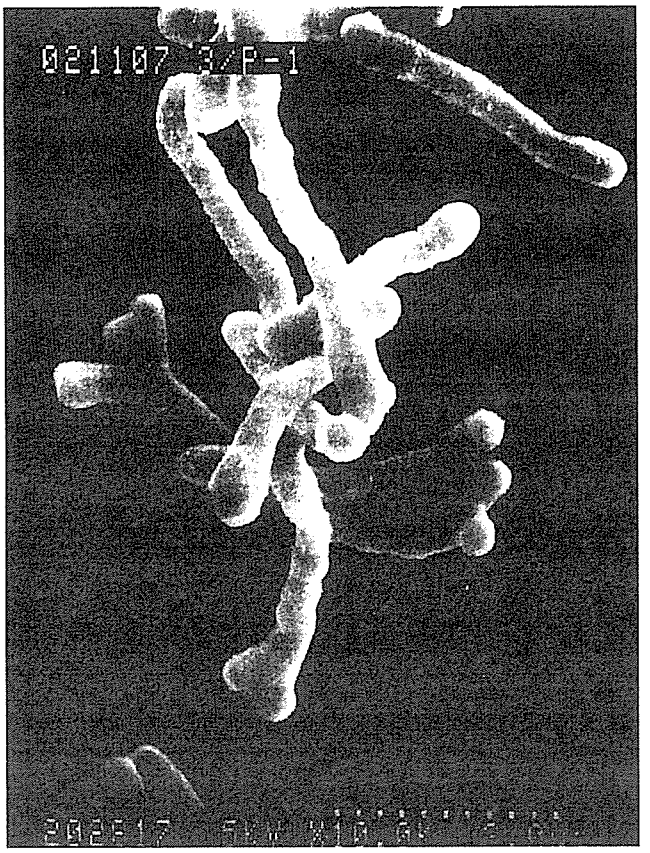


図7　大腸内のマイクロフローラ
Fig. 7　The micro flora in the colon
（上野川先生スライドより）

分の刺激が調節性（制禦性）T細胞などの発達を誘導し，その後のTh1/Th2バランスを良好にコントロールすることができるということも考えられると思います．

Kraft先生，こうした微生物の働きについてはいかが思われますか．

Kraft

バクテリアと喘息について，別の側面からでしたらコメントできます．

私は最近参加したシンポジウムで，マイコプラズマと喘息との関連について話をする機会がありました．PCR（ポリメラーゼ連鎖反応）で気道がマイコプラズマ陽性であった患者についてレトロスペクティブな調査を行ったところ，それらの患者では増悪の頻度が高い傾向にありました．また，気道に多くのマスト細胞が浸潤していました．

これは少し直感的に理解しにくい現象だと思います．なぜなら，幼少期に頻回に抗生物質を投与すると，アトピー発症が増加するのに対して，マイコプラズマに関しては，将来この微生物を抗生物質で治療するときが来るかもしれないのです．われわれは臨床試験を行いましたが，気道にマイコプラズマ感染が認められる患者においては，抗生物質を使用することにより喘息の改善がみられました．

上野川

確かにおっしゃるとおりです．ただ，さきほどKraft先生も，お話のなかで少ない量のLPSはTh2を誘導するとおっしゃいましたよね．そうすると少量のLPSではむしろアレルギー患者は増えてしまうということですね．

Kraft

確かにそうです．私も確立された喘息においてはLPSやエンドトキシンが喘息を増悪させるということについて日々考えを巡らせているのです．衛生仮説がもつパラドックスですね．

例えば，都市部のアレルギー性喘息患者は，空気中のアレルゲンだけでなく，大量のエンドトキシンに曝露されているわけです．こうした状況下では，エンドトキシンによりアレルギー症状が緩和されるといったメリットはみられません．アレルゲンやエンドトキシンだけでなく，他の多くの因子が複雑に絡み合い，わかりにくくなっているのかもしれません．

喘息の発症に関しては明確なデータが得られているものの，すでに確立されている喘息における衛生仮説の矛盾については，これから解き明かしていかなければならない部分だと思います．

上野川

私自身，衛生仮説は今後とも非常に重要だと思います．衛生仮説がなかったなら，プロバイオティクスの治療への応用という発想は生まれてこなかったでしょうからね．

最後に
～衛生仮説に残された課題と読者へのメッセージ～

近藤

衛生仮説を支持するデータの話から，衛生仮説を応用した治療の話に移ったところで，衛生仮説のはらむ矛盾点に話が落ち着きました．本当にぐるっと，一周した感じですね．ここで最後に，お一人ずつ本日のテーマについて，読者へのメッセージとして，一言コメントいただければ幸いです．

上野川

衛生仮説がいまだ「仮説」に過ぎないことはわかっています．例えば，長い間，環境中バクテリアと免疫系の相互作用につ

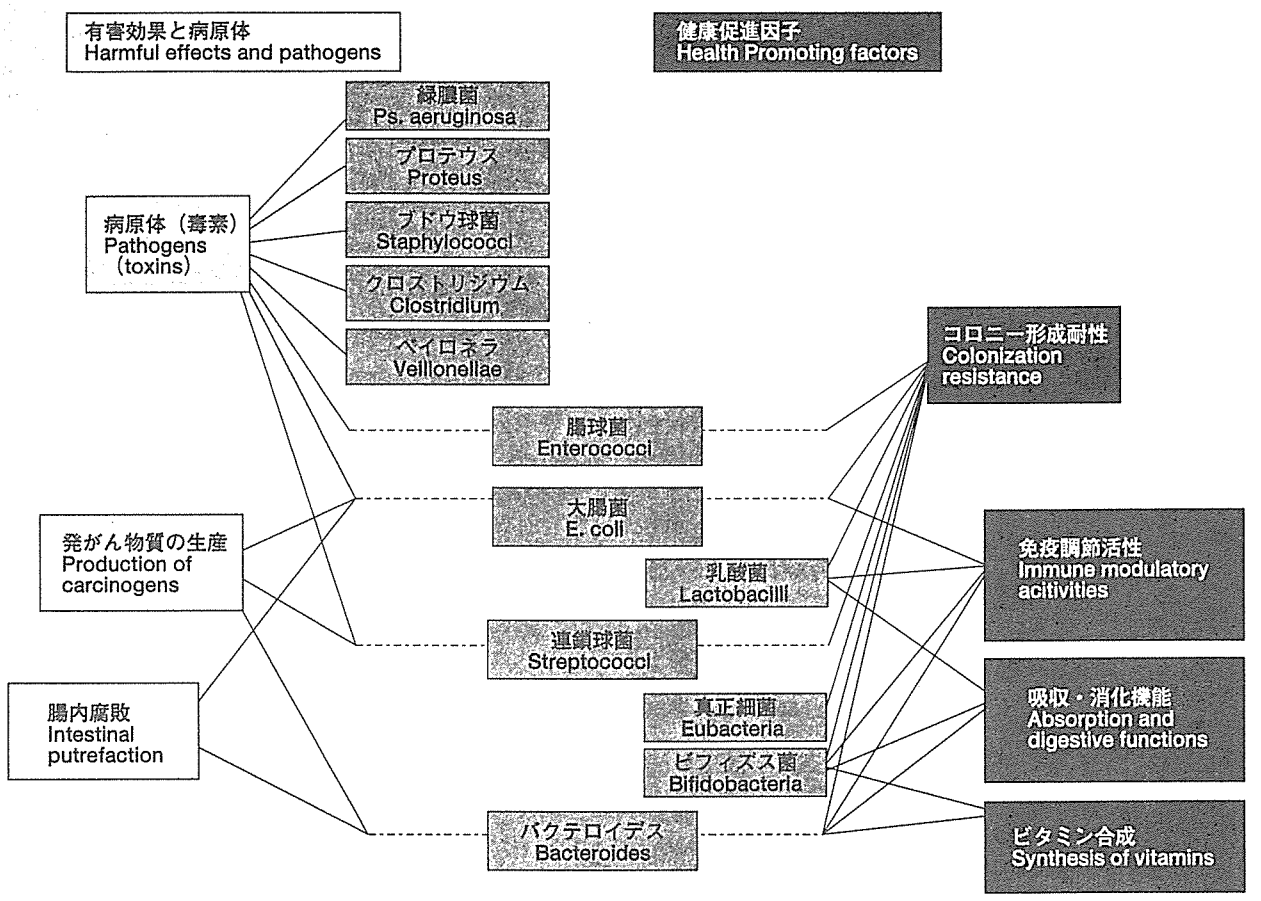


図 8　ヒトの微生物叢組成とヒトの健康への影響
Fig. 8　Composition of human microbiota and effects on man health

(Roberfroid, et al ：1995)

Kondo

Dr. Kraft, do you know about probiotics?

