平成16年度 厚生労働科学研究 免疫アレルギー疾患等予防・治療研究事業
免疫アレルギー疾患に係わる胎内・胎外因子の同定に関する研究
研究課題名：正常ヒト肺線維芽細胞におけるEotaxin発現に対する
デキサメサゾンの影響とその分子メカニズムの検討

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**研究要旨**：感染やアレルゲン曝露による気管支喘息の急性増悪時には、ステロイドの投与が行われる。その作用は、多岐にわたるが、気道上皮に対しEotaxin産生を低下させ、好酸球性炎症を抑制する可能性が示唆されている。しかしながら、線維芽細胞に対する効果は不明である。今回、正常ヒト肺線維芽細胞（NHLF）におけるIL-4依存性のEotaxin産生に対するステロイドの影響を検討した。NHLF培養液中にデキサメタゾン（DEX）を添加し、同時にIL-4で刺激した。24, 48, 72時間後の培養上清中のEotaxin蛋白質、mRNAをELISA法、real-time PCR法にて測定した。また、プロモーターアッセイにより、分子機序を検討した。NHLFにおいて、DEXは、IL-4刺激によるEotaxin蛋白質およびmRNA産生を添加後24時間では抑制したが、48時間、72時間後では反対に増強した。一方、DEXは細胞増殖には影響を及ぼさなかった。プロモーターアッセイでは、Stat6結合領域を介してIL-4の刺激で活性化を受けるが、DEXの添加により活性が低下し、濃度依存性がみられた。DEXは線維芽細胞のEotaxin産生に対して2相性の反応を呈した。その分子機序として、あとから生成される何らかの蛋白質の関与が推定された。

研究協力者
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## A. 研究目的

気管支喘息における気道炎症においては、好酸球が重要な役割を果たしており、IL-5やEotaxinなどのサイトカインがメディエーターとして介在している。肺線維芽細胞はEotaxinを産生する細胞のひとつであり、また、末梢気道では、上皮下に好酸球の集積が強いことが知られている。

また、気管支喘息の増悪時の治療にステロイドの全身投与がしばしば用いられている。ステロイドは上皮細胞においてEotaxinの産生を低下させるといわれているが、上皮下に存在する線維芽細胞に対する効果は不明である。

IL-4は、IL-4受容体複合体に結合し、JAK, STAT系を介してEotaxinの転写を起こす。JAK1，3ががリン酸化し、このJAKにより、STAT6（signal transducers and activators of transcription）がリン酸化を受け、二量体を形成し、核内に情報を伝達する。STAT6は、STAT6 binding siteに結合して、Eotaxinの転写が始まる。

正常ヒト肺線維芽細胞において、IL-4依存性の Eotaxin 産生がみられるかどうか、また、Eotaxin 産生に対するステロイドの効果について検討する。

## B．研究方法

正常ヒト肺線維芽細胞(normal human lung fibroblast;NHLF)を培養し、IL-4(10ng/mL)で刺激し、同時に $10^{-4}$～$10^{-8}$M のデキサメサゾン（DEX）を添加したあとに、Eotaxin 蛋白質、mRNA、を測定し、プロモーターアッセイを行った。Eotaxin 蛋白質の定量は、IL-4、DEX の添加後 24, 48, 72 時間後の培養上清中の Eotaxin 蛋白質を ELISA 法にて測定した。Eotaxin mRNA の測定は、IL-4、DEX の添加後 12, 24, 48, 72 時間後に Isogen にて total RNA を抽出し、Reverse Transcriptase で mRNA から cDNA を作成し、eotaxin およびβアクチンの転写量を Real-Time PCR 法で測定した。

プロモーターアッセイによる Eotaxin プロモーター活性の測定は、NHLF に、リポトランスフェクション法にて各種エオタキシンプロモーターの下流にホタルルシフェラーゼ遺伝子を有するプラスミドを導入し、培養液中に IL-4、DEX を添加し、その 8 時間後に Luciferase 活性を測定した。なお、同時に EF1α プロモーターの下流にウミシイタケルシフェラーゼを有するプラスミドを導入し、内部標準とした。

Cell count kit-8 を用いて細胞数の測定を行った。

## C．研究結果

Eotaxin 蛋白質は、IL-4 の刺激により発現が増強した。しかし、IL-4 と同時に DEX を添加すると、Eotaxin 蛋白質は、24 時間後では抑制されたが、48 時間後、72 時間後ではさらに増強していた。

Eotaxin mRNA は、IL-4 の刺激により発現が増強した。DEX を添加すると、24 時間後までは抑制されたが、48 時間以降は増強していた。また、この DEX の濃度を $10^{-5}$～$10^{-8}$M と変えて添加した実験では、IL-4, DEX 添加 72 時間後において、DEX $10^{-6}$M で Eotaxin mRNA は最も強く発現していた。

Eotaxin のプロモーターアッセイでは、IL-4 の刺激で増強したルシフェラーゼ活性が、DEX の添加により減少し、濃度依存性が見られた。

48 時間以後の Eotaxin mRNA、蛋白質の発現の増強の理由を調べるため、IL-4, DEX の添加した後、12 時間または 24 時間後に、転写を阻害する Actinomycin D （ACD）（100 nM）や、蛋白合成を阻害する Cycloheximide (CHX)（100 nM）を添加し、72 時間後に Eotaxin mRNA を抽出し測定した。すると、IL-4 と DEX を添加により発現が増強した Eotaxin mRNA は、IL-4 と DEX を添加した 12 時間後に CHX や ACD を添加すると、その発現は抑制された。ACD を添加したもののほうが CHX を添加したものよりも強く抑制された。

IL-4 や DEX の添加により、細胞の増

殖があるかどうか調べるため、Cell Counting Kit-8 を用いて細胞数を測定したところ、細胞数に変化はなく、DEX は細胞増殖に影響を及ぼさなかった。

また他のステロイド、hydrocortisone(HC), prednisolone (Pred)についても同様に IL-4 と同時に添加し、Eotaxin 蛋白質、Eotaxin mRNA の測定を行った。IL-4 により増強した Eotaxin mRNA は、HC や Pred の添加により 48 時間以後ではさらに増強していた。Eotaxin 蛋白質の測定では、IL-4 の刺激により増加した Eotaxin 蛋白質は、HC の添加では明らかな傾向は示さなかったが、Pred の添加後は 48 時間以後で増加していた。ほぼ DEX と同様の傾向を示していた。

## D. 考察

IL-4 刺激下においては、48 時間後以降では、それ以前では抑制されていた Eotaxin mRNA や蛋白質が、逆に増強していた。この機序に関しては、12 時間以後に生成される何らかの蛋白質により、Eotaxin の産生が増加することが推定された。

## E. 結論

Th2 バランス（IL-4 刺激下）においては、肺線維芽細胞からも気道上皮と同様に Eotaxin を産生し、喘息の気道炎症に関与する可能性が示唆された。デキサメサゾンは 2 面性の効果を示し、気道炎症に対する短期抑制効果は確認されたが、中止後にリバウンドが生じる可能性が示唆された。

## F. 研究発表

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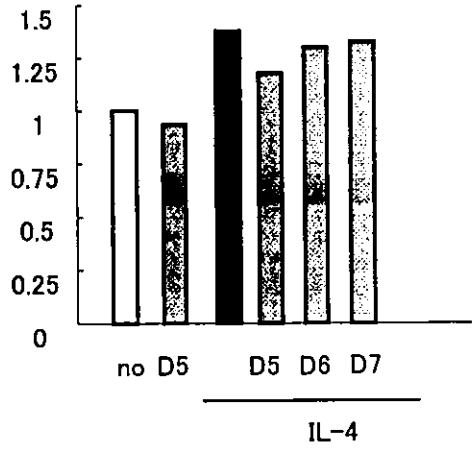
## G. 知的財産権の出願・登録状況

