先端医学講座 31

ヒスタミン，メサコリン，アセチルコリンを用いた気道過敏性試験の相違

The differences of the bronchial provocation test using histamine, methacholine, acetylcholine

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Key words：気管支喘息，気道過敏性，アセチルコリン，メサコリン，ヒスタミン

Abstract

気道過敏性検査には間歇吸入法と連続吸入測定法があり，非特異的収縮物質としてメサコリン(Mch)，アセチルコリン(Ach)，ヒスタミン(Hist)などがある。これらの相違については当センターにおける日常臨床から得られた臨床研究においてAch気道過敏性は血清ECP値と相関し，吸入ステロイドの反応性が良好であることから好酸球性炎症を反映すること，またHist気道過敏性は生理学的により末梢気道を反映していること，さらにAch，Hist気道過敏性をほぼ同時期に行った結果からは気道過敏性にはheterogeneityが存在することが明らかとなったが，根本的な違いは未だに充分には証明されていない。

はじめに

気管支喘息は気道の慢性炎症性疾患であり，気道過敏性の発現は喘息の基本的な病態のひとつであり喘息発症の必要条件である。気道過敏性とは「非喘息の人にとって刺激とならない微細な物質や変化に対して容易，かつ過剰に反応し，気道が狭窄する」状態のことをいい，気道収縮をきたす刺激としては第一に気道平滑筋への直接的な刺激としてメサコリン(Mch)，アセチルコリン(Ach)，ヒスタミン(Hist)などが挙げられ，第二には肥満細胞や無随知覚神経末端からの化学伝達物質放出（ロイコトリエン，プロスタグランジン，Histなど）による間接的な平滑筋刺激が挙げられる。Mch，Ach，Histは気道収縮をきたす非特異的収縮物質であるが，生体における意義についてはまだ充分には解明されていない。

また気道過敏性検査は喘息の診断だけでなく，重症度，治療経過，長期予後など喘息経過中にも臨床応用が可能である。当センターでは成人喘息患者を対象とし，日常臨床においてほぼ同一時期にAchとHistの標準法による気道過敏性検査を行い，喘息の診断とともに治療経過，予後について検討している。ここでは気道過敏性検査の方法とそれに用いる非特異的収縮物質であるMch，Ach，Histに

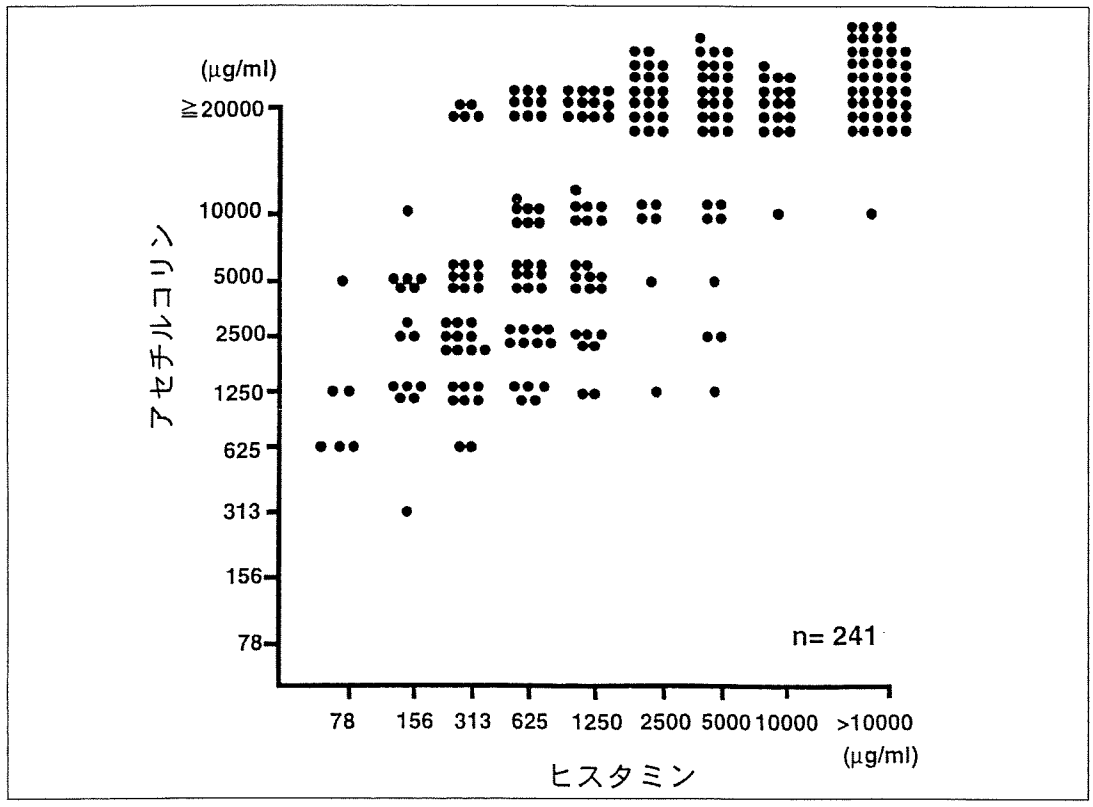

図1　アセチルコリンとヒスタミンの閾値

よる違いについて，発症年齢別の差違や治療効果など臨床成績から言及することにする。

1. 気道過敏性検査の方法

気道過敏性検査に用いられる薬剤吸入誘発検査ではいろいろな濃度のエロゾールを吸入させ，その吸入ごとにFEV1を測定する間歇吸入法と，濃度の異なるエロゾールを吸入させながら呼吸抵抗を連続的に測定する連続吸入測定法がある。間歇吸入法にはAsthma and Allergic Disease Center (AADC)[1]による標準法，Hargreave[2]らによるtidal breathing法と吸入標準化研究会による吸入試験の標準法[3]があり，連続吸入測定法としてはアストグラフ法[4]が広く用いられている。非特異的収縮物質については，AADC，tidal breathing法においてはMch，Histが，標準法ではAch，Histが用いられている。アストグラフ法では連続吸入測定のため咳嗽を誘発しにくいMchが用いられる。AchとMchは構造式が類似しており，Mchでメチル基がひとつ増える以外の違いはない。

2. アセチルコリン，ヒスタミンのheterogeneity

当センターでは成人喘息患者を対象とし，日常臨床においてAch，Histの標準法による気道過敏性検査を行っている。この2つの検

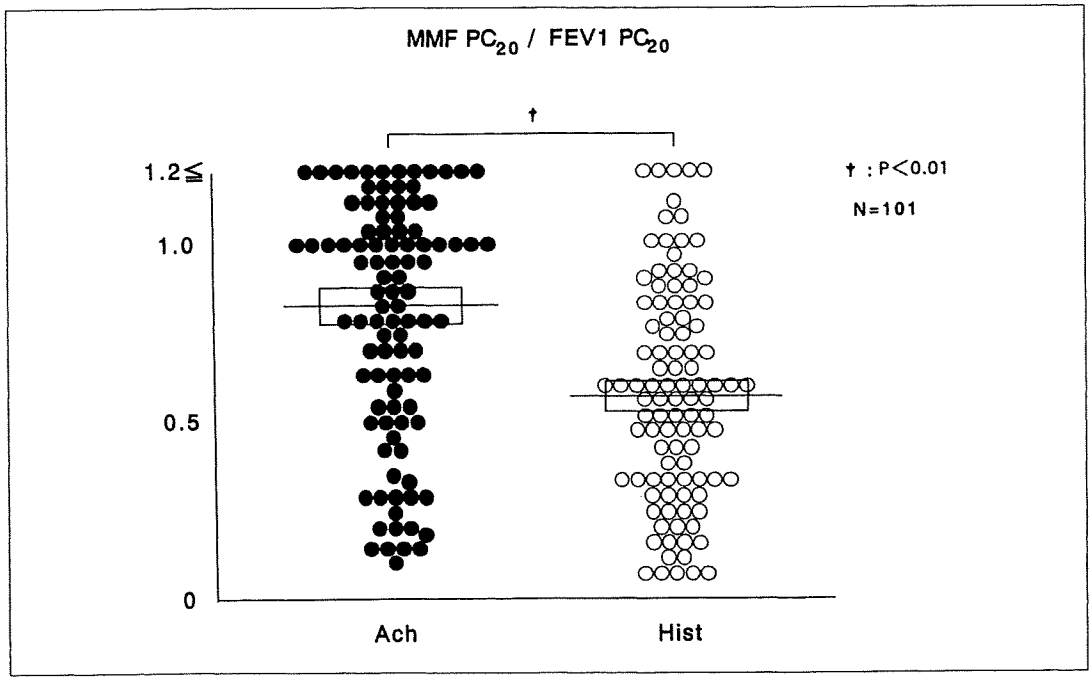

図2　Hist気道過敏性はAch気道過敏性と比べてより末梢気道を反映する

査は1週間以上の間隔を空け，1ヶ月以内に行っており，ほぼ同時期の状態を反映していると考えている。図1は同時期にAch，Histの気道過敏性検査を行った241症例の閾値を示す[5]。Ach，Histともに過敏性のある患者群では両者に相関があり，かつHist閾値がAch閾値と比較して低値である。しかし，Ach閾値>10,000μg/mL，すなわちAch気道過敏性が軽度である症例のHist気道過敏性をみると313μg/mLから>10,000μg/mLと幅広く分散している。AchやHistは一般的にロイコトリエンやPAF等を含めた非特異的収縮物質といわれているが，それぞれが固有のreceptorを介しての反応であり，気道におけるreceptorの存在部位が異なることを考えると，Ach，Histに対する反応性のheterogeneityが存在することが示唆される。