Kraft

Yes, I do. Although this field is still in its infancy, many American scientists, including me, are very interested in this area. I have seen a study that showed that alimentary therapy using probiotics changes the flora in the human intestine. A small study in asthma suggested a favorable effect of probiotics in asthma. Large-scale studies may be conducted on asthma as well in the near future. It would be very interesting.

Kondo

The concept that "probiotics" can prevent allergy is also important for understanding the hygiene hypothesis. What studies have been conducted on allergic diseases?

Kaminogawa

First, in 1998, we showed that lactobacilli suppressed serum IgE/IgG and systemic anaphylactic symptoms using a food allergy mouse model (Fig. 11)[7].

Then, in 2002, a Finnish group led by Kalliomaki M showed that giving lactobacilli to pregnant or lactating mothers and infants (up to 6 months old) reduced eczema symptoms in the first 2 years of life (Fig. 12)[8].

Kraft

What mechanism do you think, are behind these phenomena?

Kaminogawa

This is just a hypothesis, but I think it is through the lipoteichoic acid and peptide glycan that exist in the cell walls of gram-positive bacteria such as lactobacilli and bifidobacteria. I think that when these bacteria meet immune cells, first these materials transmit the signal

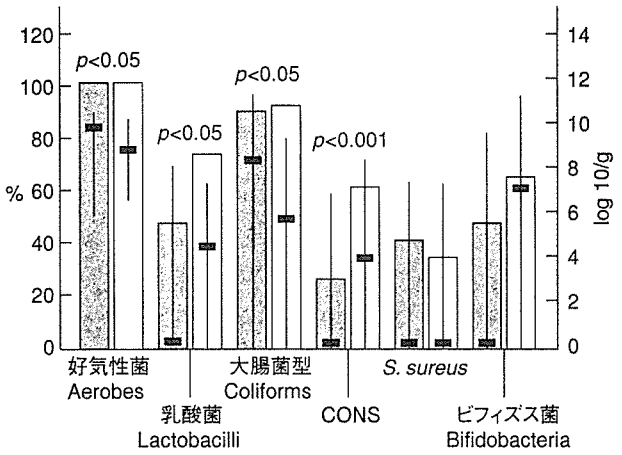


図9　エストニアの小児アレルギー患者13人(グレー)と非アレルギー児童16人(白)における腸内マイクロフローラ
結果は，コロニー形成率(%，カラム)と数(log CFU/g，範囲と中央値，線と色付きシンボル)で示してある．

Fig. 9　Intestinal microflora of 13 allergic (grey) and 16 nonallergic (white) Estonian children
The results are presented as colonization rate (% , columns) and counts (log CFU/g, range and median, lines and filled symbols)

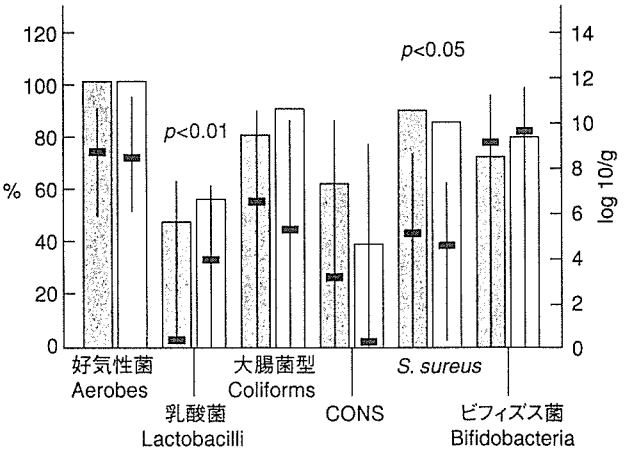


図10　スウェーデンの小児アレルギー患者14人(グレー)と非アレルギー児童19人(白)における腸内マイクロフローラ
Fig. 10　Intestinal microflora of 14 allergic (grey) and 19 nonallergic (white) Swedish children
結果は，コロニー形成率(%，カラム)と数(log CFU/g，範囲と中央値，線と色付きシンボル)で示してある．
The results are presented as colonization rate (% , columns) and counts (log CFU/g, range and median, lines and filled symbols).

いては不明なところが多かったと思います．しかし，最近では両者に強い相互作用があることが明らかとなっています．ですから体内の免疫的バランスは，多くのバクテリア感染を通じることにより確立されていると考えられます．そして現在は，両者

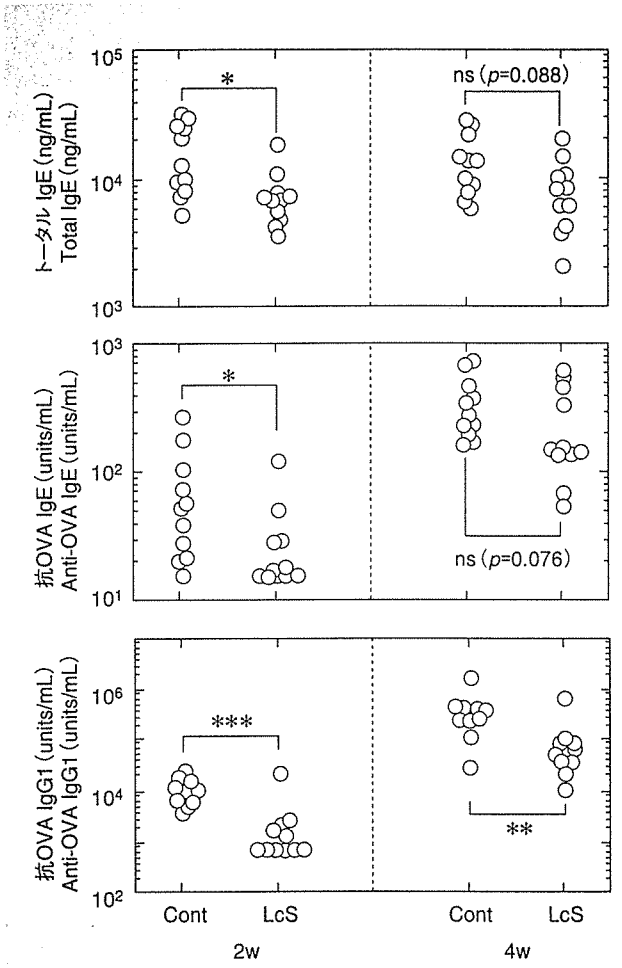


図 11　LcS 投与による血清抗体反応の阻害
Fig. 11　Inhibition of serum antibody responses by LcS administration
OVA-TCR-Tg マウスに OVA 飼料を4週間与え，intraperitoneally により200 μg の LcS あるいは生理食塩水(コントロール)を3回投与(1，3，5日目)した．血清中のトータル IgE，OVA 特異的 IgE，OVA 特異的 IgG1レベルを2週目と4週目に ELISA 法で測定した．個々のマウスの値を示してある(n=11)．*p < 0.05; **p <0.01; ***p <0.001, Mann-Whitney *U*-test で計算．
OVA-TCR-Tg mice were fed an OVA diet for 4 weeks, and 200 μg LcS or saline (Cont) was administered intraporitenoally three times (on days 1,3and5). The serum levels of total IgE, OVA-specific IgE and OVA-specific IgG1 at week 2 and week 4 were determined by ELISA. The values for individual mice (n=11) are expressed. *p <0.05; **p < 0.01; ***p <0.001, calculated by Mann-Whitney *U*-test.
(文献7より引用)