1、特許取得
　特になし

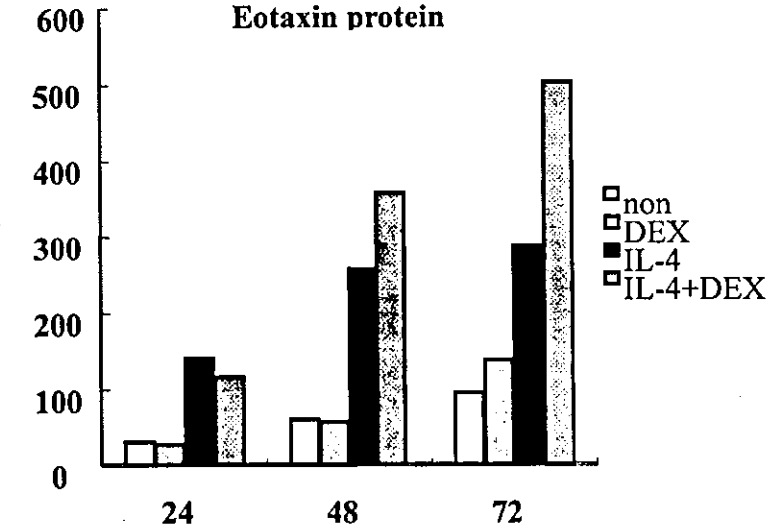
2、実用新案登録
　特になし

**Eotaxin promoter assay**

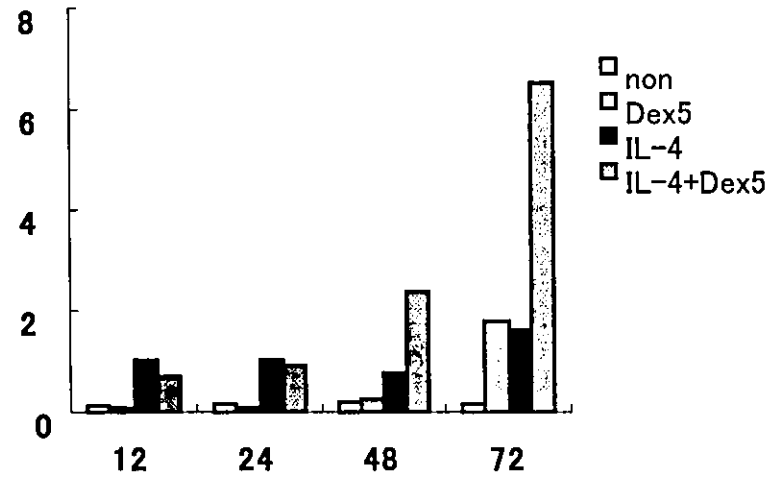



ng/ml

**Eotaxin protein**




**Eotaxin mRNA**



平成16年度 厚生労働科学研究 免疫アレルギー疾患等予防・治療研究事業

# 免疫アレルギー疾患に係わる胎内・胎外因子の同定に関する研究

（課題名） 周産期に起因する呼吸障害と乳児喘息

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**研究要旨：**
早産児の慢性肺疾患(CLD)は、NICU入院中のみならず、長期にわたり医療を必要とする点で依然として未熟児医療の大きな問題点である。我が国においては、CLDはRDS先行型と胎内感染型など7型に分類されている。今年度の研究では1)CLDと早産に関連するといわれているUreaplasma保菌に関連性はあるのか、2)新生児期の慢性肺疾患の重症度を規定する因子は何か、3)退院後の呼吸器予後を規定する因子は何かを検討した
1) 感染型CLDは非感染型CLDに比べて、胎盤のUreaplasma保菌((28.36、2.11-38.14)、出生時の白血球数(1.21、1.06-1.39)と有意に関連して発症した。
2)CLDの重症度は、CLDの型には関連せず、在胎週数、出生体重に関連していた。3)退院後にwheezingのepisodeが2回以上あるいは喘息と診断されたれいでは、新生児のUreaplasma保菌例が多く、酸素投与日数が長かった。また、CLDⅢ型はCLD非発症型に比べて喘息発症が有意に多かった。

研究協力者
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## A. 研究目的

早産児の慢性肺疾患(Chronic lung disease; CLD)は在胎32週未満の早産児に限られ、幼若であるほど発症頻度は高い。1967年にNorthwayが報告したBronchopulmonary dysplasia(BPD)は、比較的大きな早産児の呼吸窮迫症候群(RDS)に高濃度の酸素と高い陽圧換気が原因で、未熟肺に生じた肺損傷とその修復過程の異常であるとされてきた(old BPD)[1]。近年、超早産児の全身管理が進歩し、サーファクタント補充療法が導入され、人工呼吸管理法も進歩し、非常に幼若な早産児が生存するようになり、慢性肺疾患の概念も変化してきた[2]。酸素、陽圧などによる生後の炎症性変化と同時に出生前の胎内感染による気道の炎症性変化がその原因として強調されるようになってきた[3]。本邦においては、藤村らは、生後RDSがなく絨毛膜羊膜炎があり、血清IgMが増加しているCLDの存在を主張[4]、それに基づいて厚生省研究班では、CLDを7タイプに分類した[5]。

この中でも感染先行型のCLDは、呼吸障害が重症化、遷延化する例があり、NICU退院後も在宅酸素を必要とし、再入院する例が多い。一方、近年女性生殖器の*Ureaplasma urealyticum*保菌と早産、絨毛膜羊膜炎(CAM)、pretermPROMとの関連が数多く報告されるようになった[6]。*Ureaplasma urealyticum*は、広く動植物界に存在し疾患の原因となる*Mycoplasma*の一種で、ヒトでは泌尿生殖器に常在する菌であり、泌尿器感染のみで

なく、妊婦の下部生殖器培養では40～80%が陽性であるからである。*Ureaplasma*とCLDの関連性も報告されるようになった[6]。細菌学的に*Mycoplasma*は大きさが0.3～0.8μmの多形性を示すグラム陰性菌で、光学顕微鏡では見えず細菌濾過器を通過するが、ウイルスとは異なり、無細胞培地で増殖し小さなコロニーを形成する。我々は、*Ureaplasma*培養は10B液体培地とA8平板培地がセットになったビオメリュー社のMycoplasma-lyoを用いて行っている。近年ではPCR法が開発され、その検出率は従来の培養法とほぼ同じあるいはむしろ鋭敏であることが報告され、また、DNAの相同性から、*U.urealyticum*は*U.parvum*(serovar1、3、14、6)と*U.urealyticum*(serotype1,2,3)とに分類されるようになった[7]。我々の検討では、母体の膣培養からPCRで同定された*Ureaplasma*は*U.parvum*が93%でserovar3が多かったが、特定のserovarが早産を起こしやすいということはなかった。

今年度の課題としては、1)CLDと*Ureaplasma*保菌に関連性はあるのか、2)新生児期の慢性肺疾患の重症度を規定する因子は何か、3)退院後の呼吸器予後を規定する因子は何か、を研究課題として取り上げ、以下の方法で検討した。

## B. 研究方法

対象は1997年11月から2001年8月までに自治医大総合周産期母子医療センターNICUに入院した32週未満の単胎である。178例入院し、27例は死亡、生存152例中、胎盤の病理学的検索、胎盤の培養検査、生後の新生児の培養検査が行われた110例を対象として検討した。日齢28で胸部X線写真上変化が認められ酸素を必要としているCLDは44例で、17例は非感染型、22例は感染型、5例は混合型なので、前者の2タイプ39例について検討した。

## C. 研究結果

1) CLDと*Ureaplasma*保菌の関連性

CLDを2種類に分類((感染先行型；CAM+IgM＞15mg/dl、非感染型；RDS+/-、CAMなし)、Logistic回帰分析で、CAMは、PROM(OR 10.19, 95%信頼限界3.10-33.56)と胎盤の*Ureaplasma*保菌(6.73、1.89-23.91)に関連し、感染型CLDは非感染型CLDに比べて、胎盤の*Ureaplasma*保菌((28.36、2.11-38.14)、出生時の白血球数(1.21、1.06-1.39)と有意に関連して発症した。感染型CLDで白血球は非感染型に比べて有意に多いが、*Ureaplasma*保菌陰性例でも高値であり、おのおのがindependentに感染型CLD発症に関連していることがグラフからも伺える(図1)。

2) 新生児期の慢性肺疾患の重症度規定因子

図2に示したのはCLDの型別による酸素投与日数、人工呼吸(MV)日数である。Ⅲ型が酸素日数、MV日数ともに長いが、症例数が少なく、また、各症例の変動が大きく有意差はなかった。CAMの有無、胎盤保菌の有無、新生児保菌の

有無に関しても、酸素投与日数、MV 日数ともに差は見られなかった。また、新生児期の呼吸障害の重症度を修正36週時に酸素投与が必要な症例と定義すると、単変量解析で、在胎週数と出生体重のみが有意であった。

3) 退院後の呼吸器予後の規定因子-1

NICU退院後2回以上wheezingのエピソードあるいは気管支喘息と診断された例を BA 群として周産期因子との関連性を検討した。BA群は酸素投与期間が有意に長く、新生児保菌例が多かった(表2)。

4) 退院後の呼吸器予後の規定因子-2

1997年1月から2003年12月までにNICU に入院した32週未満の新生児で、胎盤の病理学的検索が行われ生存退院した新生児 174 名について、呼吸器予後を調査した。1)～3)の対象と大部分が重複してはいるが、*Ureaplasma* の胎盤検索が行われなかった例もこの検討には含まれている。BA(+)は上記と同様、2回以上のwheezing episodeがあるか喘息として治療されている症例である。Ⅰ型12名、Ⅱ型41名、Ⅲ型23名、Ⅲ'型16名、CLD(-)例82例で、Ⅰ型、Ⅱ型は約1/4がwheezingを発症、Ⅲ型は 48%とほぼ半数が罹患していた。CLD(-)例とⅢ型の発症頻度に有意差が認められたが、CLD(-)とⅠ型、Ⅰ型とⅢ型の間に発症頻度の差は認められなかった。

*Ureaplasma*が感染型のCLD発症と有意に関連性があることが証明された。また、CLD の病型により新生児期の呼吸不全の重症度に差は認められなかったが、退院後、乳幼児期に 2 回以上のwheezing episode があるか喘息として治療されている症例は *Ureaplasma* 保菌、感染型のCLD症例に多かった。生後CLDを発症する児は臍帯血の炎症性サイトカインが上昇していると報告されており[7]、全身性の炎症性反応(fetal inflammatory response syndrome)を経由してCLDを発症している可能性があり、次年度は *Ureaplasma* 保菌と臍帯血の炎症性サイトカインの変化、CLDとの関連を検討し、さらに *Ureaplasma* 保菌とその後の喘息発症をさらに詳細に検討する予定である。

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表1. CLDの分類

| 病型 | RDS | IgM高値 絨毛膜羊膜炎 | 胸部X-P28日以上 泡沫状/気腫状陰影 |
|---|---|---|---|
| I | + | - | + |
| II | + | - | - |
| III | - | + | + |
| III' | - | + | - |
| IV | - | 不明 | + |
| V | - | - | - |
| VI | いずれの型にも分類されないもの | | |