3. アセチルコリン，ヒスタミンの気道収縮反応の差違

Ach，Histの気道収縮反応がどのように異なるのかは充分には解明されていない。そこで1999-2001年に受診した成人喘息患者のうち抗炎症薬をしていない初診患者101症例を対象としほぼ同時期にAch，Hist気道過敏性検査を行い，末梢気道の評価を行った。Ach，Hist標準法において一般的な評価である$AchPC_{20}$や$HistPC_{20}$はFEV1が20％低下した時点でのAch濃度を指すが，ここではより末梢の指標として考えられているMMFのレベルで20％低下した濃度を$MMF\text{-}PC_{20}$として$FEV1\text{-}PC_{20}$と$MMF\text{-}PC_{20}$の両方を測定し，その比について検討した[6]。結果，$MMF\text{-}PC_{20}$と$FEV1\text{-}PC_{20}$の比はAchと比較してHistがより低値であった（図2）。これはHist気道過敏性ではFEV1が20%低下するHist濃度より，

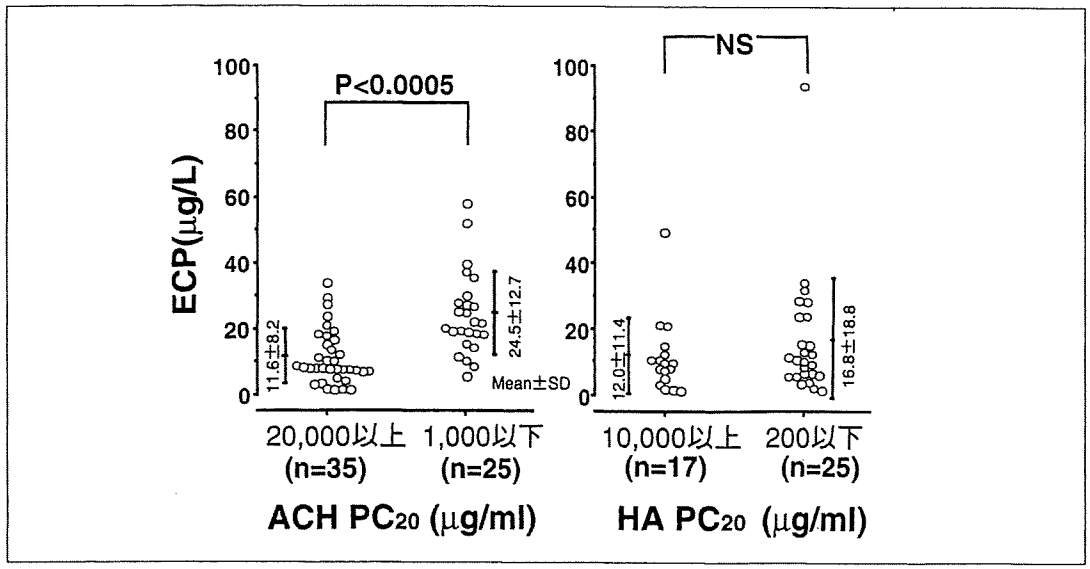

図3　気道過敏性と血清ECP値との関連

より少ないHist濃度でMMFレベルではすでに20%以上低下することを示している。MMFはより末梢気道を反映することからHist気道過敏性はAch気道過敏性と比較してより末梢気道病変を反映することが示唆される。

4. 気道過敏性の炎症としての評価

Eosinophilic cationic protein(ECP)は好酸球性炎症を反映する一つの指標である。当院では1997年に外来通院中の成人喘息患者で減感作療法以外には無治療で，過去2年以上無症状の患者を対象としbeclomethasone dipropionate(BDP) CFC 200-600μg／日による治療を行い，経時的に気道過敏性試験を実施，また検査直前に血清ECP値を測定し，$AchPC_{20}$と血清ECP値の相関について検討した[7]。結果，Ach気道過敏性を$AchPC_{20} \geqq 20,000\mu g/mL$の正常域群と$AchPC_{20} \leqq 1,000\mu g/mL$の高度亢進群で比較をすると高度亢進群で血清ECP値が有意に高かった（図3）。一方，Hist気道過敏性では$HistPC_{20} \geqq 10,000\mu g/mL$の正常域群と$HistPC_{20} \leqq 200\mu g/mL$の高度亢進群で比較をすると血清ECP値は有意差を認めなかった。このことからAch気道過敏性はHist気道過敏性と比較して，好酸球性炎症をより反映することが示唆された。

5. 発症年代別の気道過敏性
—小児発症喘息と成人発症喘息の比較—

成人喘息は発症年代別に小児発症，成人再発，成人発症に分類されるが[8]，発症年齢別の気道過敏性の差違については充分に解明されていない。そこで2003年時に外来通院中の成人喘息患者275名を対象とし，外来初診時に一週間以上の間隔をあけてかつ一ヶ月以内にAch, Hist気道過敏性を行い発症年代別に検討した[9]。またアトピー素因の有無から病型分類を追加した。発症年齢別の内訳は成人

発症187名（アトピー型113名，非アトピー型74名），成人再発型53名，小児発症35名であった。小児発症は成人発症と比較して$AchPC_{20}$, $HistPC_{20}$が有意に低値であり気道過敏性が亢進していた。また成人再発と比較すると小児発症は$HistPC_{20}$では有意に低値であったが，$AchPC_{20}$とは統計学的に有意差を認めなかった。成人発症，成人再発では$AchPC_{20}$, $HistPC_{20}$ともに差を認めなかった。さらに成人発症をアトピー型，非アトピー型に分類すると非アトピー型では$AchPC_{20}$, $HistPC_{20}$ともにアトピー型と比較して気道過敏性が軽度であった。

アトピー型が非アトピー型と比較して気道過敏性が亢進していることはこれまでの知見からも伺えることであるが，今回検討した小児発症（成人喘息）は全例がアトピー型であること，成人再発型の多くはアトピー型であることをふまえるとアトピー素因の有無だけでは気道過敏性亢進のメカニズム，また$AchPC_{20}$, $HistPC_{20}$の違いを充分には説明できないと考えられる。

6. 成人喘息の吸入ステロイド治療後の気道過敏性の反応性

気道過敏性検査は治療効果判定として有用であろうか？ 2001年時に外来通院中の成人喘息患者260名を対象とし，抗炎症薬である吸入ステロイド（CFC-BDP）を使用し，Ach, Hist気道過敏性がどのように変化するかについて，気道過敏性をそれぞれ$AchPC_{20}$, $HistPC_{20}$と評価し，治療前後でその比（post $AchPC_{20}$ / pre $AchPC_{20}$）について検討した[10]。

結果，吸入ステロイドを使用しない場合は小児発症喘息，成人再発型喘息，成人発症喘息ともに気道過敏性の臨床的に有意な改善と考えられる4倍以上の改善を認めなかった。また吸入ステロイド使用した場合では，成人発症喘息および成人再発型喘息ではAch気道過敏性が前値と比較して約7倍以上改善したが，小児発症喘息では約3倍の改善であり臨床的に有意な改善を認めなかった。またHist気道過敏性については成人発症喘息，成人再発型喘息および小児発症喘息ともに吸入ステロイド治療後も有意な改善を認めなかった。このことからAch気道過敏性はHist気道過敏性と比較して吸入ステロイドの反応性が良好，すなわち炎症性の気道過敏性を反映しているといえるであろう。

7. アセチルコリン気道過敏性が正常化する症例

吸入ステロイドが普及するとともに近年では喘息の臨床症状だけではなく，気道過敏性が正常域まで改善する症例が増加している。Achが正常域（$AchPC_{20}$>20,000μg/mL）まで改善した237症例のHist気道過敏性を検討すると約半数は正常域まで改善していたが，残りの約半数はHist気道過敏性が残存していた[11]。吸入ステロイドは抗炎症薬であり，好酸球性炎症を鎮静化させることを考えると，Hist気道過敏性は炎症以外の過敏性を反映している可能性も推測される。

おわりに

Ach気道過敏性とHist気道過敏性の根本的な違いはまだ充分には証明されていない。しかし，当センターの臨床成績からAch気道過敏性とHist気道過敏性にはheterogeneityがあ

ること，Ach気道過敏性は好酸球性炎症を反映し，吸入ステロイドの反応性が良好であることが明らかである。Hist気道過敏性は生理学的により末梢気道を反映していることが示唆された。発症年齢別の気道過敏性の検討では特に小児発症で亢進しており，これは遺伝素因が関与しているのか，長期間の罹病期間が関与しているのか今後さらなる検討を必要とする。