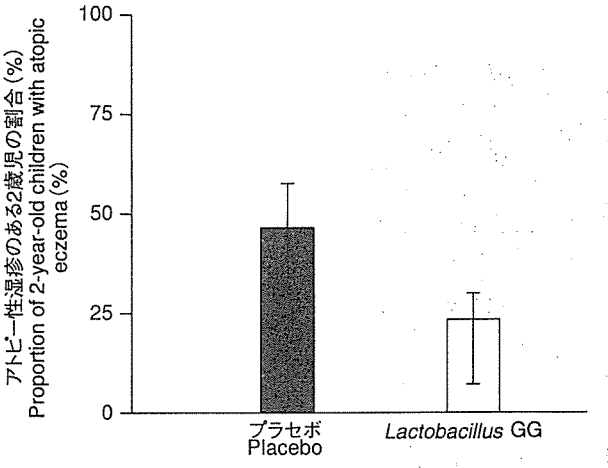


図 12　*Lactobacillus* GG のアトピー性疾患に対する治療効果
Fig. 12　Treatment effect of *Lactobacillus* GG on atopic disease
棒は95%信頼区間を示す．
Bars are 95% CI.
(文献8より引用)

through TLR2 on antigen-presenting cells, resulting in induction of Th1 cells and suppression of allergic reactions. (Fig. 13).

Kondo

That is a very interesting study. It is also reasonable to consider that, as a mechanism of allergy suppression using probiotics, exposure to these stimuli and stimuli from their components in the perinatal/neonatal period induces the development of regulatory T-cells, leading to better control of the balance between Th1 and Th2.

Dr. Kraft, what do you think about the roles of these bacteria?

Kraft

I can comment from a different perspective regarding bacteria and asthma.

In a symposium that I participated in recently, I had the opportunity to discuss the relationship between mycoplasma and asthma. We have conducted a retrospective review of patients who tested positive for mycoplasma in their airways by PCR. We found that their symptoms tended to be exacerbated more frequently. They also had more mast cells in the airway.

I think this might be a bit counterintuitive because frequent antibiotic use may actually contribute to the development of atopy in the young. With regard to mycoplasma, we may see a day when we actually treat this organism using an antibiotic. We have performed a clinical trial and found improvement in asthma with antibiotics in those patients who demonstrated mycoplasma in their airways.

Kaminogawa

I agree with you. But I think that you said that low-dose LPS might induce Th2. That means that low-dose LPS increases allergy in patients.

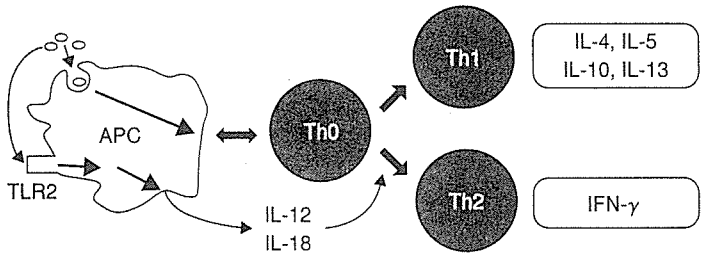


図 13　グラム陽性菌(乳酸菌，ビフィズス菌)
Fig. 13　Gram positive bacteria (lactobacillus, Bifidobacterium)
(上野川先生スライドより)

の相互作用は免疫細胞例の TLR と細胞成分との間で成立されることが明らかとなっています．その相互作用が崩れるとさまざまな病気になるのです．長い進化の間で，両者の相互関係は生きていく上で必須のものになっているのではないでしょうか．

Kraft

昔は，感染症は世界的にも主要な死因のひとつだったわけですが，衛生状態が発達し，抗生物質の使用が可能な現代においては，状況が異なります．しかしながら，衛生状態の改善により，アレルギー性疾患は増えてしまったのです．早期感染，免疫システムの発達，アレルギー性疾患の発症の相互作用に関しては，学ばなければならない点が多いと思います．

近藤

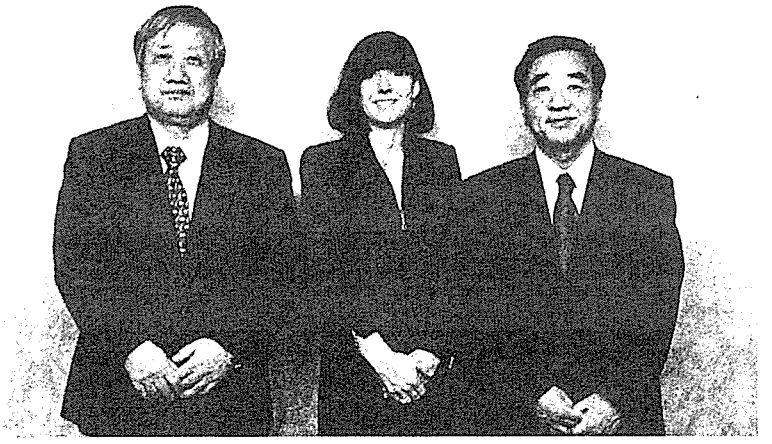
まだまだ衛生仮説に残された課題は多いようですが，先にご紹介いただいたプロバイオティクスの応用や CpG DNA 療法，減感作療法など，この理論を利用した最新の治療法についての研究もさかんに行われるようになってきており，今後どのような知見がこの分野で得られるのか，非常に楽しみです．

衛生仮説が広まるにつれ，不潔な食物と農村へ引っ越すことが喘息などアレルギー疾患の予防のためにはよいのでは，という意見も出てくるようになりました．これに対し，今日のお話を含めて衛生仮説が意味するところは，人間が改めて不潔な環境に後戻りすることを推奨するものではなく，人間が生を受けた本来の原点に戻って，良好な地球規模的自然環境の中で生きることの重要性を示唆しているものということを強調して，終わりの言葉に代えさせていただきたいと思います．

今日は，衛生仮説の成り立ちから， pros and cons，そして最新治療への応用に至るまで，幅広いお話をいただけたと思います． Kraft 先生，上野川先生，本日はどうもありがとうございました．

Kraft・上野川

ありがとうございました．



Kraft

Indeed. I am also thinking about some of the paradoxes that we see with the hygiene hypothesis in established asthma, LPS or endotoxin can make asthma worse.

For example, asthma patients with allergy in inner cities have exposure not only to allergens, but also to a lot of endotoxin. Yet they don't seem to be deriving much benefit from the endotoxin exposure. Many factors other than allergens and endotoxins are also involved which may confound this observation.

So, although we have more definitive information in terms of the development of asthma, we need to elucidate the paradoxes of the hygiene hypothesis in established asthma in future studies.

Kaminogawa

I think that the hygiene hypothesis remains an important issue. If it had not been for the hygiene hypothesis, the idea of applying probiotics for treatment would never have come along.

FINALLY -FUTURE ISSUES OF HYGIENE HYPOTHESIS AND MESSAGES FOR READERS-

Kondo

We have talked about evidence that supports the hygiene hypothesis, then treatment that utilizes the hygiene hypothesis where we found paradoxes of the hygiene hypothesis. It is really like coming full circle. Finally, I would appreciate it if each of you could make a comment on today's topics as a message for the readers.