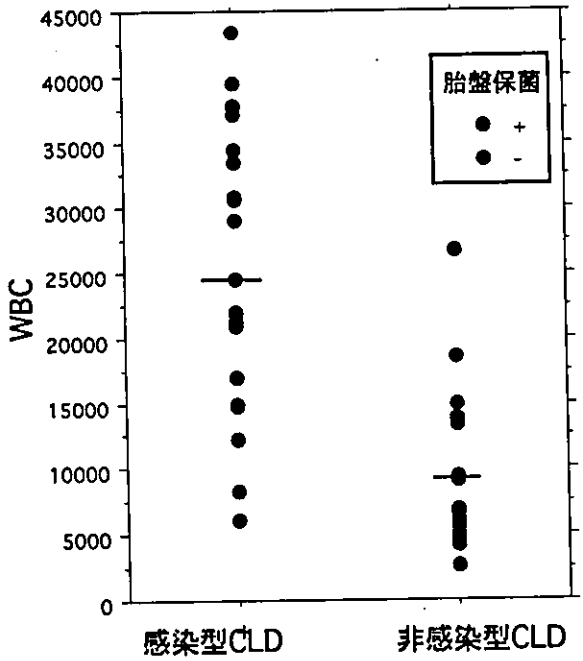


図1 感染型CLDと非感染型CLDの入院時白血球数、Ureaplasma胎盤保菌の有無

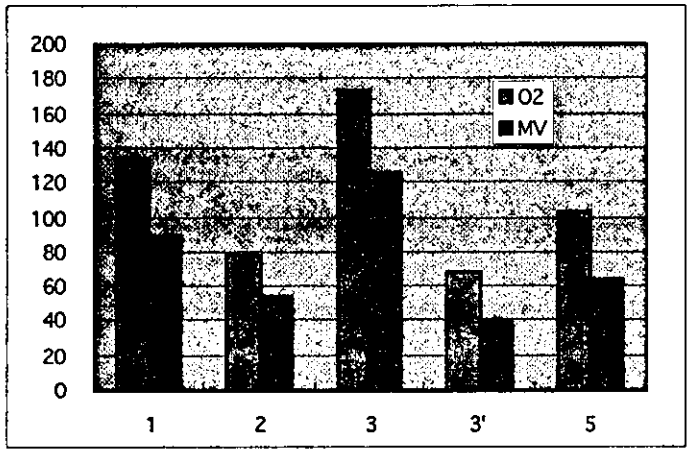


図2　CLDの病型別の酸素投与日数
人工呼吸(MV)日数

表２　退院後の呼吸器予後

| | BA(+) | BA(-) | p |
|---|---|---|---|
| n | 11 | 25 | 36 |
| GA(W) | 27.4(2.54) | 27.1(1.83) | 0.747 |
| BW(g) | 1067(295) | 958(298) | 0.315 |
| Ap1 | 4.09(2.59) | 4.56(2.66) | 0.627 |
| Ap5 | 7.30(1.34) | 6.72(2.15) | 0.428 |
| WBC | 17045(11527) | 18696(12310) | 0.71 |
| MV | 137(256) | 61(32.7) | 0.144 |
| O2 | 167(194) | 85(41.9) | 0.048 |
| 新生児+/- | 8/3 | 8/17 | 0.028 |
| CAM+/- | 7/4 | 11/14 | 0.24 |
| 胎盤+/- | 5/6 | 9/16 | 0.59 |
| 感染/非感染CLD | 8/3 | 12/13 | 0.156 |

3例：追跡不可

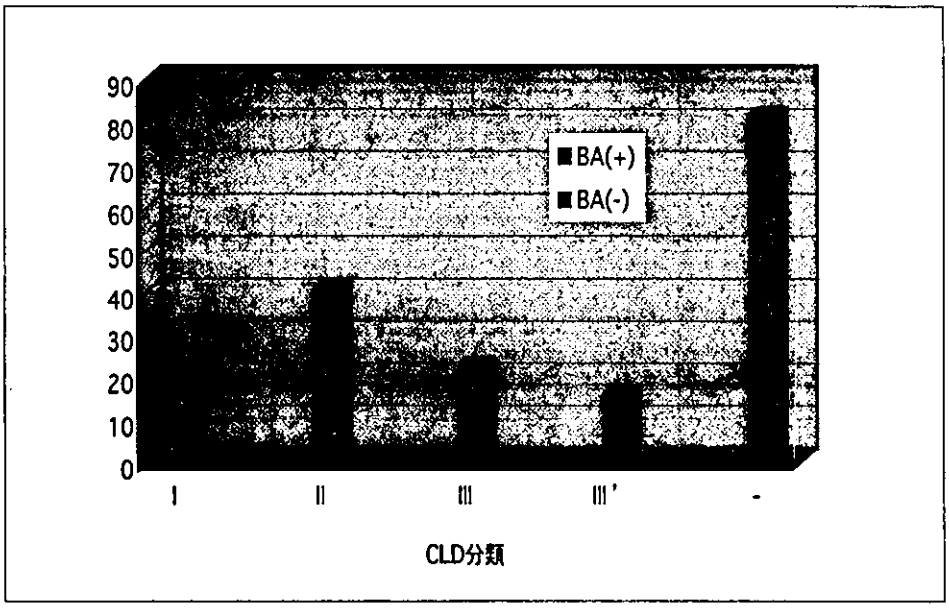


図４　CLD の病型別による喘息発症

# 研究成果の刊行に関する一覧

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻名 | ページ | 出版年 |
|---|---|---|---|---|---|
| Kondo N, Matsui E, Kaneko H, Aoki M, Kato Z, Fukao T, Kasahara K, Morimoto N | RNA editing of interleukin-12 receptor β 2, 2451 C-to-U (Ala 604 Val) conversion, associated with atopy | Clin Exp Allergy. | 34 | 363-368 | 2004 |
| Kawaguchi M, Kokubu F, Odaka M, Watanabe S, Suzuki S, Ieki K, Matsukura S, Kurokawa M, Adachi M, Huang SK | Induction of granulocyte-macrophage colony-stimulating factor by a new cytokine, ML-1 (IL-17F), via Raf I-MEK-ERK pathway | J Allergy Clin Immunol. | 114 | 444-450 | 2004 |
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| 本間 洋子 | Ureaplasma と新生児感染 | 日本未熟児新生児学会雑誌 | 17 | 15-20 | 2005 |

Clin Exp Allergy 2004; 34:363–368

# RNA editing of interleukin-12 receptor β2, 2451 C-to-U (Ala 604 Val) conversion, associated with atopy

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## Summary

*Background* The production of IgE in B lymphocytes is down-regulated by IFN-γ. IL-12 induces IFN-γ production by T lymphocytes and natural killer cells by binding to its specific receptor. RNA editing is a post-transcriptional modification.

*Objective* Here we show that the RNA editing of IL-12 receptor (R) β2 is associated with atopy.

*Methods* Atopic patients and non-atopic healthy controls were studied. Fragments of IL-12R β2 cDNA and genomic DNA were amplified and sequenced. Furthermore, the function of the IL-12R β2 chain was investigated.

*Results* Sequence analysis of the cDNA clones representing IL-12R β2 mRNA transcripts revealed a C-to-U conversion at nucleotide 2451 (Ala 604 Val) on exon 13 in some atopic patients. Surprisingly, sequence analysis of their genomic DNA showed no 2451 C-to-T (Ala 604 Val) mutation. We concluded that the observed C-to-U mismatch in the cDNA clone is due to a post-transcriptional modification, RNA editing. The C-to-U conversion was observed in 21 (20.6%) of 102 atopic patients, whereas this conversion was observed in only 4 (3.8%) of 104 non-atopic subjects ($P<0.001$). IFN-γ production by peripheral blood mononuclear cells (PBMCs) stimulated with IL-12 in the subjects with the C-to-U conversion was significantly lower than that in the subjects without the C-to-U conversion. In atopic patients with the C-to-U conversion, PBMCs faintly showed the tyrosine phosphorylation of Stat4, and the IgE production by PBMCs was not suppressed by IL-12 whereas it was suppressed by IFN-γ.

*Conclusions* The RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion causes impairment of the IL-12 signal cascade and the subsequent reduction in IFN-γ production, resulting in the impaired down-regulation of IgE production. This is the first report indicating that atopy is associated with RNA editing.

**Keywords** atopy, IL-12 receptor β2, RNA editing

*Submitted 30 December 2002; revised 8 August 2003; accepted 17 November 2003*

## Introduction

Atopy is characterized by enhanced immunoglobulin E (IgE) responses to common environmental antigens and leads to clinical disorders such as asthma, eczema and rhinitis. IL-4 promotes a class switch to IgE in B lymphocytes and Th2 $CD4^+T$ lymphocyte differentiation [1]. IgE production by B lymphocytes is down-regulated by IFN-γ that is one of the Th1 cytokines [1]. IL-12 induces IFN-γ production by T lymphocytes and natural killer (NK) cells by binding to its specific receptor [2–4].

The receptor of IL-12 is composed of two distinct subunits, β1 and β2, that assemble to form a high-affinity IL-12 receptor (R) complex [5]. While the β2 chain of the IL-12R is expressed only in Th1 lymphocytes, the β1 chain is expressed in both Th1 and Th2 lymphocytes. Thus, the expression of both β1 and β2 chains accounts for the responsiveness of T lymphocytes to IL-12 and mediates Th1 lymphocyte differentiation [6]. On binding to its receptor, IL-12 induces activation of specific members of the Stat family of transcription factors, which then translocate to the nucleus and bind to genomic promoter regions. Stat4 is particularly important in this respect, since Stat4-deficient mice manifest impaired IFN-γ production [7]. Furthermore, the phenotype of the IL-12p40-deficient mouse is similar to that of the Stat4-deficient mouse [8]. We reported that reduced IFN-γ production by peripheral blood mononuclear cells (PBMCs) following stimulation with IL-12 but not with phytohemagglutinin (PHA) is associated with the heterozygous IL-12 β2 chain gene mutations, 1577 A-to-G (Arg 313 Gly), 2496 del 91, and 2799 A-to-G (His 720 Arg), in some atopic subjects [9].