気道過敏性検査は喘息の診断だけではなく，日常臨床において治療効果判定や，予後の推測にも有用であり，それ故にAch気道過敏性とHist気道過敏性のより明確な違いが解明されることが気道過敏性の発現の解明の糸口になり，喘息発症の根本的なメカニズムの解明に寄与するものと考えられる。

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Q&A

Question

気道過敏性テストが正常化した患者さんの病態は？

客観的指標である気道過敏性テストが正常化した患者さんの病態について教えてください．

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Answer

気道過敏性とは「非喘息の人にとって刺激とならない微細な物質や変化に対して容易，かつ過剰に反応し，気道が狭窄する」状態のことをいい，気道過敏性の発現は喘息の基本的な病態の1つであり喘息発症の必要条件であると考えられています[1)]

気道過敏性検査はメサコリン（Mch），アセチルコリン（Ach），ヒスタミン（Hist）などの非特異的な気道平滑筋収縮物質を低容量から吸入し，そのときの気道反応（収縮）を呼吸機能検査（一秒量，呼吸抵抗，気道コンダクタンスなど）によって検出します．気道過敏性検査は喘息の診断だけでなく，重症度，治療経過，長期予後など喘息経過中にも臨床応用が可能です．

成人喘息の治療における抗炎症薬の第一選択薬として吸入ステロイド（ICS）の位置づけにはもはや異論のないところです．かつてのCFC-BDP（chlorofluorocarbon－beclomethasone dipropionate）を使用していた時代には，成人喘息の中でも成人発症アトピー型においてのみAch気道過敏性（AchPC$_{20}$）が臨床的に有意と考えられる4倍以上の改善を示すことがわかりましたが，成人発症非アトピー型，小児発症（寛解なし）では有意な改善を認めませんでした[2)]．

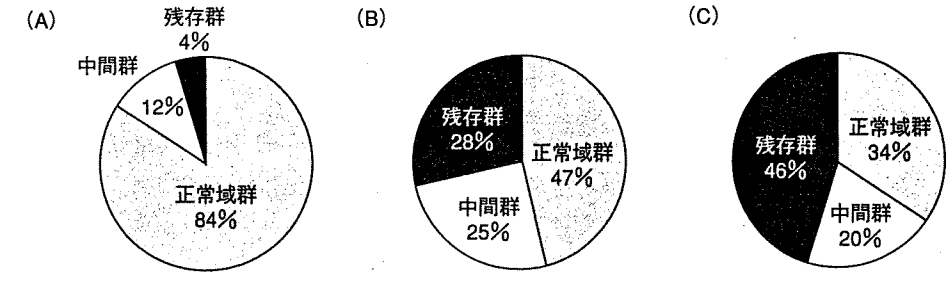

図1 初診時アセチルコリン（Ach）気道過敏性重症度別の治療後Ach気道過敏性

（A）初診時軽度亢進（AchPC$_{20}$＞5,000 γ）158症例，（B）初診時中等度亢進（625 γ＜AchPC$_{20}$＜5,000 γ）151症例，（C）初診時高度亢進（AchPC$_{20}$＜625 γ）99症例を対象とした．治療後Ach気道過敏性正常域群（AchPC$_{20}$＞20,000 γ），中間群（5,000 γ＜AchPC$_{20}$＜20,000 γ），残存群（AchPC$_{20}$＜5,000 γ）の割合を検討した

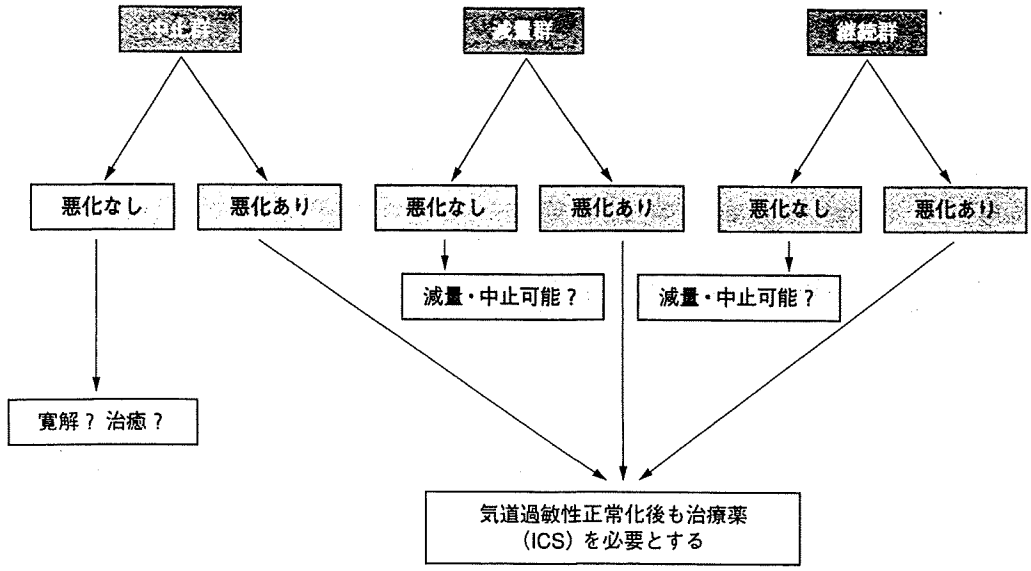

図2 アセチルコリン気道過敏性が正常化する症例のタイプ

しかし，最近のFP（fluticason propionate），BUD（budesonide），HFA-BDP（hydrofluoroalkane beclomethasone dipropionate）などICSは質や吸入効率が開発され，これらのICSを使用することで臨床症状が消失し，Ach気道過敏性が臨床的に有意な改善を示すだけでなく，正常域（すなわち$AchPC_{20}$>20,000 μg）まで改善する症例が増加しました．

当センターでの最近の臨床研究から気道過敏性検査を2回以上受験し，Ach気道過敏性が正常域まで改善した237症例を後ろ向きに解析し，初診時$AchPC_{20}$を重症度別（軽度亢進（$AchPC_{20}$>5,000 γ），中等度亢進（625 γ<$AchPC_{20}$<5,000 γ），高度亢進（$AchPC_{20}$<625 γ））の3群に分類し，治療後のAch気道過敏性を検討すると初診時軽度亢進158症例のうち133症例（84.1%），中等度亢進151症例のうち71症例（47.0%），高度亢進99症例のうち34症例（34.3%）が正常域まで改善しました（**図1**）[3]．この結果は初診時のAch気道過敏性が軽度であるほど治療後の正常化する割合が高いことを示しています．

さらにAch気道過敏性が正常域まで改善した成人喘息患者約300症例のその後の臨床症状を検討すると，約20%はAch気道過敏性が正常域まで改善した後も臨床症状が残存しており，治療薬を継続あるいはその後も増量せざるを得ない症例でした．一方，約80%の症例は臨床症状も消失しており，治療薬を継続（症状消失後も治療薬継続を希望する症例），または減量（もともと使用していたICS量がFP換算で400 μg以上では半量に減量），または中止（FP換算で200 μg以下の症例ではICSを中止）し，その後の臨床経過を追跡しました．継続群，減量群，中止群ではそれぞれ45%，39%，46%の症例で臨床症状の悪化を認めました．一方，約半数の症例では悪化を認めませんでした．ICS継続，減量，中止後に症状悪化を認める症例ではAch気道過敏性が正常化後もICSを含めた治療薬を必要とすることを示唆しています（**図2**）．一方，ICS中止後，臨床症状の悪化を認めない症例は将来の寛解・治癒予備群である可能性を示唆しています．成人喘息に寛解・治

癒がありうるのかについてはまだ十分には証明されていません．過去の当センターでの寛解調査[4]では，受診歴があるがその後通院しなくなった成人喘息患者を対象としアンケート調査を行い，回収できた症例の20.7%は3年間以上無治療で無症状である，いわゆる臨床的寛解状態にあることを報告しました．これらの寛解群は初診時Ach気道過敏性が軽度であることが明らかとなり，これまでの臨床研究をあわせると，Ach気道過敏性が軽度である症例が，治療後気道過敏性正常化しやすく，そのうちの一部がICSを中止できる可能性を示唆しています．

このように気道過敏性が正常化した症例には臨床症状が消失している症例と残存している症例があること，またICSを減量，中止後も悪化を認める症例が存在することから，気道過敏性の消失＝寛解・治癒を意味していません．罹病期間の長い喘息症例や重症例では**気道リモデリング**が生じると考えられており[5]，今回の結果からAch気道過敏性が正常域まで改善した後，臨床症状が消失し，ICS減量，中止が可能であるためには早期受診，早期診断，早期にICSが導入されることが必要であると考えられます．ICSによる早期治療が将来の気道リモデリングを抑制し，良好な予後が得られると考えられます．

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KEY WORD

気道リモデリング：気道粘膜の線維化，平滑筋肥厚，粘膜下腺過形成などにより不可逆的な気流制限が生じることをいいます．気道リモデリングの形成は気道過敏性を亢進させるといわれています．