Kaminogawa

I understand that the hygiene hypothesis is still just a hypothesis. For example, for a long time, the relationship between environmental bacteria and the immune system was not very clear. But recently, it was shown that these two strongly interact. Therefore, it is feasible to think that the immunological balance of the body is established by various bacterial infections. And we now know that the interaction is established between the TLR of the immune cells and cell constituents. When the interaction loses its balance, we get various diseases. It is probably during the long history of evolution that this interaction became essential.

Kraft

There was a time when infectious diseases were the major cause of death throughout the world. This is not longer true thanks to improvement in hygiene status and the use of antibiotics. However, now we are faced with more allergic disease because of the hygenie conditions. I think we have much to learn about the interaction between early infection, the development of the immune system and the presentation of allergic disease.

Kondo

It seems that we still have a lot to learn about hygiene hypothesis. Applications of hygiene hypothesis to treatment such as the aforementioned probiotics, CpG DNA therapy, and desensitization therapy are being vigorously studied. I am very interested in seeing the results of these studies.

As hygiene hypothesis diffuses, some people start to believe that a dirty environment and moving to a rural area may be good for the prevention of allergic diseases such as asthma. I would like to finish my talk by emphasizing that including today's comments, what hygiene hypothesis suggests is the importance of going back to the origin of the human species and living in a good natural global environment, rather than returning to a dirty environment.

Today, I think that we covered a wide variety of aspects of hygiene hypothesis, history, pros and cons, and application to the latest treatment. Dr. Kraft, Professor Kaminogawa, thank you very much.

Kraft · Kaminogawa

Thank you very much.

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ORIGINAL ARTICLE

IL-12B Promoter Polymorphism Associated with Asthma and *IL-12B* Transcriptional Activity

Koji Tatebayashi[1], Eiko Matsui[1], Hideo Kaneko[1], Toshiyuki Fukao[1], Kimiko Kasahara[1] and Naomi Kondo[1]

ABSTRACT

Background: The interleukin-12B gene (*IL-12B*) encodes the p40 chain of interleukin-12 (IL-12), which promotes cell-mediated Th1 responses and the production of interferon-gamma (IFN-γ) that downregulates IgE production. Chromosome 5q31-q33 near the *IL-12B* locus is significantly linked to asthma, as determined by a genome-wide search in the Japanese population.

Methods: We sequenced exons 1-8 including parts of the introns and promoter region of *IL-12B* in asthmatic patients and healthy controls. We examined plasma IL-12 concentrations, IL-12 production by Derf1-stimulated peripheral blood mononuclear cells (PBMCs) and the *IL-12B* transcriptional activity.

Results: *IL-12B* promoter polymorphism existed as $^{-2703}$CTCTAA/GC and $^{-2403}$T/C alleles, which were linked to each other. Homozygosity for haplotype 1 ($^{-2703}$CTCTAA /$^{-2403}$T) was associated with asthma susceptibility in Japanese children ($P < 0.001$). Both plasma IL-12 concentrations and IL-12 production by Derf1-stimulated PBMCs in the subjects with homozygotes for haplotype 1 were lower than those with homozygotes for haplotype 2 ($^{-2703}$GC /$^{-2403}$C) ($P < 0.001$). The transcriptional activity of the construct with haplotype 1 was lower than that of the construct with haplotype 2, and the *IL-12B* transcriptional activity was influenced by the $^{-2403}$T/C allele rather than by the $^{-2703}$CTCTAA/GC allele.

Conclusions: Homozygosity for haplotype 1, which is associated with reduced *IL-12B* transcriptional activity and reduced IL-12 production, is a predisposing factor for asthma susceptibility in Japanese children.

KEY WORDS

asthma, IgE, interferon-gamma, interleukin-12B promoter polymorphism

INTRODUCTION

Interleukin-12 (IL-12) is a heterodimeric molecule that is composed of two disulfide-linked subunits, p35 and p40. It is produced by macrophages, B cells and other antigen-presenting cells (APCs),[1,2] and plays important roles in interferon-gamma (IFN-γ) production by T cells and natural killer (NK) cells.

Genome-wide linkage screens, in which the genetic factors of the diseases can be identified, have been performed for asthma and recognized many regions linked to asthma.[3] Asthma is associated with Th2 cytokines, such as IL-4, IL-5, IL-9, IL-13, which are mapped to chromosome 5q31-q33. Polymorphisms of the IL-4 receptor α chain and IL-13 are associated with asthma.[4-6] Yokouchi *et al.* have reported significant evidence for linkage of asthma to 5 q31-33 near the *IL-12B* locus but not the IL-4 and IL-13 loci in the Japanese population.[7] Therefore, *IL-12B* is one of the candidate genes for asthma. Several polymorphisms have been identified in *IL-12B*,[8,9] including a single-nucleotide polymorphism in the 3' untranslated region, which has been associated with the susceptibility to type 1 diabetes and atopic dermatitis[10,11] but not to asthma and allergic rhinitis in the Japanese population.[12] Recently, it has been reported that the polymorphism exists in the *IL-12B* promoter region.[13,14]

In this study, we sequenced exons 1-8 including parts of the introns and region 3 kb upstream from

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Received 10 November 2004. Accepted for publication 26 January 2005.

Table 1 Sequence of oligonucleotides for PCR.

Primer	Sequence	Primer	Sequence	position
1S	5'-GAGAAGCATTCAGAAGCTCT-3'	1A	5'-GTCCCACTTCACAATCCAGA-3'	promoter
2S	5'-GTTTGTCAGCAGACCTTCCT-3'	2A	5'-GGAACAGGGCTCTGAATTGT-3'	promoter
3S	5'-GACAAGTGATTTCACTGCGG-3'	3A	5'-GGGCTAGTCCTATATGAAAG-3'	promoter
4S	5'-GGTATCCAGCTCTCTAACTC-3'	4A	5'-GACTTTGCCTTTTAGCCTTC-3'	promoter
5S	5'-GCAATCTGCTTTGTCCACTT-3'	5A	5'-GCTAAGAGGTATGCAAAGGT-3'	promoter
6S	5'-GCAGGTACATGTTCCTGTTC-3'	6A	5'-GGTTCTTCCCAAGTCAGAGA-3'	promoter
7S	5'-GCCAAGATGGGTGGTAAATA-3'	7A	5'-GAGGAGGGAACATAGACATC-3'	promoter
8S	5'-GCATCTCCATCTCCTTCCTT-3'	8A	5'-GCACACTAACGGTTTCTACA-3'	exon1
9S	5'-GGCTTAAAGGGGCCAAGT-3'	9A	5'-AGGGAGCACTATCCCTCAGC-3'	intron1-1
10S	5'-ATGTTATCTCATTGCCTTTC-3'	10A	5'-AAGTGGTTCTGAAACCACTG-3'	intron1-2
11S	5'-GTATCAGATGGCTTGCCTTA-3'	11A	5'-GTGCATGGTTGCCCATTTCA-3'	exon2
12S	5'-GGGAAGACTAAGCTCTACTG-3'	12A	5'-CAACGAACCAAGACTGTCAT-3'	intron2
13S	5'-GTCTTGTGCTGTTTGCAGTT-3'	13A	5'-GCATCTCCAAACTCTTTGAC-3'	exon3-1
14S	5'-GTGACACCCCTGAAGAAGAT-3'	14A	5'-GAGGCTAAGCATTCAGACTG-3'	exon3-2
15S	5'-GATAGTGTATCACTCTGCAC-3'	15A	5'-GCTGAGAAACCAGAGCAGTT-3'	exon4
16S	5'-TACTTCTGCTGACACCACTA-3'	16A	5'-GAACTAGGATCAAATTGTATAC-3'	intron4-1
17S	5'-GGTTACATAATCATATGTA-3'	17A	5'-GTTAGGATTTCAGGTGTGAG-3'	intron4-2
18S	5'-TCCAGAGACATGTAAGTGC-3'	18A	5'-GAGATGATGCTTGTCAACCA-3'	exon5
19S	5'-GCATCTCTCAGATCCTGCAA-3'	19A	5'-GCACCTGAATCACTTCTTAC-3'	exon6
20S	5'-GCTAGAAAGATGAAAGCTGG-3'	20A	5'-GTTTCTGATTCTGGCAACTG-3'	exon7
21S	5'-TAGCTCATCTTGGAGCGAAT-3'	21A	5'-GCTTGCCAGAGGCTTTCTTG-3'	intron7
22S	5'-GCAAGCTTGCAGGACTCAGA-3'	22A	5'-GATGGATCAGGTCATAAGAG-3'	exon8-1
23S	5'-GCCAGGATGTATGGAATGTT-3'	23A	5'-GACAGGGTCTCATTCTGTCA-3'	exon8-2
24S	5'-GCCTAGGTGACAGAATGAGA-3'	24A	5'-GCAAGCAGAGTACTCAAATC-3'	exon8-3