RNA editing is a post-transcriptional modification that results in the generation of nucleotides within an RNA transcript that do not match the bases present within the genome [10]. Mammalian RNA editing events, often represented by cytidine-to-uridine (C-to-U) and adenosine-to-inosine

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(A-to-I) conversions, are predominantly mediated by base deaminations [10]. Here we show that the RNA editing of the IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion on exon 13 is associated with the reduction in the extent of IL-12 signalling, leading to insufficient IFN-γ production and atopy. This is the first report indicating that atopy is associated with RNA editing.

## Methods

### *Atopic and control subjects*

One hundred and two atopic patients (9.3 ± 8.2 years old) with major allergic diseases such as bronchial asthma and/or atopic dermatitis having elevated levels of serum IgE and/or specific IgE antibodies, were studied. The diagnosis of bronchial asthma was made according to the criteria of the American Thoracic Society, and that of atopic dermatitis was made according to the criteria of Hanifin. The levels of specific IgE antibodies against house dust, mite, hen's egg, and cow's milk were measured by fluoroenzyme immunoassay. Scores of 3+ to 6+ were considered positive. None of the patients had been receiving systemic steroids. One hundred and four healthy controls (11.5 ± 13.7 years old) had no history of atopic diseases and their serum IgE levels were within normal limits for their age. The ethics committee of Gifu University School of Medicine approved the research project, and informed consent was obtained from all the subjects or their parents.

### *Cell preparation and culture*

PBMCs were isolated from the heparinized blood of the controls and atopic subjects by Ficoll-Paque (Pharmacia, Sweden) gradient centrifugation. The cells were suspended to give a density of $10^6$ cells/mL in the culture medium which consisted of RPMI1640 supplemented with 10% heat-inactivated fetal calf serum. PBMCs were cultured at 2 mL per tube in culture test tubes in the presence or absence of 5 IU/mL IL-12 (R&D systems, Germany), 400 ng/mL IL-18, or 10 μg/mL PHA for 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$ [9].

### *Assays for cytokines*

The culture supernatants incubated for 24 h in test tubes were spun to remove the cells after the cultures. The IFN-γ concentrations of the supernatants were measured with a human enzyme-linked immunosorbent assay (ELISA) kit (Ohtsuka, Japan). The detection limit was 20 pg/mL [9].

### *Sequence analysis of cDNA and genomic DNA of IL-12R β2 chain*

Total cellular RNA was extracted from PBMCs cultured with PHA for 24 h using an Isogen kit (Nippon Gene, Japan). Fragments of IL-12R β2 cDNA were amplified by reverse transcription-polymerase chain reaction (RT-PCR), ligated to a T-vector (Novagen) and sequenced using an autosequencer [5]. The conditions for RT-PCR were 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, for 40 cycles. For amplification of exon 13 of IL-12R β2 cDNA, the sense primer 5′-GATGACAGCTCTGACAGCTG-3′ and the anti-sense primer 5′-GGCCTGATGACCTTGGAIT-3′ were used. Genomic DNA was extracted from leukocytes. Exon 13 and the flanking region of IL-12R β2 genomic DNA were amplified by PCR with the sense primer 5′-GATGA CAGCTCTGACAGCTG-3′ and the anti-sense primer 5′-CATTGTCTCCAGGAAGATAG-3′ [11]. The conditions used for PCR were 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, for 30 cycles.

### *Expression constructs encoding IL-12R β2 and transfected Ba/F3 cell clones*

The expression constructs encoding human wild-type IL-12R β2 or variant-type (2451 C-to-U conversion) IL-12R β2 were prepared in the PEF-BOS expression vector, as described elsewhere [5]. Ba/F3 cells were transfected by electroporation with the expression constructs encoding either the wild-type IL-12R β2 or the variant-type IL-12R β2. Then, the transfected Ba/F3 cells were cloned.

### *Flow cytofluorometric analysis*

IL-12R β2 expressing Ba/F3 cell clones were detected by indirect immunofluoresence analysis using flow cytometry. Briefly, $10^6$ cells in 100 μL of staining buffer were incubated with 1 μg/mL rat anti-hu IL-12R β2(2B6) mAb or isotype control Ab for 30 min, followed by incubation with biotinylated anti-rat-Ig $F(ab)^2$ fragments for 30 min, and finally incubated with streptavidin conjugated to PE (PharMingen) for 30 min. All incubations were performed at 4 °C in a staining buffer, and the cells were washed twice between incubations. The stained cells were analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

### *Proliferative responses*

The Ba/F3 cell clones were cultured with IL-12 (0.5, 5 or 50 IU/mL) for 24 h. DNA synthesis was measured by adding 0.5 μCi $[^3H]$ thymidine per well 4 h before harvesting onto glass-fiber filters. $[^3H]$ thymidine incorporation (c.p.m.) was measured by liquid scintillation counting, and the results were expressed as the means of triplicate.

### *Immunoprecipitation assay for phosphorylated Stat4*

PBMCs from the patients and controls were stimulated with PHA and IL-12 (5 IU/mL) or the control culture medium for 15 min. The cells were lysed in 1% Triton X-100, 150 mM NaCl, 20 mM $Na_2PO_4$, 1% aprotinin, 5 mM PMSF, 100 mM NaF and 2 mM $Na_3VO_4$, and were immunoprecipitated with rabbit antisera for Stat4 (Santa Cruz Biotechnology). Precipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to a nylon membrane, the blots were probed with antibody for phosphotyrosine. Equal loading of Stat4 was confirmed by stripping the same membranes and reprobing them with the antiserum for Stat4.

### *Suppression of* in vitro *IgE production*

PBMCs ($10^6$cells/mL) from the atopic patients with or without the 2451 C-to-U conversion were cultured at 2mL per tube in culture test tubes with Derf 1 (5μg/mL, Asahi, Tokyo, Japan) and IL-4 (500 U/mL, Genzyme/Techne, USA) for 14 days at 37°C in a humidified atmosphere containing 5% $CO_2$. For suppression of IgE production, IFN-γ (1000 U/mL, Genzyme/Techne, USA) or IL-12 (5IU/mL) was added to the culture. The IgE concentrations of the culture supernatants were measured by ELISA.

### *Statistics*

The significance of difference between groups was analysed by the Mann–Whitney's *U* test or $\chi^2$-test.

## Results

### *RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion associated with atopy*

In this study, we found that IL-12R β2 mRNA editing modifies cytidine in an alanine codon (GCU) at nucleotide 2451 in the extracellular domain to a uridine (GUU), converting to a valine codon (Ala 604 Val) in atopic patients. Fragments of IL-12R β2 cDNA were amplified by RT-PCR, ligated to a T-vector and sequenced using an autosequencer. Interestingly, sequence analysis of the cDNA clones representing IL-12R β2 mRNA transcripts revealed the C-to-U conversion at nucleotide 2451 (Ala 604 Val) on exon 13 in some of the atopic patients (Fig. 1). Very recently, van Rietschoten et al. [11] reported the genomic organization of the human IL-12R β2 chain gene. Therefore, we determined the sequence of the genomic DNA of the IL-12R β2 chain. Surprisingly, sequence analysis of the genomic DNA of the IL-12R β2 chain from the atopic patients showed neither 2451 C-to-T (Ala 604 Val) mutation on exon 13 nor mutation in the flanking region of exon 13 (Fig. 1). Therefore, it was suggested that the C-to-U mismatch observed upon comparison of IL-12R β2 genomic DNA with cDNA clones had arisen at the RNA level. RNA editing is formally defined as any RNA-processing event (excluding RNA splicing) that generates an RNA transcript with a primary nucleotide sequence different from that of its gene. Therefore, we concluded that the observed C-to-U mismatch in the cDNA clone of the IL-12R β2 chain is due to the RNA editing of this transcript.

To determine whether the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA is associated with atopy, we conducted a genetic association study on atopy. The C-to-U conversion was observed in 21 (20.6%) of the 102 atopic patients, whereas this conversion was observed in only 4 (3.8%) of the 104 non-atopic subjects. There was a significant ($P<0.001$, by $\chi^2$-test) difference in the C-to-U conversion frequency between the non-atopic subjects and the atopic subjects (Table 1). The subjects exhibited neither this conversion nor any mutations in the flanking region of exon 13 in the genomic DNA of the IL-12R β2 chain.

### *IFN-γ production by PBMCs stimulated with IL-12, IL-18 or PHA*

To determine whether the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA affects the IL-12 signal

**Table 1.** An association study of the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in IL-12R β2 chain cDNA responsible for atopy

| | C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA | | | |
|---|---|---|---|---|
| | *n* | – | + | *P*-value |
| Non-atopic subjects | 104 | 100 | 4 (3.8%) | <0.001 |
| Atopic patients | 102 | 81 | 21 (20.6%) | |

*P*-value was calculated by $\chi^2$-test.
Sequence analysis of genomic DNA of the IL-12R β2 chain showed no 2451 C-to-T (Ala 604 Val) mutation in any of the subjects tested.