PC_{20}：標準法による気道過敏性試験の評価を表します．Ach, Histなどの非特異的物質を低濃度から2分間吸入し，その後，FEV_1を測定し，吸入前後のFEV_1が20%以下に低下した濃度を閾値といい，20%低下した濃度をPC_{20}と表現します．

ADVICE

治療後もAch気道過敏性が正常化しない状態では，たとえ臨床症状が消失していてもICSの減量・中止は望ましくありません．

Transduction of Phosphatase and Tensin Homolog Deleted on Chromosome 10 into Eosinophils Attenuates Survival, Chemotaxis, and Airway Inflammation[1]

Tetsuya Adachi,[2] Satoko Hanaka, Tomoko Masuda, Hisanao Yoshihara, Hiroyuki Nagase, and Ken Ohta

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is part of a complex signaling system that affects a variety of important cell functions. PTEN antagonizes the action of PI3K by dephosphorylating the signaling lipid phosphatidylinositol 3,4,5-triphosphate. In the present study, we used a TAT fusion protein transduction system to elucidate the role of PTEN in eosinophils and airway inflammation. A small region of the HIV TAT protein (YGRKKRRQRRR), a protein transduction domain known to enter mammalian cells efficiently, was fused to the N terminus of PTEN. Flow cytometric analysis of annexin V- and propidium iodide-stained cells was used to assess eosinophil survival. A chemotaxis assay was performed using a Boyden chamber. Cell analysis in bronchoalveolar lavage fluid and histological examinations were performed using OVA-challenged A/J mice. We found that TAT-PTEN was successfully internalized into eosinophils and functioned as a phosphatase in situ. TAT-PTEN, but not a TAT-GFP control protein, blocked the ability of IL-5 to prevent the apoptosis of eosinophils from allergic subjects. The eotaxin-induced eosinophil chemotaxis was inhibited by TAT-PTEN in a dose-dependent manner. Intranasal pretreatment with TAT-PTEN, but not TAT-GFP, significantly inhibited the OVA-induced eosinophil infiltration in bronchoalveolar lavage fluid. Histological examination of the lung, including H&E and Alcian blue/periodic acid-Schiff staining, revealed that TAT-PTEN, but not TAT-GFP, abrogated eosinophilic inflammation and mucus production. Our results suggest that PTEN negatively regulates eosinophil survival, chemotaxis, and allergic inflammation. The pharmacological targeting of PTEN may constitute a new strategy for the treatment of eosinophilic disorders. ***The Journal of Immunology,*** **2007, 179: 8105–8111.**

The pathogenesis of asthma is characterized by the infiltration of tissues by inflammatory cells such as eosinophils, mast cells, and T cells. Several mediators released by these cells cause epithelial damage, leading to enhanced bronchial hyperresponsiveness and airway obstruction (1). Although eosinophils have been considered as the most important cells in this process, the results of a clinical trial using an anti-IL-5 Ab has raised questions about the role of eosinophils in bronchial hyperresponsiveness (2). A subsequent study demonstrated that treating asthmatics with anti-IL-5 Ab reduces airway eosinophil numbers and the deposition of extracellular matrix proteins in the bronchial subepithelial basement membrane, suggesting the involvement of eosinophils in airway remodeling (3). In support of this finding, eosinophil-deficient mice exhibit decreased subepithelial fibrosis and smooth muscle hyperplasia (4). Thus, the targeting of eosinophils is considered to be an attractive strategy for treating asthma.

Phosphatase and tensin homolog on chromosome 10 (PTEN)[3] is a lipid and protein tyrosine phosphatase that dephosphorylates phosphotyrosine as well as the D3 position of phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-diphosphate. The tumor suppressor gene *PTEN* is located at 10q23 and its mutation is detected in several neoplasms, including glioblastoma and breast and prostate cancers (5). The transduction of PTEN into tumor cell lines results in cell cycle arrest or apoptosis, suggesting that PTEN controls cell proliferation and survival (6–10). In *Dictyostelium* and mouse neutrophils, it has been demonstrated that the coordination of PI3K and PTEN is required for proper chemotaxis (11–13). The role of PTEN in immunity has also been investigated using PTEN-deficient mice. Suzuki et al. (14) have generated T cell-specific PTEN-deficient ($Pten^{flox/-}$) mice in which the T cells exhibit autoreactivity, enhanced proliferation, and inhibition of apoptosis. Similar phenomena are observed in the B cells derived from B cell-specific PTEN-deficient ($bPten^{flox/flox}$) mice (15). These results indicate that PTEN negatively regulates most cellular functions in the immune system. However, little is know about the role of PTEN in eosinophil function.

To elucidate the importance of intracellular signaling molecules in eosinophils, several approaches have been attempted. Although the application of pharmacological inhibitors is a common technique for targeting molecules of interest, its nonspecificity is frequently problematic. In contrast to proliferating cells, eosinophils have low turnover rates; hence, the validity of using antisense oligonucleotides or small interfering RNAs to assess signaling in eosinophils remains debatable. The overexpression of wild-type or dominant-negative protein by plasmid

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[3] Abbreviations used in this paper: PTEN, phosphatase and tensin homolog on chromosome 10; AB, Alcian blue; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff; ROCK, Rho-associated coiled-coil-forming protein kinase; VEGF, vascular endothelial growth factor.

transfection is not possible because eosinophils are short-lived and terminally differentiated cells. To overcome these problems, we used a TAT fusion protein system that facilitates cellular internalization of the protein (16). It has been shown that the dominant-negative forms of Ras or PI3K efficiently block IL-5 signaling and corresponding functions in eosinophils (17–19).

In the present study, we generated TAT-PTEN to investigate the role of PTEN in eosinophils and airway inflammation. TAT-PTEN, but not a TAT-GFP control protein, blocked the ability of IL-5 to prevent apoptosis in the eosinophils obtained from allergic subjects. The eotaxin-induced eosinophil chemotaxis was inhibited by TAT-PTEN in a dose-dependent manner. Intranasal pretreatment with TAT-PTEN, but not TAT-GFP, significantly inhibited the OVA-induced eosinophilic inflammation in a murine model of asthma.

Materials and Methods

Generation of the TAT-PTEN construct

The TAT-GFP expression vector was provided by Dr. S. Dowdy (20). The expression cassette cosmid containing the wild-type *PTEN* (21) under the control of the CAG promoter (22) was supplied by the Riken Laboratory (Tsukuba, Japan). A cDNA fragment encoding *TAT-PTEN* was amplified by PCR (each cycle was conducted at 94°C for 40 s, 56°C for 30 s, and 72°C for 1 min, for a total of 35 cycles) using an AmpliTaq Gold DNA polymerase (Applied Biosystems) from the *PTEN*-containing cosmid with the forward primer that included the *TAT* sequence at the 5′ region (5′-TACGGTCGTAAGAAACGTCGCCAGCGTCGCCGTATGACAGCCATCATCAAAGAGATCGT-3′, *TAT* sequence is underlined) and the reverse primer (5′-TCAGACTTTTGTAATTTGTGTATGCTG-3′). The PCR product was subcloned into a pCRII-TOPO cloning vector (Invitrogen Life Technologies). To extend the His tag sequences at the 5′ region, an additional PCR (each cycle was conducted at 94°C for 40 s, 58°C for 30 s, and 72°C for 1 min, for a total of 35 cycles) was performed with the pCRII-TAT-PTEN construct as a template and the following primers (forward, 5′-ATGGGCAGCAGACATCATCATCATCATCACAGCAGCGGCTACGGTCGTAAGAAACGTC GCCAGCGT-3′, *His* sequence is underlined; and reverse, 5′-TCAGACTTTTGTAATTTGTGTATGCTG-3′). The final PCR product was cloned into the pCRII-TOPO vector and the sequence of the obtained construct (pCRII-His-TAT-PTEN) was confirmed using an Applied Biosystems PRISM 3700 Genetic Analyzer.

Purification of the TAT-PTEN protein

The polyhistidine-tagged *TAT-PTEN* gene was expressed in TOP10-competent *Escherichia coli* cells. TAT-PTEN was purified by sonication (50 kHz, amplitude: 20) in buffer Z (8 M urea, 20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM PMSF, and 1 μM pepstatin A) containing 20% glycerol. Cell lysates were resolved by centrifugation, absorbed onto a Ni Sepharose High Performance column (GE Healthcare Bio-Sciences), washed, and then sequentially eluted with 50, 100, and 250 mM imidazole in buffer Z/20% glycerol. Urea and imidazole were removed from the resultant protein solution in a dialysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM 2-ME, 1 mM PMSF, and 20% glycerol) using a low-volume 10,000 MWCO Slide-A-Lyser dialysis cassette (Pierce). The protein concentration was measured using a DC protein assay (Bio-Rad). Expression of the TAT-PTEN protein was confirmed by SDS-PAGE and Western blotting. The phosphoinositide phosphatase activity of the protein was determined using a PTEN malachite green assay kit (Upstate Biotechnology) with prepared phospholipid vesicles (0.1 mM diC_8PIP_3, 0.5 mM DOPS, 20 mM HEPES (pH 7.4), 1 mM EGTA, and 4.2% ammonium molybdate in 4 N HCl). Each fusion protein was flash frozen at −80°C.