the transcriptional start site of *IL-12B* in 30 patients. Furthermore, we investigated *IL-12B* promoter polymorphism in 111 asthmatic patients and 78 controls, and examined the relationship between *IL-12B* polymorphism and asthma prevalence, IL-12 production and *IL-12B* transcriptional activity. We showed that *IL-12B* promoter polymorphism is associated with asthma and influences IL-12 production and *IL-12B* transcriptional activity.

METHODS

PATIENTS AND CONTROL SUBJECTS

One hundred and eleven asthmatic patients and 78 controls were enrolled in this study. Asthma was diagnosed on the basis of the American Thoracic Society guidelines. All the asthmatic patients showed total IgE levels above 200 IU/mL or specific sensitization to major allergens such as house dust and mite. The mean age ± SD of the asthmatic patients was 5.6 ± 2.9 years, and their mean IgE ± SD level was 906.8 ± 1347.4 IU/mL. All the controls had a negative history of atopic diseases. Their plasma IgE levels were lower than 150 IU/mL and their specific IgE levels were negative. The mean age ± SD of the controls was 4.7 ± 3.4 years, and their mean IgE ± SD level was 52.3 ± 52.4 IU/mL. Informed consent was obtained from all the subjects or their parents.

DETECTION OF *IL-12B* POLYMORPHISM

Genome DNA was extracted from neutrophils with a Sepa-gene kit (Sanko Junyaku, Tokyo, Japan). Exons 1-8 including parts of the introns and region 3 kb upstream from the transcriptional start site of *IL-12B* were amplified and sequenced using an ABI 3100 DNA sequencer (Applied Biosystems, CA, USA). We also sequenced previously identified polymorphisms in the introns.[9] The conditions for the PCR were 94 ℃ for 1 minute, 60℃ for 1 minute, and 72℃ for 1 minute. The primers used are shown in Table 1.

CELL PREPARATION

Peripheral blood mononuclear cells (PBMCs) were separated from the heparinized blood of the subjects by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden). PBMCs were suspended at a density of 1×10^6/mL in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

CELL CULTURE

PBMCs (1×10^6/mL) were cultured in the presence or absence of 5 IU/mL recombinant human IL-12 (R &D Systems, Inc, Wiesbaden, Germany) or 5 μg/mL Derf1 (Asahi, Tokyo, Japan) for 24 hours in a final volume of 1 mL in a round-bottom tube (Falcon 2059,

Becton Deckinson Labware, Franklin Lakes, NJ, USA) at 37℃ in a humidified atmosphere containing 5% CO_2.[15]

ASSAYS FOR CYTOKINES

Plasma samples isolated from heparinized blood and the supernatants of the cell culture were stored at -30℃ until assay. IL-12 concentrations in the plasma and the supernatants of the Derf1-stimulated cell culture were measured with a human IL-12+p40 ELISA kit (Bio Source International, CA, USA) ; the minimum detection limit was 7.81 pg/mL. This ELISA kit recognized both natural and recombinant human IL-12, as well as the free p40 subunit. IFN-γ concentrations in the supernatants of the IL-12-stimulated cell culture were measured with a human IFN-γ ELISA kit (Ohtsuka, Tokyo, Japan) ; the minimum detection limit was 15.6 pg/mL.[16]

IgE ASSAY

Plasma IgE levels were determined by chemiluminescent enzyme immunoassay. Specific IgE antibodies for house dust, mite, cedar pollen, cow's milk, and hen's egg were measured by using a Uni-Cap assay kit (Pharmacia, Uppsala, Sweden). A specific IgE level above 3.5 IU/mL was considered positive.

CELL LINES AND REAGENTS

Mouse macrophagic cell line RAW264 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

PLASMIDS FOR LUCIFERASE ASSAY

The -2808/-2303 distal enhancer region of *IL-12B* was obtained from genomic DNA by PCR amplification with the primers 5' -ttccggtcgACATGTTGATAA ACCTCTTCTCC-3' and 5' -ttgccggatcCGTAGCTCA CAAGGGGACATCAAAGATGAC-3' . The conditions for the PCR were 94℃ for 1 minute, 60℃ for 1 minute, and 72℃ for 1 minute. *Sal*I and *Bam*HI restriction endonuclease sites embedded in the PCR primers allowed the release of a 512-bp insert. The amplified PCR product was subcloned into the luciferase reporter plasmid PicaGene Promoter Vector 2 (PGV-P2 ; Toyo Ink Mfg. Co., Ltd., Tokyo, Japan). Furthermore, we constructed the mutant vector, which changed the T allele in the reporter vector with a 1.1 genotype into the C allele (mut-1). Circular DNA, which was the vector with a 1.1 genotype, was amplified using the sense primer 5' -GACAAGTGATTTCA CTGCGGGAAGACAATTCAGAGC-3' , and the antisense primer 5' -GCTCTGAATTGTCTTCCCGCAGT GAAATCACTTGTC-3' with native Pfu DNA polymerase (Stratagene, California, USA). These primers were oligonucleotides with one base mismatch (underline). The conditions for the PCR were 95℃ for 1 minute, 55℃ for 1 minute, and 68℃ for 10 minutes. The DNA sequence of PCR product was confirmed with an ABI 3100 DNA sequencer. We constructed another mutant vector, which changed the C allele in the reporter-vector with a 2.2 genotype into the T allele (mut-2) by the same method. The vector with a 2.2 genotype was amplified using the sense primer 5' -GACAAGTGATTTCACTGTGGGAAGACAATTCA GAGC-3' , and the anti-sense primer 5' -GCTCTGAAT TGTCTTCCCACAGTGAAATCACTTGTC-3' .

TRANSFECTION

The *IL-12B* promoter-reporter construct was transfected in RAW264. The cells were collected, resuspended at a concentration of 3×10^6 cells/10 ml in an 80 mm^2 flask, and incubated for 24 hours at 37℃ in a 5% CO_2 atmosphere. DNA (reporter construct 12 μg, CMV control vector 3 μg) was resuspended in 600 μL of OPTI-MEM (Gibco BRL, Grand Island, NY, USA) in a 15 mL Falcon tube, to which 15 μL of lipofectamine 2000 (Gibco BRL, Grand Island, NY, USA) was added ; the mixture was incubated for 20 minutes at 37℃ in a 5% CO_2 atmosphere. The cells were washed once, resuspended in 6 mL of DMEM (Gibco BRL, Grand Island, NY, USA) with 10% FCS and 600 μL of OPTI-MEM including the reporter construct in an 80 mm^2 flask, and incubated for 24 hours at 37℃ in a 5% CO_2 atmosphere. The cells were treated with recombinant murine IFN-γ (1,000 U/mL ; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 6 mL of the same medium for the stopping lipofectamine reaction for 24 hours before the addition of LPS (1 μg/mL) (Sigma, St. Louis, USA). After stimulation with LPS for 24 hours, cells were harvested and lysed by cell lysis buffer.[17] The lysates were used for luciferase assay with a PicaGene Dual SeaPansy Luminescence kit (Toyo Ink Mfg. Co., Ltd., Tokyo, Japan).