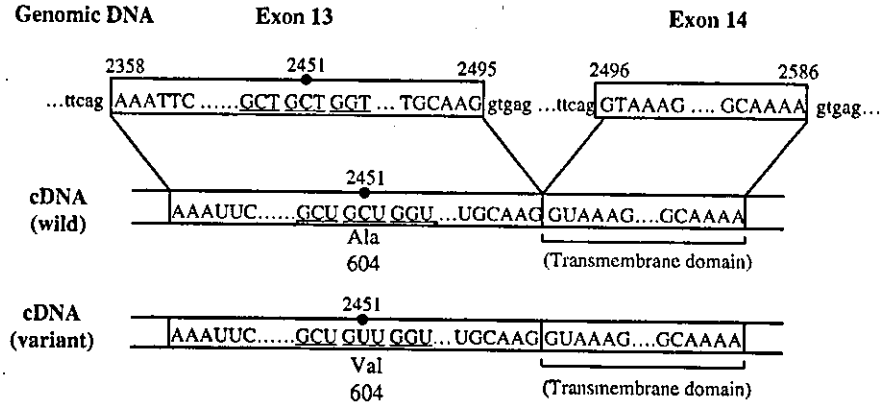


**Fig. 1.** Sequence analysis of genomic DNA and the cDNA of IL-12R β2 exons 13 and 14 and their flanking regions in non-atopic healthy controls and atopic patients. Sequence analysis of genomic DNA of the IL-12R β2 chain from any of the non-atopic healthy controls and any of the atopic patients showed no 2451 C-to-T (Ala 604 Val) mutation in exon 13. Sequence analysis of cDNA (variant) clones representing IL-12R β2 mRNA transcripts indicates the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in some of the atopic patients. Therefore, it is indicated that the observed C-to-U mismatch in the cDNA clone of IL-12R β2 chain is due to RNA editing of this transcript. The number above each sequence indicates the number of the nucleotide according to the Genbank database U 64198, and the number under each sequence indicates the number of amino acid.

 

**Table 2.** IFN-γ production by PBMCs stimulated with IL-12, IL-18 or PHA in the subjects (atopic patients and non-atopic subjects) with or without the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in IL-12R β2 chain cDNA

| | IFN-γ concentration (pg/mL)* | | |
|---|---|---|---|
| | Without C-to-U conversion† ($n = 169$) | With C-to-U conversion† ($n = 25$) | *P*-value‡ |
| Stimulated | | | |
| with IL-12 | 154.7 (34.9 ~ 685.9) | 69.3 (16.0 ~ 266.5) | 0.013 |
| with IL-18 | 68.6 (11.8 ~ 398.7) | 57.3 (9.0 ~ 364.5) | 0.962 |
| with PHA | 1568.2 (582.1 ~ 4224.5) | 1578.1 (711.8 ~ 3498.6) | 0.970 |

*Genometric means are shown, and the ranges of SD are shown in parentheses. †at nucleotide 2451 (Ala 604 Val) in IL-12R β2 chain cDNA. ‡*P*-values were calculated by Mann–Whitney's *U* test.

cascade, we, next, conducted an association study on IFN-γ production by PBMCs following stimulation with IL-12, IL-18 or PHA. After PBMCs were cultured with IL-12, IL-18 or PHA for 24 h, the IFN-γ concentration in the culture supernatants was measured (Table 2). The results revealed that IFN-γ production by PBMCs stimulated with IL-12 in the subjects with the C-to-U conversion was significantly ($P < 0.013$) lower than that in the subjects without the C-to-U conversion. In contrast, there was no significant difference in IFN-γ production by PBMCs stimulated with IL-18 or PHA between the subjects with the C-to-U conversion and those without the conversion.

### *Expression of IL-12R β2 chain*

To investigate the expression of the wild-type or variant-type IL-12R β2 chain, Ba/F3 cells were transfected by electroporation with wild-type IL-12R β2 cDNA or variant-type IL-12R β2 cDNA containing the C-to-U conversion at nucleotide 2451 in the PEF-BOS expression vector and then cloned [5]. The IL-12R β2-chain-expressing cells were detected by flow cytometry using the anti-IL-12R β2 antibody. The results revealed that the staining intensity and the percentage of cells expressing the IL-12R β2 chain in the clone with the C-to-U conversion were lower than those in the clone without the conversion (Fig. 2a). Furthermore, the degree of proliferative responses of the cells was measured. As a result, the proliferative response of the Ba/F3 cell clones transfected with variant-type IL-12R β2 cDNA containing the C-to-U conversion at nucleotide 2451 to IL-12 (0.5, 5 IU/mL) was lower than that of the Ba/F3 cells transfected with wild-type IL-12R β2 chain cDNA (Fig. 2b).

### *Tyrosine phosphorylation of Stat4*

Furthermore, to investigate the functional aspects of the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA, we examined the tyrosine phosphorylation of Stat4. Although PBMCs from the patient without the C-to-U conversion (patient 2) and the control (control 1) cultured with IL-12 and PHA showed the tyrosine phosphorylation of Stat4, PBMCs from the patient with the C-to-U conversion (patient 1) cultured with IL-12 and PHA faintly showed the tyrosine phosphorylation of Stat4 (Fig. 3 a). These results suggest that the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA is associated with reduced signal transduction of IL-12 for IFN- γ production by PBMCs.

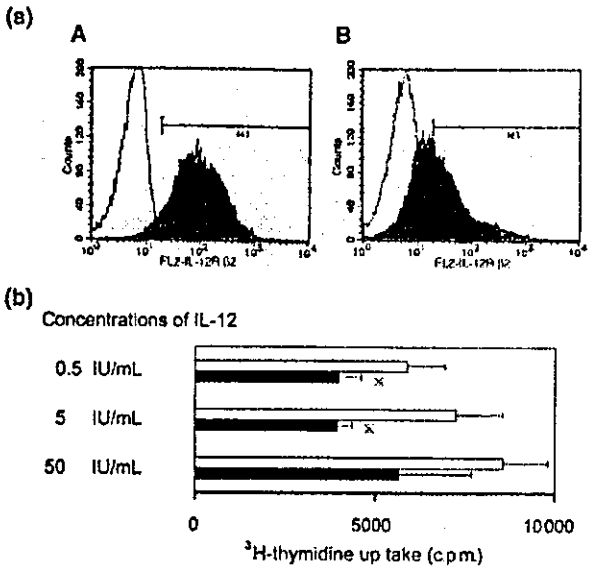


**Fig. 2.** (a) Expression of the IL-12R β2 chain without or with the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in Ba/F3 cell clones transfected with wild-type (A) or variant-type (B) IL-12R β2 cDNA. $10^6$ cells in 100 μL of staining buffer were incubated with 1 μg/mL rat anti-hu IL-12R β2(2B6) mAb (black) or isotype control Ab (white) for 30 min, followed by incubation with biotinylated anti-rat-Ig F(ab)$^2$ fragments for 30 min, and finally incubated with streptavidin conjugated to PE (PharMingen) for 30 min. IL-12R β2 expressing cells were detected by flow cytometry. The staining intensity and the percentage (50.1%) of cells expressing the IL-12R β2 chain in the clone with the C-to-U conversion were lower than those (the percentage: 94.4%) in the clone without the conversion. (b) Proliferative responses of the Ba/F3 cell clones transfected with wild-type or variant-type IL-12R β2 cDNA containing the C-to-U conversion at nucleotide 2451. Ba/F3 were stimulated with IL-12 (0.5, 5 or 50 IU/mL) for 24 h. The proliferative responses of the Ba/F3 cell clones transfected with variant-type IL-12R β2 cDNA (closed column) to IL-12 (0.5 IU/mL, 5 IU/mL) were significantly (*$P < 0.05$ for each) lower than those of the Ba/F3 cell clones transfected with wild-type IL-12R β2 chain cDNA (open column). Means+SD (c.p.m.) of triplicate are shown.

### In vitro *IgE production suppressed by IL-12*

Next, we examined the effects of IL-12 on *in vitro* IgE production by PBMCs from the atopic patients with the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA (Fig. 3 b). IgE production by PBMCs cultured with IL-4 and Derf 1 for 14 days was suppressed by IL-12 as well as by IFN-γ in the atopic patients without the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA. In contrast, in the

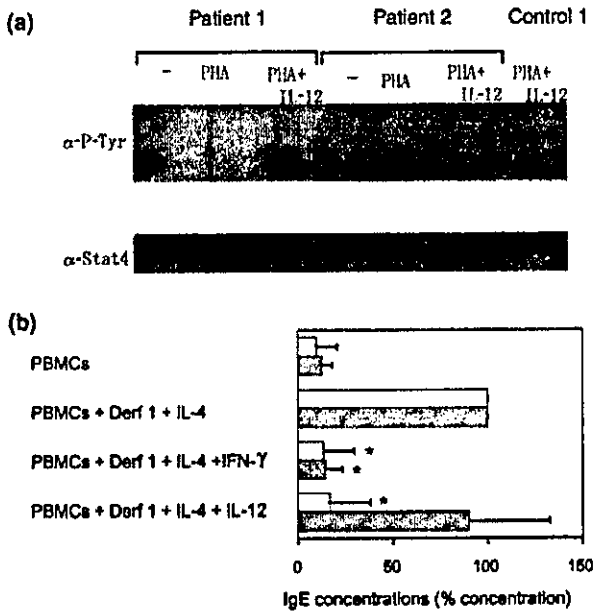


**Fig. 3.** (a) Tyrosine phosphorylation of Stat4 in PBMCs. PBMCs from the patients with or without the C-to-U conversion at nucleotide 2451 (Ala 604 Val) on the IL-12R β2 chain and the control subject were cultured with PHA and IL-12. Cell lysates were immunoprecipitated with anti-Stat4, resolved by SDS-PAGE, transferred to a nylon membrane and blotted sequentially with anti-phosphotyrosine (α-P-Tyr, upper panel) or anti-Stat4 (a Stat4, lower panel). No band was shown on blotting with α-P-Tyr in PBMCs from the patients cultured with or without PHA. Although PBMCs from the patient without the C-to-U conversion (patient 2) and the control (control 1) cultured with both IL-12 and PHA showed a band on blotting with α-P-Tyr, indicating phosphorylation of Stat4, PBMCs from the patient with the C-to-U conversion (patient 1) cultured with both IL-12 and PHA showed a very faint band. (b) Suppression of *in vitro* IgE production. IgE production by PBMCs was induced by Derf 1 and IL-4. Suppression by IFN-γ (1000 U/mL) and IL-12 (5 IU/mL) was represented by % concentration. % concentration = 100 X IgE concentration in the culture with IFN-γ or IL-12/IgE concentration in the culture without both IFN-γ and IL-12. In the atopic patients (open column, mean +SD, $n = 3$) without the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA, IgE production was significantly ($^*P<0.05$ for each) suppressed by IL-12 as well as by IFN-γ. In contrast, in the atopic patients (shadow column, mean+SD, $n = 3$) with the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA, IgE production was not suppressed by IL-12, whereas it was significantly ($^*P<0.05$) suppressed by IFN-γ.

atopic patients with the C-to-U conversion, IgE production by PBMCs was not suppressed by IL-12 whereas it was suppressed by IFN-γ.