Eosinophil purification

Peripheral venous blood was obtained from subjects with and without atopic characteristics. The geometric means of serum IgE from normal and atopic donors were 162 and 1479 IU/ml, respectively. Eosinophils were isolated by sedimentation with 6% dextran, followed by centrifugation on 1.088 Percoll (GE Healthcare Bio-Sciences) density gradients as modified from the method of Hansel et al. (23). The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec). The eosinophils (>99% purity) were then suspended in HBSS with 1% FCS in tubes coated with 3% human serum albumin.

Preparation of cytosolic cell extracts

Eosinophils were incubated with and without TAT-PTEN or TAT-GFP for the indicated times at 37°C. The cells were lysed in a lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10% glycerol, and 1 μg/ml aprotinin, leupeptin, and pepstain). After 20 min on ice, detergent-insoluble materials were removed by 12,000 × *g* centrifugation at 4°C. The whole cell lysates were boiled in 2× Laemmli reducing buffer for 4 min.

In some experiments, the cells were stimulated with 100 ng/ml IL-5 (R&D Systems) or 100 nM eotaxin (R&D Systems) for 3 min; the reaction was then terminated by adding nine volumes of ice-cold HBSS containing 1 mM Na_3VO_4. The cells were lysed using the Bio-Plex Cell Lysis Kit (Bio-Rad) for further quantification of phosphorylated and total Akt performed using Bio-Plex Phosphoprotein Panel (Bio-Rad) and Luminex 200 (Luminex). Data are shown as a percentage of mean fluorescence intensity of unstimulated control.

Gel electrophoresis and Western blotting

SDS-PAGE was performed using Ready Gels J (Bio-Rad). The concentration of the polyacrylamide was 7.5–10%. The electrophoresed gel was blotted onto Hybond ECL membranes (GE Healthcare Bio-Sciences). Blots were incubated in a blocking buffer containing 10% BSA in TBST buffer (20 mM Tris-HCl, 137 mM NaCl (pH 7.6), and 0.05% Tween 20) for 1 h, followed by incubation in the primary Ab (0.1 μg/ml) for 1–2 h (mouse monoclonal anti-His Ab obtained from GE Healthcare Bio-Sciences and rabbit polyclonal anti-PTEN and anti-GFP Abs obtained from Santa Cruz Biotechnology). After washing three times in TBST buffer, the blots were incubated for 30 min with a HRP-conjugated secondary Ab (0.04 μg/ml) directed against the primary Ab. The blots were developed with an ECL substrate according to the manufacturer's instructions (GE Healthcare Bio-Sciences). In some experiments, blots were reprobed with another Ab after stripping in a buffer of 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS at 50°C for 30 min.

Cellular uptake of TAT fusion proteins

TAT-PTEN and TAT-GFP were labeled with FITC (Pierce) according to the manufacturer's instructions, and excess FITC was removed by dialysis using the same cassette as that described above for protein purification. Eosinophils resuspended in HBSS with 1% FCS were incubated with 1 μM of the FITC-conjugated TAT-PTEN or TAT-GFP for 30 min at 37°C. The cells were washed with HBSS with 1% FCS and placed on glass slides using Shandon Cytospin 3 (Thermo Scientific). The intracellular distribution of TAT fusion proteins was visualized under a laser confocal microscope (LSM510; Zeiss).

Survival assay

An apoptosis detection kit (MBL) was used to quantitatively determine eosinophils undergoing apoptosis, by virtue of their ability to bind to annexin V and exclude propidium iodide. Purified eosinophils were resuspended at 0.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS. After incubation with TAT-PTEN or TAT-GFP, the eosinophils were further cultured with 1 ng/ml IL-5 (R&D Systems) in 24-well tissue culture plates for 24 h at 37°C. The cells were washed twice in cold PBS and stained with annexin V and propidium iodide according to the manufacturer's instructions. Eosinophil apoptosis was analyzed using a FACScan cytometer (BD Biosciences), gating on the live cell population.

Chemotaxis assay

Chemotaxis of eosinophils was conducted in duplicate using 5-μm polyvinylpyrrolidone-free polycarbonate membranes (Nucleopore) in Boyden chambers (NeuroProbe). Human eotaxin was diluted in HBSS containing 0.02% BSA and placed in the lower wells (100 μl) at a concentration of 10 nM. After incubation of the eosinophils with and without the TAT fusion proteins for 30 min, 100-μl aliquots of the cell suspension at 2×10^6 cells/ml were placed in the upper chambers. The loaded chambers were incubated at 37°C in humidified air containing 5% CO_2 for 1 h. The membrane was then removed, fixed, and stained for 3 min in May-Grünwald solution. The cells that migrated and adhered to the lower surface of the membrane were counted from 10 fields under the light microscope. The chemotactic response to the buffer (<40/10 fields) was subtracted from that induced with eotaxin with or without the inhibitors. The data are shown as a percentage of that induced with eotaxin alone.

Bronchoalveolar lavage fluid (BALF) cell analysis and histological examination in mice

Care and use of the animals followed the guidelines of the Principles of Laboratory Animal Care formulated by the National Society for Medical Research. Specific pathogen-free male A/J mice (10–12 wk old) were purchased from SLC. The mice were initially immunized four times with 10 μg of OVA plus 2 mg of alum on days 0, 28, 35, and 49. After the sensitization, the mice were intranasally challenged with 40 μl of 10 mg/ml OVA from days 50–53. The mice were divided into four groups for the intranasal administration of pretreatment and challenge: 1) PBS plus PBS, 2) PBS plus OVA, 3) TAT-PTEN (3 μmol/mouse) plus OVA, and 4) TAT-GFP (3 μmol/mouse) plus OVA. On day 54, BALF was obtained from the mice by intubating and washing the lungs with 1 ml of saline until 5 ml of fluid was recovered. The cells were pelleted from the lavage fluid, resuspended in 1 ml of saline, and placed on glass slides for counting and fixation using Shandon Cytospin 3 (Thermo Scientific). The slides were then stained with Diff-Quik (Sysmex) and cell differentiation was assessed microscopically. The supernatant of the BALF was concentrated 10-fold by freeze-drying to examine the cytokine/chemokine levels. The measurement of cytokine/chemokine was performed using Bio-Plex Cytokine Panel (Bio-Rad) and Luminex 200. The lungs were fully inflated using 10 cm of H_2O pressure and fixed with 20% formaldehyde for H&E and Alcian blue/periodic acid-Schiff (AB/PAS) staining.

Statistical analysis

Results are expressed as means ± SEM. The data were analyzed for statistical significance using the Mann-Whitney *U* test and ANOVA. Post hoc analysis was performed using the Bonferroni/Dunn test.

Results

Cellular internalization and in situ activity of TAT-PTEN

Dowdy and colleagues (20) have described how TAT fusion proteins were internalized into mammalian cells within 30 min in a concentration-dependent manner. Thus, we constructed TAT-PTEN to examine the role of PTEN in eosinophil function and allergic inflammation (Fig. 1). Initially, we incubated eosinophils with 1 μM TAT-PTEN or TAT-GFP for 15 s, 5 min, and 30 min at 37°C to assess the cellular uptake of the proteins. After the incubation, the eosinophils were washed with cold HBSS and lysed. The lysates were subjected to electrophoresis and Western blotting with anti-PTEN or anti-GFP Ab and then reprobed with anti-His Ab. As shown in Fig. 2*A*, the blots with both anti-PTEN and anti-His Abs revealed that TAT-PTEN efficiently entered cells in a manner that depended on the length of incubation. In contrast, internalization of TAT-GFP reached a maximum at 15 s and plateaued for 30 min (Fig. 2*B*). To further confirm these results, we observed the intracellular distribution of the TAT fusion proteins in eosinophils. The confocal microscopic analysis of transduced TAT-PTEN (Fig. 3*A*) and TAT-GFP (Fig. 3*B*) demonstrated cytoplasmic localization accompanied with intense uptakes, possibly within endosomes. Our TAT-PTEN, unlike the dominant-negative form of TAT fusion proteins reported previously (17–19), is designed to function as an in situ phosphatase. PTEN is assumed to inhibit the PI3K pathway by dephosphorylating phosphatidylinositol 3,4,5-triphosphate, leading to the down-regulation of Akt, which lies downstream of PI3K. For this reason, we investigated the effect of TAT-PTEN on Akt activation in eosinophils to detect the in situ activity of the protein. After pretreatment of the eosinophils with 1 μM TAT-PTEN or TAT-GFP for 30 min, Akt phosphorylation induced by IL-5 or eotaxin was measured. As shown in Fig. 4, internalized TAT-PTEN significantly reduced Akt phosphorylation in eosinophils stimulated with IL-5 or eotaxin (Fig. 4).

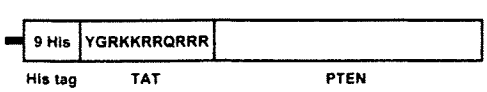

FIGURE 1. Structure of the TAT-PTEN fusion protein. Nine His residues and the 11-aa TAT peptide precede the N-terminal of the PTEN protein. The 11-aa of TAT comprise the protein transduction domain.