STATISTICAL ANALYSES

The significant difference between two groups was analyzed by the Mann-Whitney *U* test. The significant differences among three groups were analyzed by the Bonferroni/Dunn test. The frequency in *IL-12B* promoter polymorphism between the asthmatic patients and the controls was compared using the chi-square test for independence. Hardy-Weinberg equilibrium was determined by means of chi-square test for independence. Statistical significance was assumed for *p*-values less than 0.05.

RESULTS

DETECTION OF *IL-12B* POLYMORPHISMS

Exons 1-8 including parts of the introns and region 3 kb upstream from the transcriptional start site of *IL-12B* were amplified by PCR. Several polymorphisms have been reported in *IL-12B*.[8,9] We found three novel polymorphisms. The T/C allele (NP2) existed at -2403 from the transcriptional start site of *IL-12B*,

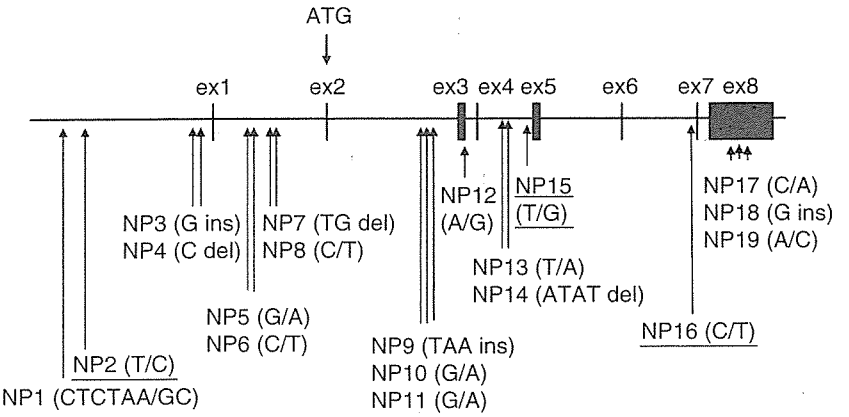

Fig. 1 Genomic organization of human *IL-12B*. By comparing the complete genomic sequence with the published cDNA sequence, the position of exons 1-8 and introns was deduced. Arrows indicate approximate positions of confirmed polymorphisms. NP2, 15 and 16 are novel polymorphisms (underlines). The other described NPs have been reported in the previous study. NP = nucleotide polymorphism

Table 2 Genotype frequencies of *IL-12B* promoter polymorphism in asthmatic patients and controls.

Genotype	Control (n=78)(%)	Asthmatic patient (n=111)(%)
1.1	15 (17.9%)	34 (30.6%)
1.2	34 (43.6%)	63 (56.8%)
2.2	29 (37.2%)	14 (12.6%)
P-value		<0.001

and the T/G (NP15) and C/T (NP16) alleles existed in introns 4 and 6 (Fig. 1). We investigated the frequency of *IL-12B* polymorphisms and plasma IL-12 concentrations in 30 subjects. Although the frequencies of all *IL-12B* polymorphisms were not significantly different between the allergic patients and the controls, plasma IL-12 concentrations in the subjects with the homozygotes for the $^{-2703}$CTCTAA/$^{-2403}$T allele (haplotype 1) were significantly different from those in the subjects with homozygotes for the $^{-2703}$GC/$^{-2403}$C allele (haplotype 2) in NP1 + 2 (data not shown). Therefore, we investigated that whether there is an association between NP1 + 2 and asthma, and whether NP1 + 2 affect IL-12 production.

ASSOCIATION BETWEEN *IL-12B* PROMOTER POLYMORPHISM AND ASTHMA

NP1 existed at −2703 from the transcriptional start site in *IL-12B*. We also identified NP2 at −2403 from the transcriptional start site, and this polymorphism was linked to NP1. We designated the homozygotes for haplotype 1, heterozygotes, and homozygotes for haplotype 2 as genotypes 1.1, 1.2, and 2.2, respectively. The frequencies of *IL-12B* promoter polymorphisms in 189 samples are shown in Table 2. Allele frequency of *IL-12B* promoter polymorphism did not deviate from expected Hardy-Weinberg equilibrium ($P > 0.1$). The frequency of a 1.1 genotype in the asthmatic patients was significantly higher than that in the controls ($P < 0.001$).

PLASMA IL-12 CONCENTRATIONS IN THE CONTROLS AND ASTHMATIC PATIENTS

As shown in Figure 2a, plasma IL-12 concentrations in the asthmatic patients were significantly lower than those in the controls. The mean ± SD of plasma IL-12 concentrations in the asthmatic patients and controls were 136.8 ± 62.0 pg/mL and 232.0 ± 84.0 pg/mL ($P < 0.001$).

ASSOCIATION BETWEEN *IL-12B* PROMOTER POLYMORPHISM AND PLASMA IL-12 CONCENTRATIONS

We examined plasma IL-12 concentrations in each genotype in both the controls and asthmatic patients. Plasma IL-12 concentrations in the subjects with a 1.1 genotype were 153.1 ± 70.0 pg/mL, which were significantly lower than those in the subjects with a 2.2 genotype (218.6 ± 85.1 pg/mL) ($P < 0.001$; Fig. 2b). Since reduced IL-12 production in the asthmatic patients might be affected by many factors such as some genetic effects and environmental factors, we also examined plasma IL-12 concentrations in each genotype only in the controls. Plasma IL-12 concentrations in the controls with a 1.1 genotype were 205.2 ± 63.8 pg/mL, which were significantly lower than those in the controls with a 2.2 genotype (255.8 ± 70.8 pg/mL) ($P < 0.05$; Fig. 2c).