## Discussion

Our results showed that RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion in atopic subjects caused impairment of the IL-12 signal cascade, and then reduced IFN-γ production by PBMCs following IL-12 stimulation, resulting in impaired down-regulation of IgE production.

Recently, it has been reported that a homozygous nonsense mutation of the IL-12R β1 chain gene causes impairment of salmonella and mycobacterial immunity [12, 13]. The development of Th1 lymphocytes is disturbed in IL-12 or IL-12R β1 knockout mice [8, 14]. In this study, atopic subjects with RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion, did not exhibit any impairment of salmonella and mycobacterial immunity. The IL-12R β2 subunit, similar to the IL-12R β1 subunit, is a member of the gp130-type subgroup of the cytokine receptor superfamily. However, each of the two IL-12R subunits itself is more closely related to gp130 than to each other. In contrast to IL-12R β1, which does not contain any tyrosine residues, the cytoplasmic region of IL-12R β2 contains three tyrosine residues, suggesting an important role of the β2 subunit in IL-12 signal transduction [5]. Presky et al. [5] reported that Ba/F3 cells transfected with IL-12R β2 alone proliferates in response to human IL-12 although the role of endogenous mouse IL-12R β1 in IL-12 signal transduction in these transfectants cannot be ruled out. Thus, IL-12R β2 is different from IL-12 β1 in both structure and function.

It has been noted that RNA editing, a post-transcriptional modification, plays an important role in achieving molecular diversity [10]. The forms of RNA editing are classified into two categories, namely, C-to-U and A-to-I conversions that occur by nucleotide deamination. The best example of C-to-U editing occurs within RNA transcripts encoding apolipoprotein B RNA [15, 16] and is mediated by the activity of cytidine deaminase. Recently, A-to-I conversions have been observed within a growing number of RNAs, including those encoding several glutamate receptor subunits [17–19] and the G-protein-coupled serotonin 2C receptor [20]. Moreover, RNA editing of WT1, that is thought to be a susceptibility gene for Wilm's tumour, converts U-to-C at nucleotide 839, transforming genomically encoded leucine into proline [21]. The leucine, non-edited form of the protein, is a more potent transcriptional repressor than the proline-containing isoform, suggesting that this editing might be associated with the development of Wilm's tumour. The neurofibromatosis type-1 gene product neurofibromin, associated with an increased risk of neurofibromatosis type 1 (NF1), is thought to serve as a tumour suppressor [10]. Although editing of C 2914 of this gene occurs at low levels (<2%) in control subjects, patients with NF1 show almost eight times the level of editing at this position. The editing at this site converts a CGA (Arg) codon into a UGA (stop) codon, suggesting that NF1 patients lack sufficient quantities of neurofibromin [22]. Furthermore, it is suggested that a reduction in the amount of this potential tumour suppressor may prevent appropriate regulation of the Ras signalling pathway, leading to unchecked cellular proliferation and cancer [22].

The expression levels of IL-12R β2 and the proliferative responses to IL-12 in variant-type IL-12R β2 transfected Ba/F3 were lower than those of wild-type. The RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion, associated with atopy in this study may disturb conformational binding of IL-12 to IL-12R, although the possibility that the antibody is affected by the conformational change cannot be excluded.

In the immunological system, RNA editing has not been reported. An immunological system as well as a neurological system require molecular diversity. From this viewpoint, it is natural that lympocytes utilize RNA editing for the regulation of the function on the cytokine and cytokine receptor. Activation induced cytidine deaminase (AID), which is the causative gene for the hyper-IgM syndrome, is homologous to that of mammalian RNA editing deaminase, APOBEC1.

AID had deaminase activity when tested for deoxycytidine deamination. Therefore, AID may be another RNA editing

deaminase although its substrate has not yet been identified. AID or other enzymes may be the candidate for RNA editing in an immunological system. It is indicated that the regulatory spacer and mooring sequences (such as UGAUAC, AAUU, UGAUCAGUAUA, respectively in human apolipoprotein B) may provide binding sites for distinct components of the cellular editing machinery: once bound, the factor(s) would be correctly positioned to edit nucleotides within a certain distance upstream from the binding site [10, 23]. Therefore, we investigated these sequences in the IL-12R β2 chain gene. However, we were not able to find any motifs.

Atopic disorders develop by a combination of genetic risk factors and environmental factors. Very recently, Karcher et al. found the temperature sensitivity of RNA editing reaction in the plastid ndhB transcript [24]. Therefore RNA editing, one of post-transcriptional modifications, in atopic patients may be induced by a combination of genetic factors and environmental factors. Experiments along these lines are now under way. Our results indicate that several candidate genes that have failed to show association should be investigated at the mRNA level. Although several polymorphisms or mutations of the genes associated with atopy have been reported [9, 25–27], this study is the first report indicating that atopy is associated with RNA editing, a post-transcriptional modification.

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# Induction of granulocyte-macrophage colony-stimulating factor by a new cytokine, ML-1 (IL-17F), via Raf I-MEK-ERK pathway

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**Background:** ML-1 (IL-17F) is a recently discovered cytokine, and its function remains elusive. GM-CSF is a crucial cytokine for the maturation of various cell types and regulates allergic airway inflammation.

**Objective:** The functional effect of ML-1 in the expression of GM-CSF was investigated.

**Methods:** The levels of gene and protein expression in normal human bronchial epithelial cells (NHBEs) in the presence or absence of various kinase inhibitors or, in some cases, of a Raf1 dominant-negative mutant were determined by RT-PCR and ELISA, respectively. Western blotting was performed to investigate kinase activation.

**Results:** The results showed first that ML-1 induces, in a time-dependent and dose-dependent manner, the gene and protein expression for GM-CSF NHBEs, which are associated with activation of Raf1 and MAP kinase kinase (MEK) kinases. Selective MEK inhibitors, PD98059 and U0126, and Raf1 kinase inhibitor I significantly inhibited ML-1–induced GM-CSF production. Furthermore, overexpression of Raf1 dominant-negative mutants inhibited IL-17F–induced GMCSF expression. The combination of PD98059 and Raf1 kinase inhibitor I completely blocked GM-CSF production, whereas 2 protein kinase C inhibitors, Ro-31-7549 and GF109203X, and a phosphatidylinositol 3-kinase inhibitor, LY294002, showed no inhibitory effect.

**Conclusion:** These findings suggest that ML-1 induces GM-CSF expression through the activation of the Raf1-MEK–extracellular signal-regulated kinase 1/2 pathway. (J Allergy Clin Immunol 2004;114:444-50.)

*Key words: Bronchial epithelial cells, ERK1/2, GM-CSF, MAP kinase, Raf1*

*Abbreviations used*
DMSO: Dimethyl sulfoxide
ERK: Extracellular signal-regulated kinase
MEK: MAP kinase kinase
NF-κB: Nuclear factor κB
NHBE: Normal human bronchial epithelial cell
PI3K: Phosphatidylinositol 3-kinase
PKC: Protein kinase C

Airway allergic inflammation is characterized by activation and migration of various inflammatory cells, such as eosinophils, granulocytes, and macrophages. The accumulation of these cell types causes bronchoconstriction, lung damage, and fibrosis. Bronchial epithelial cells play an important role in the regulation of airway inflammation through their ability to express a wide range of proinflammatory cytokines, including GM-CSF.[1] Investigation of the regulatory pathway involving modulation of airway epithelium by cytokines is thus important to uncover the pathogenic mechanisms of airway inflammation.

GM-CSF plays a pivotal role in inflammatory and immunologic processes. Release of GM-CSF in the airway can mediate acute inflammatory responses as well as initiate and perpetuate local immune responses. Elevated levels of GM-CSF have been well demonstrated in bronchoalveolar lavage fluid, endobronchial biopsy, and sputum samples from asthmatics.[2-4] Elevated levels of GM-CSF, mainly derived from epithelial cells, have been demonstrated to increase eosinophil activation and survival in asthmatics.[5-7] Bronchial asthma is an inflammatory disease of the airways characterized by eosinophil infiltration of the lung and airway hyperreactivity. GM-CSF stimulates the recruitment and activation of eosinophils via β-integrin–mediated adhesion to epithelial and endothelial cells[8] and prolongs eosinophil survival via inhibition of apoptosis.[9-11] Moreover, mouse models of asthma and diesel-induced hyperresponsiveness have demonstrated an association between epithelial cell-derived GM-CSF and airway hyperresponsiveness.[12,13] These findings suggest that GM-CSF is 1 of the critical cytokines in the pathogenesis of allergic airway inflammation, and airway epithelium plays a pivotal role in causing and perpetuating airway inflammation via the induction of GM-CSF. However, the regulatory mechanisms of GM-CSF expression in airway epithelium have not been well understood.