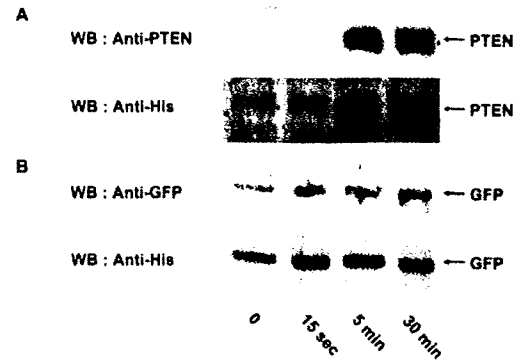

FIGURE 2. Kinetics of (*A*) TAT-PTEN and (*B*) TAT-GFP transduction into eosinophils. Eosinophils were incubated with 1 μM TAT-PTEN or TAT-GFP at 37°C for the indicated times. The TAT fusion protein in cell lysates was examined by Western blotting (WB) using anti-PTEN or anti-GFP Ab and then reprobed with anti-His Ab.

Effect of TAT-PTEN on eosinophil survival and chemotaxis

PTEN has been demonstrated to promote apoptosis in a number of tumor and immune cells (7–10, 24, 25). Thus, we investigated the role of PTEN in the maintenance of eosinophil survival. After incubating with TAT-PTEN or TAT-GFP for 30 min, eosinophils were stimulated with IL-5 for 24 h and viability was assessed by annexin V and propidium iodide staining. After 24 h, in the absence of IL-5, almost one-half of the eosinophils had undergone apoptosis. In contrast, eosinophil viability was >80% after stimulation with IL-5. Incremental increases in the concentration of TAT-PTEN, but not TAT-GFP, significantly abrogated the survival of eosinophils from atopic donors (Fig. 5). Interestingly, however, the effect of TAT-PTEN was not observed in the eosinophils from normal subjects (Fig. 5). We next studied the effect of TAT-PTEN on eotaxin-induced eosinophil chemotaxis since PTEN has been observed to be involved in the migration of *Dictyostelium* and mammalian cells (11–13). Eosinophils were incubated with and without the TAT fusion proteins and then applied to the upper Boyden chambers. The lower chambers contained 10 nM eotaxin. The cells that migrated and adhered to the lower surface of the membrane were counted under the light microscope. TAT-PTEN, but not TAT-GFP, dose-dependently reduced the chemotaxis of eosinophils, regardless of their atopic state (Fig. 6).

Effect of TAT-PTEN on allergic inflammation

Kwak et al. (26) have reported that the intratracheal administration of adenovirus-carrying PTEN cDNA significantly inhibits eosinophilic inflammation and bronchial hyperresponsiveness. We therefore investigated the in vivo effect of TAT-PTEN in a murine model of asthma. The OVA-sensitized mice were intranasally pretreated with and without TAT-PTEN or TAT-GFP, followed by intranasal OVA challenge. Airway inflammation was assessed by BALF cell analysis and histological examination. Following OVA challenge, the number of cells in the BALF increased due largely to an increase in eosinophils (Fig. 7). The increase in the numbers of BALF cells, particularly eosinophils, was significantly abrogated in mice pretreated with TAT-PTEN, but not in those treated with TAT-GFP (Fig. 7). A histological examination revealed that airway inflammation and the production of purple-stained mucus were augmented by OVA challenge (Fig. 8, *C* and *D*) compared with the PBS control (Fig. 8, *A* and *B*). The H&E staining revealed

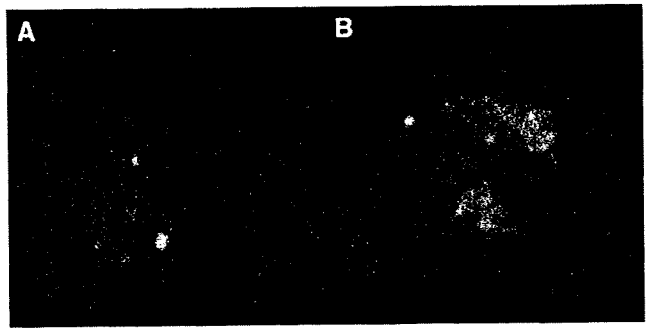

FIGURE 3. Intracellular localization of (*A*) TAT-PTEN and (*B*) TAT-GFP in eosinophils. Eosinophils were incubated with 1 μM FITC-conjugated TAT-PTEN or TAT-GFP for 30 min at 37°C. After preparation of a cytospin specimen, the cells were visualized under a confocal microscope.

that airway inflammation was attenuated by the administration of TAT-PTEN (Fig. 8*E*). Pretreatment with TAT-PTEN markedly reduced the mucus-producing epithelial cells stained with AB/PAS (Fig. 8*F*). The effect elicited by TAT-PTEN was not observed in the case of TAT-GFP administration (Fig. 8,*G* and *H*). To study the effect of TAT-PTEN on Th1/Th2 balance, we measured the levels of cytokine/chemokine in BALF. The increase in the IL-5 level caused by OVA challenge was significantly inhibited by TAT-PTEN, but not by TAT-GFP (Fig. 9*A*). In contrast, the administration of TAT-PTEN significantly increased the RANTES level in BALF (Fig. 9*B*). Since the down-regulation of vascular endothelial growth factor (VEGF) expression in allergen-induced asthmatic lung by PTEN has recently been demonstrated (27), we also measured the VEGF concentration in BALF. Although TAT-PTEN tended to decrease the VEGF level, the difference was not significant (data not shown).

Discussion

In the present study, we developed a novel TAT fusion protein to investigate the role of PTEN in eosinophils and airway inflammation. TAT-PTEN, but not TAT-GFP, reduced the IL-5-induced survival of eosinophils obtained from allergic subjects. The eotaxin-induced eosinophil chemotaxis was inhibited by TAT-PTEN. The intranasal administration of TAT-PTEN, but not TAT-GFP, significantly inhibited the OVA-induced eosinophilic inflammation. This is the first report to clarify the role of PTEN in

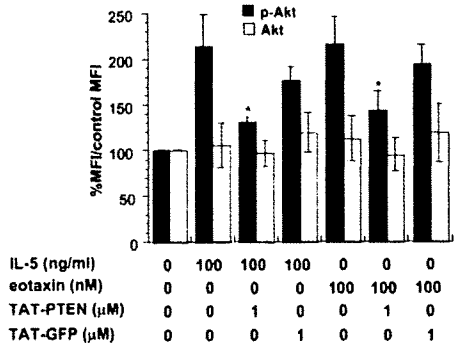

FIGURE 4. The in situ phosphatase activity of TAT-PTEN in eosinophils stimulated with IL-5 or eotaxin. Eosinophils were incubated with 1 μM TAT-PTEN or TAT-GFP for 30 min and then stimulated with 100 ng/ml IL-5 or 100 nM eotaxin for 3 min. Quantification of the phosphorylated and total Akt was performed using the Luminex System. The data are shown as a percentage of mean fluorescence intensity of unstimulated control and are expressed as means ± SEM ($n = 4$). *, $p < 0.05$ vs without the protein (ANOVA).

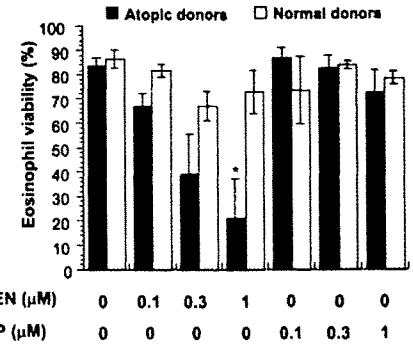

FIGURE 5. Effect of TAT-PTEN on eosinophil survival. Eosinophils were incubated with and without the TAT fusion proteins for 30 min. The cells were further cultured with IL-5 (1 ng/ml) for 24 h and then the viability of eosinophils was assessed by annexin V and propidium iodide staining. The data are expressed as means ± SEM ($n = 3$). *, $p < 0.05$ vs without the protein (ANOVA).

eosinophils. Moreover, we demonstrated the utility of TAT-PTEN as a therapeutic modality for the treatment of allergic diseases.