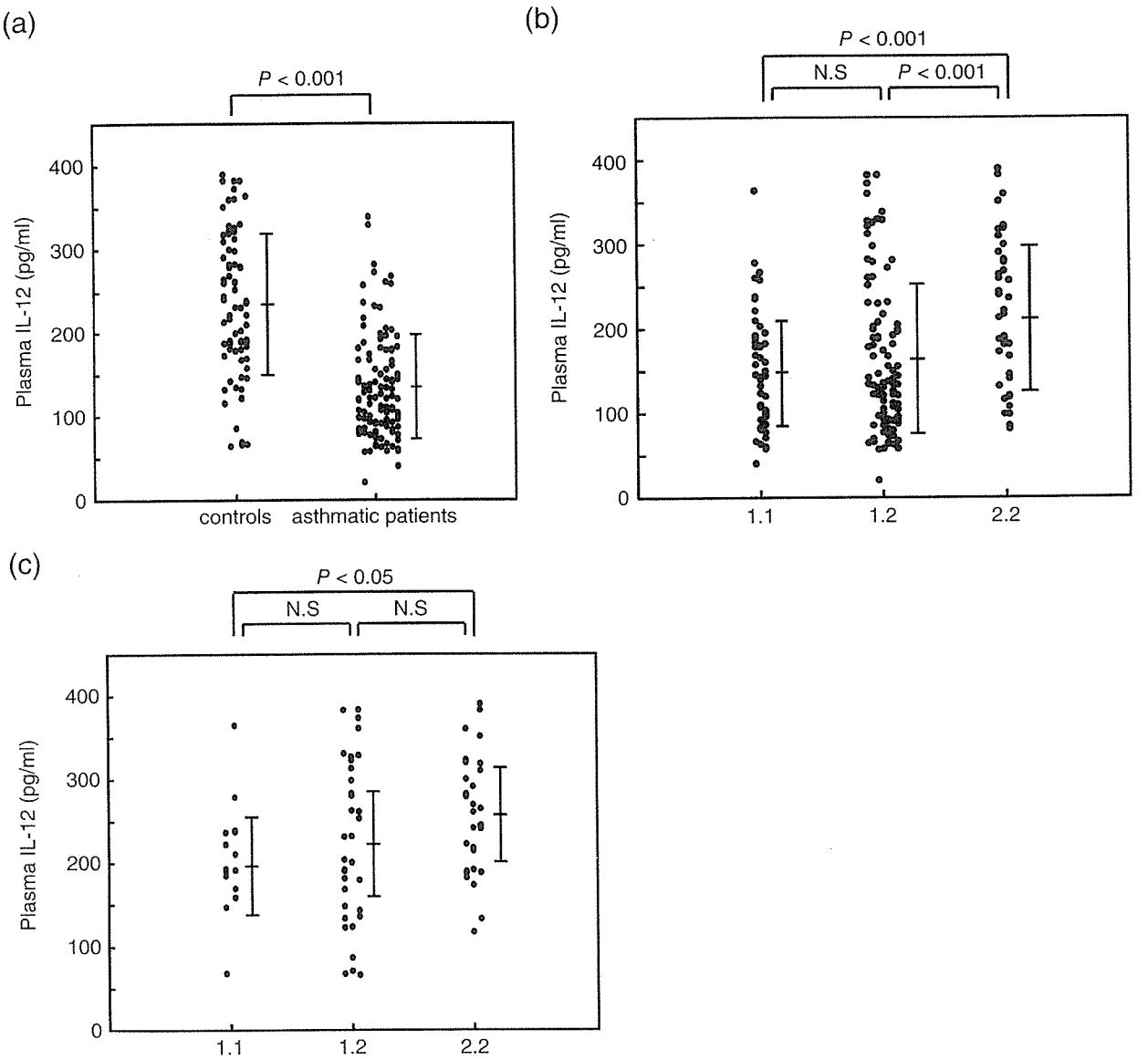

Fig. 2 (**a**) Plasma IL-12 concentrations in asthmatic patients and controls. The plasma IL-12 concentrations in the asthmatic patients were significantly lower than those in the controls (P<0.001). (**b**) Relationship between *IL-12B* promoter polymorphism and plasma IL-12 concentrations in both the controls and asthmatic patients (n=189). The plasma IL-12 concentrations in the subjects with a 1.1 genotype were significantly lower than those in the subjects with a 2.2 genotype (P<0.001). (**c**) Relationship between *IL-12B* promoter polymorphism and plasma IL-12 concentrations in the controls (n=78). The plasma IL-12 concentrations in the controls with a 1.1 genotype were significantly lower than those in the controls with a 2.2 genotype (P<0.05).

IL-12 PRODUCTION BY DERF1-STIMULATED PBMCS IN THE CONTROLS AND ASTHMATIC PATIENTS

Since most asthmatic patients had positive CAP-RAST scores for D farinae (Derf1), their PBMCs were cultured with the specific antigen Derf1 for 24 hours. IL-12 production by Derf1-stimulated PBMCs in the asthmatic patients (378.0 ± 271.4 pg/mL) was significantly lower than that in the controls (663.0 ± 364.2 pg/mL) (P < 0.001 ; Fig. 3a).

ASSOCIATION BETWEEN *IL-12B* PROMOTER POLYMORPHISM AND IL-12 PRODUCTION BY DERF1-STIMULATED PBMCS

We examined IL-12 production by Derf1-stimulated PBMCs in each genotype in both the controls and asthmatic patients. The subjects with a 1.1 genotype (347.2 ± 229.2 pg/mL) had a lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype (690.2 ± 331.1 pg/mL) (P < 0.001 ; Fig. 3b). In the controls, the subjects with a 1.1 genotype

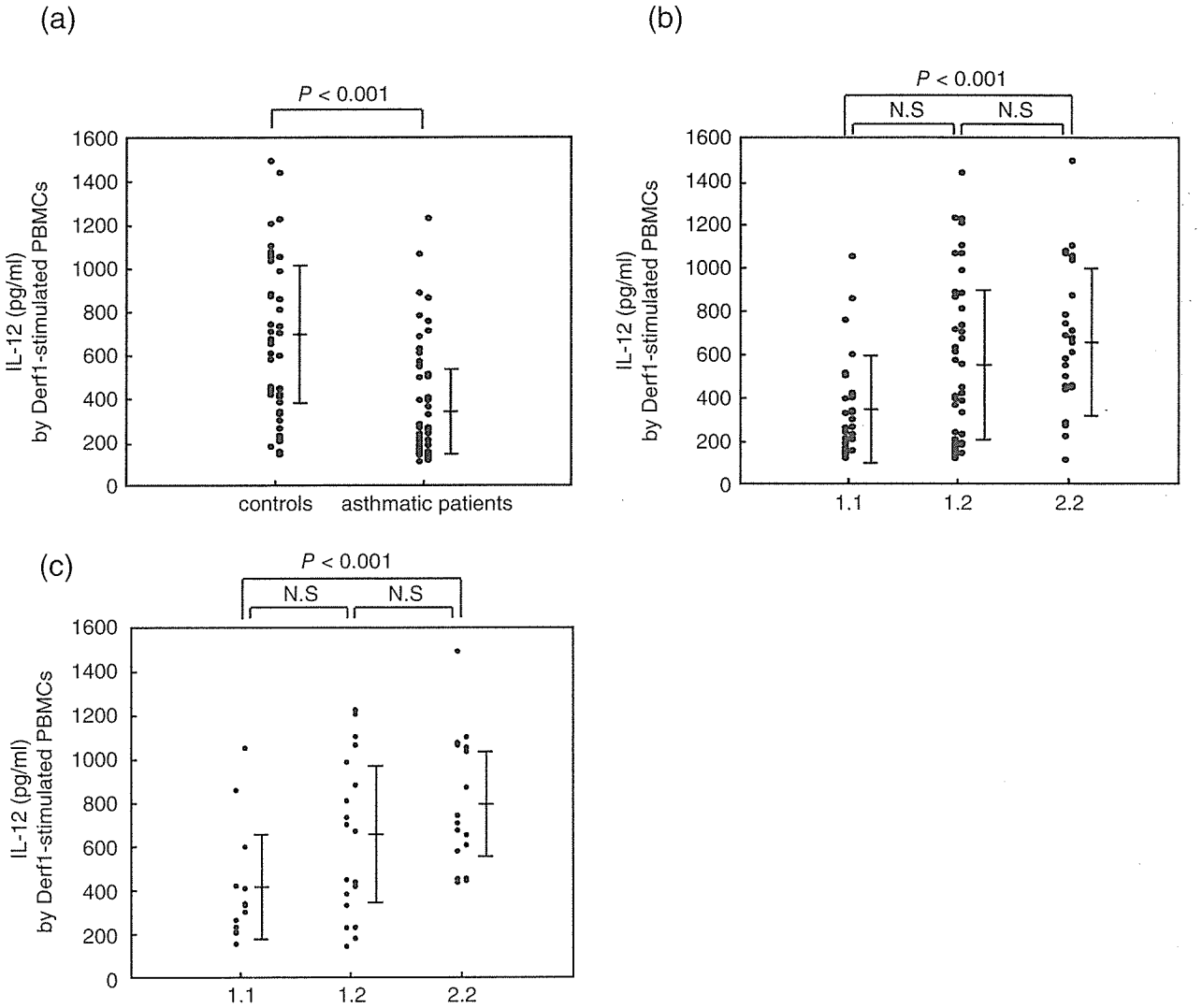

Fig. 3 (a) IL-12 production by Derf1-stimulated PBMCs in asthmatic patients and controls. The IL-12 production in the asthmatic patients was significantly lower than that in the controls ($P < 0.001$). (b) Relationship between *IL-12B* promoter polymorphism and IL-12 production by Derf1-stimulated PBMCs in both the controls and asthmatic patients ($n = 189$). The IL-12 production in the subjects with a 1.1 genotype was significantly lower than that in the subjects with a 2.2 genotype ($P < 0.001$). (c) Relationship between *IL-12B* promoter polymorphism and IL-12 production by Derf1-stimulated PBMCs in the controls ($n = 78$). The IL-12 production in the controls with a 1.1 genotype was significantly lower than that in the controls with a 2.2 genotype ($P < 0.001$).