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Recently, we and others independently discovered a novel cytokine, ML-1[14] (IL-17F[15,16]), belonging to the IL-17 gene family, but its function and signaling pathways remain to be defined. ML-1 is expressed in activated $CD4^+$ T cells, basophils, and mast cells, 3 important cell types involved in allergic inflammation.[14] Significantly, upregulated ML-1 gene expression is seen at sites of allergen challenge in the airways of asthmatics,[14] but its involvement in pulmonary allergic inflammation is unclear. To gain further understanding of the function and signaling pathways of ML-1, the role of ML-1 in the expression of GM-CSF was investigated. In this article, we provide evidence that ML-1 is able to induce GM-CSF expression in bronchial epithelial cells, involving the activation of the Raf1–MAP kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) 1/2 signaling pathway.

## METHODS

### Cell culture and human recombinant ML-1

Normal human bronchial epithelial cells (NHBEs) were purchased from Clonetics (San Diego, Calif) and cultured in bronchial epithelial basal medium (Clonetics) containing 0.5 ng/mL human recombinant epidermal growth factor, 52 μg/mL bovine pituitary extract, 0.1 ng/mL retinoic acid, 0.5 μg/mL hydrocortisone, 5 μg/mL insulin, 10 μg/mL transferrin, 0.5 μg/mL epinephrine, 6.5 ng/mL tri-iodothyronine, 50 μg/mL gentamicin, and 50 μg/mL amphotericin-B (Clonetics). The cells were cultured for no more than 3 passages before the analysis. In the case of the experiments using Raf1 dominant-negative mutants, a bronchial epithelial cell line, BEAS-2B, was used instead of NHBE for efficient transfection and was cultured in Hanks' F12/Dulbecco modified Eagle medium (Biofluids, Rockville, Md) with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin (Life Technologies-BRL, Gaithersburg, Md). Human recombinant ML-1 was generated as described previously.[14] Endotoxin levels were tested by using Kinetic-QCL Chromogenic Limulus amebocyte lysate (Bio Whittaker, Walkersville, Md). Endotoxin levels were undetectable. The cells were treated with ML-1 (10 and 100 ng/mL) for various periods.

### Gene expression of GM-CSF

Total RNA was extracted by using RNeasy (Qiagen, Chatsworth, Calif) from $1 \times 10^6$ cells at 1, 2, 4, 12, and 24 hours after stimulation with 10 and 100 ng/mL ML-1. cDNAs were synthesized from 500 ng of total RNA in the presence of Moloney murine leukemia virus reverse transcriptase (1 U/reaction; Sigma, St Louis, Mo), oligo (dT) primer, and reaction buffer at 42°C for 90 minutes, followed by PCR. The sequences of PCR primers for GM-CSF were forward, 5′-GTGGCCTGCAGCATCTCT-3′, and reverse, 5′-AAAGGGGATGACAAGCAGAA-3′; and for G3PDH were forward, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. The amplification reaction was performed for subsaturating cycles with denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds. The expected size for GM-CSF was 375 bp and for G3PDH was 450 bp. The PCR products were detected by ethidium bromide staining and quantified by video densitometry with Image 1.61 software (National Institutes of Health Public Software, National Institutes of Health, Bethesda, Md). The level of GM-CSF gene expression was quantified by calculating the ratio of densitometric readings of the band intensity for GM-CSF and G3PDH from the same cDNA sample. The values are expressed as means ± SDs (n = 3 experiments). In all cases, the induction of GM-CSF by ML-1 was clearly evident, allowing quantitative comparison by RT-PCR.

### Protein levels of GM-CSF

GM-CSF protein levels in the supernatants and cell lysates of ML-1–stimulated cells were determined with a commercially available ELISA kit (Biosource, Camarillo, Calif) according to the manufacturer's instructions. Cell supernatants were harvested from cultures in the absence or presence of 10 or 100 ng/mL ML-1 at 2, 6, 12, 24, or 48 hours after stimulation. The amount of secreted GM-CSF was determined by the ELISA and expressed as the amount recovered per $10^6$ cells. Cells corresponding to the supernatant samples described were lysed in 0.5 mL Nonidet P-40 lysis buffer (20 mmol/L Tris [pH 7.4], 4 mmol/L EDTA, 1 mmol/L phenylmethanesulfonyl fluoride, 100 mg/mL aprotinin, 200 mg/mL leupeptin, 50 mmol/L NaF, 5 mmol/L $Na_4P_2O_7$, and 1% Nonidet P-40 [all Sigma]). The GM-CSF concentration of cell lysate was reported as the amount recovered per $10^6$ cells. The values are expressed as means ± SDs (n = 6 experiments).

### ML-1–induced Raf1 and MEK activation and the effect of inhibitors

For analysis of activation of the Raf1-MEK-ERK1/2 pathway, NHBEs were treated with ML-1 (100 ng/mL) and in some cases with or without the treatment with a Raf1 kinase inhibitor I or the vehicle control ($Me_2SO$), for 1 hour. The final concentration of $Me_2SO$ did not exceed 0.1% (vol/vol). After treatment and lysis of the cells, the cellular extracts ($1 \times 10^6$ cell equivalents/lane) were suspended with an equal volume of 2 times loading buffer (0.1 mol/L Tris-HCl, pH 6.8; 4% SDS; 0.005% bromphenol blue; and 20% glycerol) containing 2-mercaptoethanol (0.7 mol/L), subjected to 4% to 20% Tris-glycine gel electrophoresis (NOVEX, San Diego, Calif), and probed with various antibodies: rabbit anti-MEK1/2 antibody, antiphospho-MEK1/2 antibody (Cell Signaling Technology, Beverly, Mass), anti-Raf1 antibody, and antiphospho-Raf1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), followed by incubation with peroxidase-linked donkey anti-rabbit Ig antibody (Amersham Biosciences, Piscataway, NJ) and visualized with enhanced chemiluminescence.

For analysis of the effects of kinase inhibitors, the cells were treated in the presence or absence of the following kinase inhibitors at varying doses: MEK1/2 inhibitors, PD98059 (2′-amino-3′-methoxyflavone; Calbiochem, La Jolla, Calif) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis[o-aminophenylmercapto] butadiene; New England Bio Labs, Beverly, Mass); p38 inhibitor, SB202190 (4-[4-fluoro-phenyl]-2-[4-hydroxyphenyl]-5-[4-pyridyl] 1H-imidazole; Calbiochem); a Raf1 kinase inhibitor I (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone; Calbiochem); a c-Jun N-terminal kinase inhibitor, SP600125 (Anthra [1,9-cd]pyrazol-6[2H]-one; Calbiochem); protein kinase C (PKC) inhibitors, Ro-31-7549 (2-[1-(3-aminopropyl) indol-3-yl]-3-[1-methylindol-3-yl] maleimide acetate; Calbiochem) and GF109203X (3-[1-(dimethylaminopropyl) indol-3-yl]-4-[indol-3-yl] maleimide, 3-[1-(3-[dimethylamino]propyl)-1H-indol-3-yl]-4-[1H-indol-3-yl]-1H-pyrrole-2,5-dione; Sigma); a phosphatidylinositol 3-kinase PI3K inhibitor, LY294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one; Calbiochem); and a vehicle control, dimethyl sulfoxide (DMSO; $Me_2SO$) for 1 hour before treatment with ML-1 (100 ng/mL). The cells and cell supernatants were harvested at 2 and 24 hours after stimulation for analyses of RT-PCR and ELISA, respectively. The final concentration of DMSO did not exceed 0.1% (vol/vol). GM-CSF gene expression and protein levels in the supernatants were determined as described. The values are expressed as means ± SDs (n = 4 experiments).

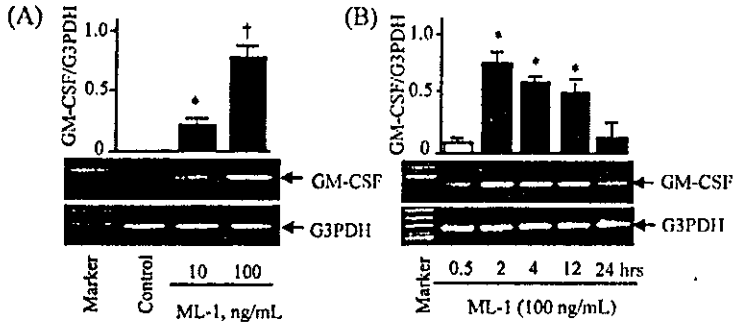


FIG 1. A, Induction of GM-CSF gene expression by different doses of ML-1. *$P$ < .05 was considered significant versus control. †$P$ < .05 was considered significant versus 10 ng/mL ML-1–stimulated cells. B, Time course study of GM-CSF gene expression. The values are expressed as means ± SDs ($n$ = 3). *$P$ < .05 was considered significant versus the intensity of 0.5-hour time point.