Eosinophils play a pivotal role in the pathogenesis of airway inflammation and remodeling in asthma. Therefore, it is of paramount importance to investigate eosinophil signaling and its functional relevance. Pharmacological inhibitors of intracellular signaling molecules have been widely used for this purpose. However, the data are often difficult to interpret because of the lack of inhibitor specificity. Although eosinophils are nonproliferating and terminally differentiated cells, transfection with plasmids or transduction with inhibitory nucleotides, such as antisense oligonucleotides and small interfering RNAs, may be unrealizable. To solve the above-mentioned problems, the TAT fusion protein system has become the focus of scientific attention. The first description of this system was made independently by Green and Loewenstein (28) and Frankel and Pabo (29), who demonstrated that the 86-aa HIV TAT protein could rapidly enter cells and subsequently transactivate the viral long terminal repeat promoter. Later, Fawell et al. (16) expanded on these findings by revealing that chemically cross-linking a 36-aa domain of TAT to heterologous proteins facilitated cell internalization. According to their results, the TAT fusion proteins were predominantly localized in the cell surface area following incubations of up to 20 min, with progressive accumulation in diffuse cytoplasmic, nuclear, and nucleolar regions with incubations of 30 min to 6 h. Several groups

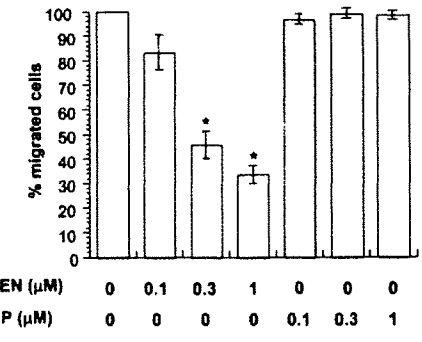

FIGURE 6. Effect of TAT-PTEN on eosinophil chemotaxis. Eosinophils were incubated with and without the TAT fusion proteins for 30 min. After incubation, the cells were subjected to the chemotaxis assay using Boyden microchambers. Eotaxin (10 nM) was used as the chemoattractant. The data are expressed as means ± SEM ($n = 4$). *, $p < 0.05$ vs without the protein (ANOVA).

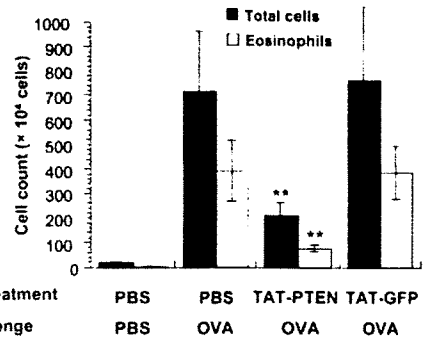

FIGURE 7. Effect of TAT-PTEN on BALF cells in a murine model of asthma. TAT-PTEN or TAT-GFP was intranasally administrated in OVA-sensitized A/J mice. The mice were intubated for lavage with a total of 5 ml of saline. Cells were pelleted from the lavage fluid and the cell number was counted under the light microscope following Diff-Quik staining. The data are expressed as means ± SEM ($n = 5$). **, $p < 0.05$ vs PBS-treated/OVA-challenged mice or TAT-GFP-treated/OVA-challenged mice (Mann-Whitney U test).

FIGURE 8. Effect of TAT-PTEN on the histological characteristics of lung tissue. Sections of lung were stained with H&E (*A, C, E,* and *G*) or AB/PAS (*B, D, F,* and *H*) stain. PBS-treated/PBS-challenged mouse (*A* and *B*), PBS-treated/OVA-challenged mouse (*C* and *D*), TAT-PTEN-treated/OVA-challenged mouse (*E* and *F*), and TAT-GFP-treated/OVA-challenged mouse (*G* and *H*). Original magnification, ×200.

have demonstrated that the TAT fusion proteins block specific signaling molecules such as Ras and PI3K in eosinophils (17–19). In the present study, we generated TAT-PTEN that was efficiently incorporated in eosinophils. It has been demonstrated that the expression of wild-type PTEN in PTEN-deficient tumor cell lines reduces phosphatidylinositol 3,4,5-triphosphate levels and inhibits Akt phosphorylation (30). We therefore examined the in situ phosphatase activity of TAT-PTEN in eosinophils and found that the Akt phosphorylation induced by IL-5 or eotaxin was inhibited by TAT-PTEN.

PTEN appears to play a particularly important role in regulating apoptosis in a variety of cell types. Somatic deletions or mutations of the *PTEN* gene are commonly detected in a large fraction of tumors (5) and overexpression of wild-type PTEN in $PTEN^{-/-}$ cell lines induces apoptosis (7–10). PTEN also inhibits Ag receptor signaling and cell survival in B cells and T cells (24, 25). Our results revealed that the transduction of PTEN inhibited the survival of eosinophils derived from atopic donors, but not those from normal subjects. Although mechanisms regarding the differential response of eosinophils based on atopic state are unclear, one possible explanation is as follows. We have previously found that Lyn, Jak2, and Raf-1, but not MAPK, are essential for the survival of eosinophils stimulated with IL-5 (31, 32). However, the role of PI3K in signaling between IL-5 and GM-CSF in eosinophil apoptosis remains controversial (33, 34). IL-5 and GM-CSF share a βc receptor that is critical for signal transduction. These results indicate that the Lyn (Jak2)-Ras-Raf-1 pathway may be common in survival signaling in the eosinophils from both atopic and normal subjects. In contrast, eosinophils from atopic donors, or primed in vivo in the allergic condition, possibly utilize the PI3K pathway as well as the Ras-Raf-1 pathway. In support of this finding, Pinho et al. (35) have established a model of allergic pleurisy in mice and found that treatment with the PI3K inhibitor abrogated the accumulation of eosinophils associated with an increased number of apoptotic events. Thus, it is reasonable that TAT-PTEN accelerates the apoptosis of atopic eosinophils by antagonizing the effect of PI3K.

The role of PTEN has recently been elucidated in *Dictyostelium* and mouse neutrophils (11–13). In the resting condition, PI3K is uniformly distributed in the cytoplasm, whereas PTEN is localized in the cell periphery. PI3K is translocated to the leading edge of the cell associated with delocalization of PTEN from the front in response to chemoattractants. Recently, Li et al. (36) have demonstrated that activated RhoA in the rear of leukocytes activates Rho-associated

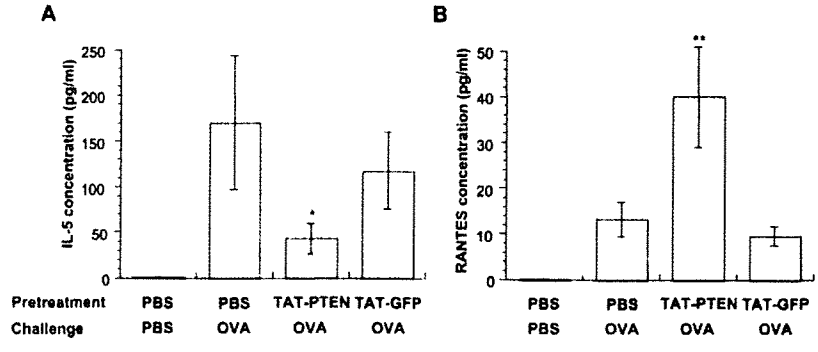

FIGURE 9. Effect of TAT-PTEN on cytokine/chemokine production. The concentration of IL-5 (*A*) and RANTES (*B*) in the BALF supernatant was measured using Bio-Plex Cytokine Panel and Luminex 200. The data are expressed as means ± SEM ($n = 5$). *, $p < 0.05$ vs PBS-treated/OVA-challenged mice; **, $p < 0.05$ vs PBS-treated/OVA-challenged mice or TAT-GFP-treated/OVA-challenged mice (Mann-Whitney U test).

coiled-coil-forming protein kinase (ROCK), subsequently forming a complex with and phosphorylating PTEN. Activated PTEN located at the back and lateral sides restricts phosphatidylinositol 3,4,5-triphosphate accumulation in the front of the cells, which is required for proper chemotaxis. Our previous report demonstrated that the Rho-ROCK pathway is activated by eotaxin and critical for eosinophil chemotaxis (37). Thus, the activation of Rho and ROCK may be responsible for endogenous PTEN activation in eosinophils. In some cells, however, overexpression of PTEN inhibits cell migration (38). Expression of the PTEN mutant G129E, which is deficient in lipid phosphatase activity but retains protein phosphatase activity, inhibits the migration of U87MG glioblastoma cells (39). This effect is likely to be mediated through the direct interaction of PTEN with FAK, followed by its dephosphorylation. In chemotactic PTEN-deficient Jurkat T cells, ectopically expressed PTEN was distributed homogenously in the cytoplasm (40). Nonetheless, in contrast to the results in *Dictyostelium* and murine neutrophils (11–13), PTEN attenuated actin polymerization and cell motility in Jurkat cells stimulated with stromal-derived factor 1 (40). In the present study, we observed that TAT-PTEN is largely distributed in the cytosol of eosinophils. Thus, the findings of Lacalle et al. (40) support our data demonstrating that TAT-PTEN blocks eotaxin-induced eosinophil chemotaxis. Taken together, it is possible that PTEN localization in migrating cells depends on cell type-specific factors.