(412.2 ± 315.0 pg/mL) had a lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype (807.2 ± 292.2 pg/mL) ($P < 0.001$; Fig. 3c). IL-12 production by Derf1-stimulated PBMCs positively correlated with IFN-γ production by IL-12-stimulated PBMCs (data not shown). Therefore, the subjects with a 1.1 genotype showed lower IL-12 and IFN-γ productions than those with a 2.2 genotype.

EFFECTS OF PROMOTER POLYMORPHISM ON PROMOTER ACTIVITY

To examine the functional activity of promoter polymorphism, we cloned the −2808/−2303 region of *IL-12B* into the PGV-P2 firefly luciferase reporter plasmid. The activity of the *IL-12B* promoter-reporter constructs was assessed in a transient transfection assay using RAW264 cells. As shown in Figure 4, the luciferase activity of the PGV-P2 plasmid with the *IL-12B* promoter-reporter constructs was significantly higher than that of the PGV-P2 plasmid only. A significantly lower luciferase activity was observed for haplotype 1 construct than for haplotype 2 construct (32.8% decrease ; $P = 0.0083$). Furthermore, we constructed mutant vectors with homozygotes for mut-1 (the CTCTAA/C allele) and mut-2 (the GC/T allele). The luciferase activity of the construct with mut-2 was

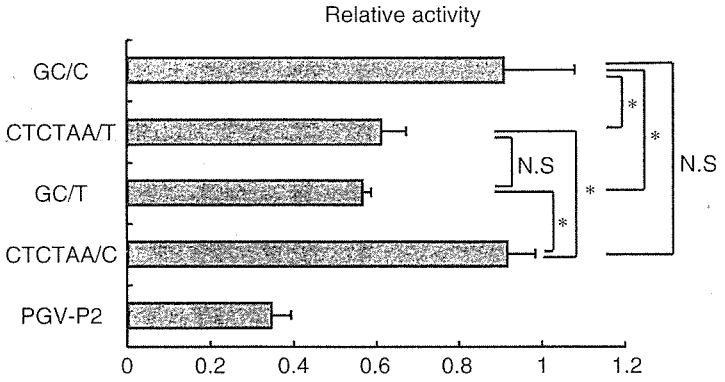

Fig. 4 Effects of *IL-12B* promoter polymorphism on promoter activity. RAW 264 cells were transfected by lipofectamine with reporter-constructs and stimulated with IFN-γ and LPS. To examine whether NP1 or NP2 affect the *IL-12B* transcriptional activity, mutated vectors (mut-1 and mut-2) were constructed. Luciferase activity was measured using the Dual-Luciferase reporter assay system. The presented results are mean ± SD from three independent experiments. * $P < 0.05$.

significantly lower than that of the construct with mut-1 and haplotype 2. This result indicates that *IL-12B* promoter polymorphism, that is, not the CTCTAA/GC allele but the T/C allele, has a major influence on the basal transcription rate of *IL-12B*.

DISCUSSION

IL-12 is a key mediator of immune responses. IL-12 is a heterodimeric molecule composed of two disulfide-linked subunits, a 35-kd subunit encoded by *IL-12A* on chromosome 3p12-q13.2 and a 40-kd subunit encoded by *IL-12B* on chromosome 5q31-q33.[18] A previous study has reported the association between *IL-12B* and asthma as determined by a genome-wide search.[7] We sequenced exons 1-8 and region 3 kb upstream from the transcriptional start site of *IL-12B*, and found three novel polymorphisms. In nineteen polymorphisms, NP1 + 2 that existed in the promoter region was shown to have a significant difference between the asthmatic patients and the controls in the genome frequency analysis. The frequencies of *IL-12B* polymorphisms in the coding region (NP3 and NP19) were not significantly different between the controls and asthmatic patients (data not shown). This result is similar to the result of Noguchi *et al.*[12]

Furthermore, the subjects with a 1.1 genotype had significantly lower plasma IL-12 concentrations and the lower IL-12 production by Derf 1-stimulated PBMCs than those with a 2.2 genotype. IL-12 plays an important role in the inhibition of Th2 cytokine production and the promotion of IFN-γ production by binding to plasma membrane receptors on activated T cells or NK cells.[2] IFN-γ inhibits IgE synthesis by human PBMCs *in vitro*.[19-22] Reduced IL-12 production and IL-12-dependent IFN-γ concentrations have been reported in asthmatic patients.[23-25] Therefore, IL-12 was shown to be associated with atopic dermatitis and asthma.[26-28] According to our result, *IL-12B* promoter polymorphism is likely to be associated with asthma prevalence by reducing IL-12 production.

Since the IL-12 levels measured during an exacerbation of asthma or bacterial/viral infection are analyzed in relation to the polymorphism, the functional consequences of the polymorphism cannot be fully explored. Hence, we investigated the functional activity of *IL-12B* promoter polymorphism. The transcriptional activity of the construct with a 1.1 genotype was lower than that of the construct with a 2.2 genotype. The transcriptional activity with mut-2 was significantly lower than that with mut-1 and haplotype 2. These results indicate that *IL-12B* promoter polymorphism, not NP1 but NP2, reduces the *IL-12B* transcriptional activity and IL-12 production. Since the NF-IL6 binding site in the *IL-12B* promoter region is shown to be in the -2405 to -2397 area of the 5'-upstream region, the T/C allele at this point may affect *IL-12B* transcriptional activity.[29] The transcriptional activity of the 296-bp construct from the transcriptional start site was reduced to half compared with that of the 3.3-kb construct.[17] This result shows that there are significant binding sites influencing the transcriptional activity from -296 to -3.3 kb of the transcriptional start site. The sequence near the T/C allele may be one of the binding sites affecting *IL-12B* transcriptional activity.

Morahan *et al.*[13] reported the association between asthma severity and a 4-bp microinsertion, which exists at a region 3 kb upstream from the transcriptional

start site of *IL-12B*. Since we identified only NP1 around the region 3 kb upstream from the transcriptional start site, the 4-bp microinsertion is likely to be NP1. According to their report, heterozygosity for *IL-12B* promoter polymorphism is associated with asthma severity, reduced *IL-12B* transcription level and decreased IL-12 secretion. Inconsistent results may have occurred due to differences in methods and population. In another study, Morahan *et al.* also reported that a 1.1 genotype is associated with mortality from cerebral malaria and with reduced production of nitric oxide in Tanzanian children, and that the *IL-12B* mRNA expression in the subjects with a 1.1 genotype is lower than that in the subjects with a 2.2 genotype.[30] These results are consistent with our results.

Khoo *et al.*[14] have recently shown that *IL-12B* promoter polymorphism is not associated with asthma prevalence, but that there is an association was between 1.1 genotype and elevated serum IgE levels in male subjects, and reduced pulmonary function in female subjects in childhood. In our data, the subjects with a 1.1 genotype had high IgE levels, however, there was no correlation with sex (data not shown). The subjects with a 1.1 genotype had reduced IL-12 production and *IL-12B* transcriptional activity compared with those with a 2.2 genotype. Therefore, homozygosity for haplotype 1 may elevate serum IgE by reducing IL-12 production.

In conclusion, the frequency of homozygosity for haplotype 1 in asthmatic patients was significantly higher than that in controls. The subjects with a 1.1 genotype had reduced plasma IL-12 concentrations and IL-12 production by Derf1-stimulated PBMCs compared with those with a 2.2 genotype. The *IL-12B* transcriptional activity was reduced by the $^{-2403}$T allele, not by the $^{-2703}$CTCTAA allele. Hence, *IL-12B* promoter polymorphism ($^{-2403}$T/C) can be a risk factor for the development of asthma.

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