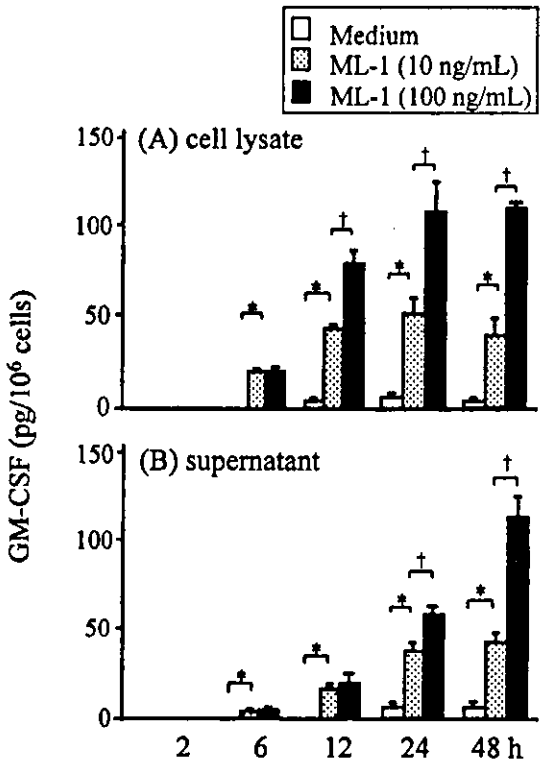


FIG 2. GM-CSF protein levels in lysates (A) and supernatants (B) of NHBEs. The values are expressed as means ± SDs ($n$ = 6). *$P$ < .05 was considered significant versus control. †$P$ < .05 was considered significant versus 10 ng/mL ML-1–stimulated cells.

## Overexpression of Raf1 dominant-negative mutant in BEAS-2B cells

The plasmid encoding pCMV-RafS621A vector (dominant-negative mutant of Raf1) cloned into pCMV and a control vector were purchased from Clontech (Palo Alto, Calif). The plasmids were prepared by using the Qiagen plasmid DNA preparation kit. NHBEs were difficult to use for transfection experiments. An epithelial cell line, BEAS-2B, was used instead. The cells were cultured on 100-mm plates and were transfected by an Effectene Reagent (Qiagen) according to the manufacturer's instructions. The cells were selected with 500 ng/mL Geneticin (G418; Gibco/BRL, Grand Island, NY). After selection, the cells were seeded into 6-well culture plates. The cells were near confluent, and the cells and cell supernatants were then harvested at 2 and 24 hours after stimulation with 100 ng/mL ML-1 for analyses of RT-PCR and ELISA, respectively. GM-CSF gene expression and protein levels in the supernatants were determined as described. The values are expressed as means ± SDs ($n$ = 3 experiments).

## Data analysis

The statistical significance of differences was determined by ANOVA. The values are expressed as means ± SDs from independent experiments. Any difference with $P$ values <.05 was considered. When ANOVA indicated a significant difference, the Scheffe $F$ test was used to determine the difference between groups.

## RESULTS

To examine whether ML-1 was able to induce GM-CSF expression, NHBEs were stimulated with varying doses of ML-1, and the levels of gene expression and protein production in cell lysates and supernatants were analyzed. The results showed first that although GM-CSF gene expression was not seen in control culture, ML-1 induced, in a dose-dependent manner, the expression of GM-CSF at the 2-hour time point (Fig 1, *A* and *B*). In the time course experiments, ML-1–induced GM-CSF gene expression peaked at the 2-hour time point in ML-1 (100 ng/mL)–treated NHBEs (Fig 1, *C* and *D*) and returned to baseline 24 hours after stimulation. To analyze the protein expression for GM-CSF, NHBEs were cultured in the absence or presence of varying concentrations of ML-1 at 5 different time points. GM-CSF proteins were not detected in the absence of ML-1 in NHBEs. The levels of GM-CSF in cell lysates were significantly increased 6 hours after stimulation with 10 and 100 ng/mL ML-1 and reached plateau at the 12-hour and 24-hour time points, respectively (Fig 2, *A*), whereas the levels of secreted GM-CSF were significantly elevated 24 hours after stimulation and were increased further at 48 hours (Fig 2, *B*).

Bronchial epithelial cells are exposed to numerous environmental stimuli, which can activate intracellular

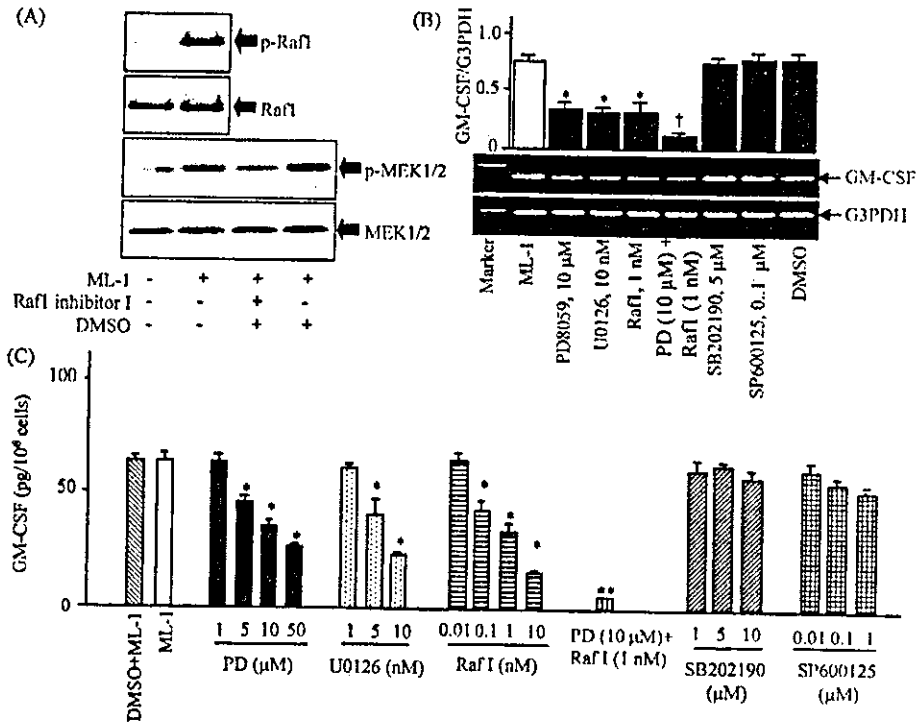


**FIG 3. A,** Western blotting analysis of Raf1 and MEK activation. The results shown are representative of 3 separate experiments. Effect of kinase inhibitors on GM-CSF gene **(B)** and protein **(C)** expression in NHBEs. The values are expressed as means ± SDs (n = 4 experiments). $^{*}P < .05$ was considered significant versus ML-1–stimulated cells. $^{\dagger}P < .05$ was considered significant versus single inhibitor.

signaling cascades, leading to upregulated gene expression and a wide range of responses, such as cytokine expression, proliferation, and apoptosis.[17] The mitogen-activated protein kinase family is likely to be central to these processes because they are known to regulate intracellular signal transduction in response to many agonists, including growth factors, cytokines, hormones, oxidants, and environmental stress factors.[18] To investigate ML-1–mediated signaling events leading to the induction of GM-CSF, the activation of the Raf1-MEK-ERK1/2 pathway was investigated. First, Western blotting analysis revealed activation of Raf1 and MEK1/2 in ML-1–stimulated cells (Fig 3, *A*), and preincubation of the cells with a Raf1 kinase inhibitor I (1 nmol/L) for 1 hour diminished the activation of MEK1/2 in comparison with that seen in the vehicle control culture (Fig 3, *A*). Furthermore, analysis of various kinase inhibitor effect on ML-1–induced GM-CSF expression showed that pretreatment of the cells for 1 hour with each of the selective MEK inhibitors, PD98059 (10 μmol/L), U0126 (5 nmol/L), and Raf1 kinase inhibitor I (1 nmol/L), significantly decreased the levels of ML-1–induced GM-CSF gene expression in NHBEs (Fig 3, *B*). As shown in Fig 3, *C*, 1 hour pretreatment of the cells with PD98059, U0126, and Raf1 kinase inhibitor I significantly attenuated, in a dose-dependent manner, the production of GM-CSF, whereas 1 hour pretreatment of the cells with vehicle alone (0.05% DMSO) did not affect GM-CSF release. In

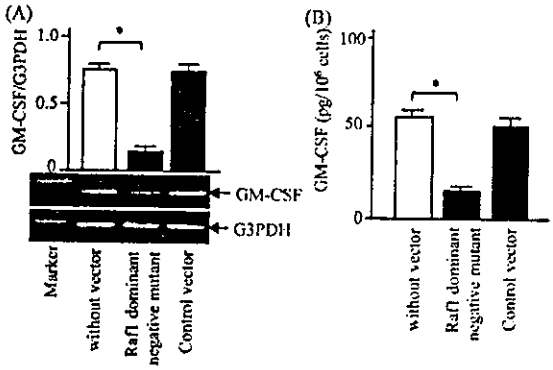


**FIG 4.** Effect of overexpression of Raf1 dominant-negative mutants on GM-CSF gene **(A)** and protein **(B)** expression in BEAS-2B cells. The values are expressed as means ± SDs (n = 3). $^{*}P < .05$ was considered significant versus the intensity of ML-1–stimulated cells without vector.

addition, the protein levels of GM-CSF were unchanged in ML-1–treated cells in the presence of a p38 kinase inhibitor, SB202190, and a JNK inhibitor, SP600125, even at a dose of 10 μmol/L and 1 μmol/L, respectively (Fig 3, *C*). Although induction of GM-CSF is partially inhibited by PD98059, U0126, or Raf1 kinase inhibitor I even at relatively high doses (50 μmol/L, 10 nmol/L, and 10 nmol/L, respectively), the combination of 10 μmol/L