Homozygosity for the null mutation of PTEN ($PTEN^{-/-}$ mice) results in early embryonic lethality (7, 40–42). $PTEN^{+/-}$ mice frequently develop a variety of cancers and autoimmune diseases (42–44). To overcome the lethality of $PTEN^{-/-}$, various cell-specific PTEN mutations have been generated using the Cre-loxP system. The T cell-specific PTEN-deficient ($Pten^{flox/-}$) T cells exhibit hyperproliferation, autoreactivity, secretion of increased levels of Th1/Th2 cytokines, and resistance to apoptosis (14). Similar phenomena, including hyperproliferation, resistance to apoptosis, and enhanced migration, are observed in PTEN-deficient ($bPten^{flox/flox}$) B cells (15). These results suggest inhibitory roles for PTEN in neoplasm formation and immune regulation. In a murine model of allergy, Kwak et al. (26) have established an elegant system for examining the role of PTEN. The intratracheal administration of adenovirus carrying PTEN cDNA significantly reduced airway eosinophil infiltration and bronchial hyperresponsiveness, indicating that the effect of PTEN is suppressive. The application of TAT fusion proteins, such as the dominant-negative forms of Ras and PI3K, has been reported in a murine model of allergy (45, 46). In the present study, we generated TAT-PTEN that efficiently blocks eosinophilic inflammation, mucus production, and IL-5 production in vivo. These results are consistent with those of Kwak et al. (26). We also observed less IL-5 production in the TAT-GFP-treated mice than in the mice not treated with TAT proteins. It has been shown that TAT transduction causes a phosphatidylserine flip from the inner to the outer cell membrane (47), which is commonly observed during cell apoptosis. Although the mechanism underlying this is unclear, the electrostatic interaction between TAT-GFP and the cell membrane may account for the reduced IL-5 production in BALF. Lung expression of RANTES mRNA is up-regulated in the Th1-skewed condition of a murine asthma model following treatment with CpG oligodeoxynucleotides or the adoptive transfer of Th1 clones (48, 49). Our results demonstrated the increase in RANTES level in BALF induced by TAT-PTEN, suggesting that TAT-PTEN modulates allergic inflammation by inducing the Th1 condition. Lee et al. (27) have recently demonstrated the down-regulation of VEGF expression in allergen-induced asthmatic lung by PTEN, which may indicate a further mechanism of TAT-PTEN action.

In conclusion, we generated TAT-PTEN that efficiently blocks eosinophil survival, chemotaxis, and airway inflammation. Targeting PTEN can be a therapeutic modality in the treatment of several cancers and autoimmune diseases. However, the molecular regulation of PTEN in eosinophilic inflammation remains to be clarified. Further studies are necessary to elucidate the detailed signaling complex around PTEN in allergy, the outcome of which may lead to the development of new molecular targeting therapy.

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Disclosures

The authors have no financial conflict of interest.

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IgE- and FcεRI-Mediated Enhancement of Surface CD69 Expression in Basophils: Role of Low-Level Stimulation

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Key Words
Basophils, CD69 · Der f 2 · Interleukin-3

Abstract
Surface-expressed CD69 is a recently recognized activation marker for basophils and is reported to be strongly induced in vitro by IL-3. In this study, we investigated whether IgE- and high-affinity receptor for IgE (FcεRI)-dependent stimuli can affect basophil CD69 expression. Highly purified basophils were cultured for 24 h in the presence of anti-FcεRI α-chain mAb, CRA-1 and IL-3, and surface CD69 expression was analyzed by flow cytometry. CRA-1 mAb at 1 ng/ml or lower concentrations, levels too low to provoke direct histamine release, dose-dependently enhanced surface CD69 expression in the presence of IL-3, although low-dose CRA-1 mAb failed to induce CD69 expression in the absence of IL-3. Recombinant Der f 2 at 10 to 100 pg/ml enhanced CD69 levels in the presence of IL-3 in basophils from mite-sensitive subjects. These results suggest that allergens may influence basophil CD69 expression even when the levels of the antigens are too low to trigger direct degranulation. Upregulated CD69 expression on locally accumulated basophils in bronchial asthma may be attributed at least in part to a combination of local cytokines, especially IL-3, plus exposure to low levels of IgE-crosslinking allergens.

Introduction

Basophils comprise the least abundant circulating leukocytes, but they demonstrate abundant expression of high-affinity receptor for IgE (FcεRI) on their cell surface. Cross-linking of IgE by antigen induces activation of basophils, and various mediators secreted by activated basophils are thought to be important participants in the pathogenesis of IgE-mediated allergic diseases, such as asthma [1].

Accumulation of basophils occurs in local tissues during antigen-induced late-phase reactions and chronic allergic diseases [2, 3]. Both basophils and basophil-derived mediators have been identified in late-phase reactions induced by experimental antigen challenge of various organs [4, 5]. Based on the findings of previous reports, it is thought that locally migrated basophils possess an acti-

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vated phenotype, as suggested by basophil surface marker analysis including CD69 [6, 7]. For example, we recently reported that basophils, retrieved by bronchoalveolar lavage from patients with asthma, showed elevated levels of surface CD69 compared to their peripheral blood basophils, and that strong induction of surface CD69 expression occurs in vitro in the presence of relatively high doses of IL-3 [7]. Thus, CD69 was thought to be a useful marker of basophil activation with potentially clinical relevance, although we failed to observe any functional significance for basophil CD69. However, in that study, there was no apparent induction of basophil CD69 expression by IgE-cross-linking stimuli. In this study, we investigated whether IgE- and FcεRI-dependent stimuli can modulate basophil CD69 expression.

Materials and Methods

Reagents

The following reagents were purchased: FITC-conjugated anti-CD69 mAb (IgG1, clone FN50, Pharmingen, San Diego, Calif., USA), FITC-conjugated mouse IgG_1 mAb (Coulter Immunotech, Marseille, France), recombinant Der f 2 (Asahi Breweries, Tokyo, Japan), and IL-3 (PeproTech, London, UK). Anti-FcεRI α-chain mAb (CRA-1) was prepared as previously described.

Determination of Basophil Surface CD69 in vitro

CD69 expression on basophils was analyzed as previously described [7]. Briefly, basophils were purified from venous blood obtained from consenting volunteers with no history of atopic diseases. Briefly, basophils were semipurified by means of Percoll density gradient centrifugation; this was followed by negative selection through use of a MACS Basophil Isolation Kit (Miltenyi BioTech, Bergisch-Gladbach, Germany). The mean purity of the basophil preparations was 97.4%. Cells (2×10^4/200 μl) were cultured in RPMI 1640 (Gibco, Grand Island, N.Y., USA) supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2 for 24 h in flat-bottomed 96-well culture plates (Iwaki, Chiba, Japan). IL-3 and/or CRA-1 mAb were included in the medium throughout the culture period.

For flow cytometry, cells were incubated first with human IgG (5 mg/ml) and then with 10 μg/ml of FITC-labeled anti-CD69 mAb. An isotype-matched FITC-labeled mouse IgG was used as a negative control. Stained cells were analyzed through use of an EPICS XL SYSTEM II (Coulter, Miami, Fla., USA). The median values of fluorescence intensity were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as described previously. Surface receptor levels were calculated with the following formula: (MESF of cells stained with anti-CD69 mAb) – (MESF of cells stained with control IgG).

In a part of the experiments, basophils were purified from mite-sensitive subjects with allergic asthma after obtaining informed consent. Cells were then cultured in the presence of IL-3 and/or various concentrations of Der f 2.

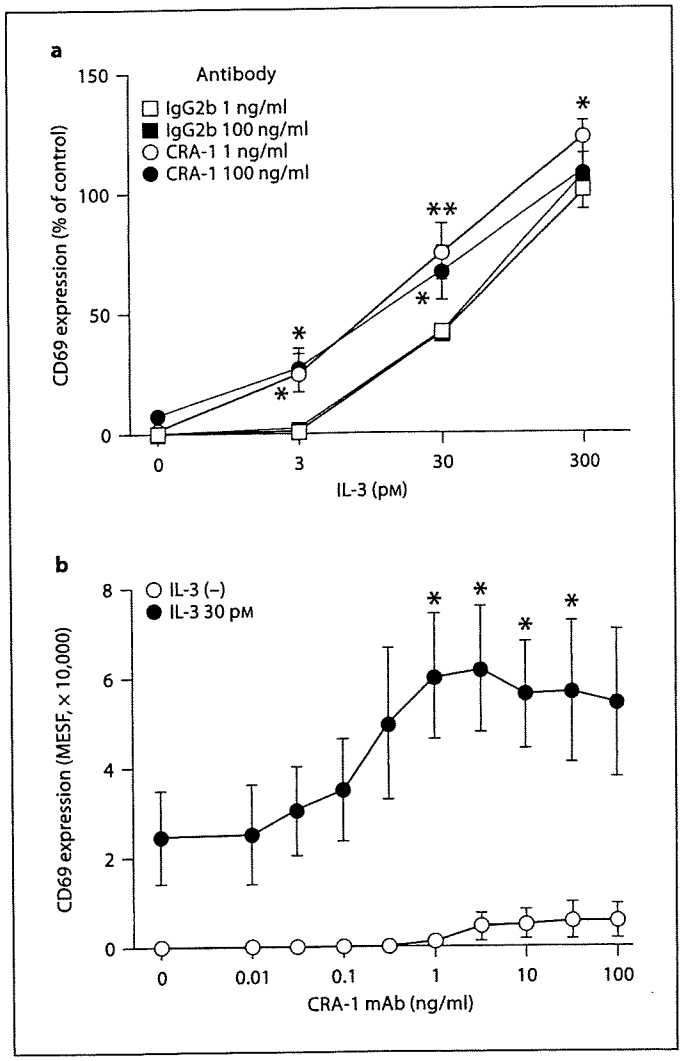
Statistics

All data are expressed as mean ± SEM. Differences between values in the in vitro experiments were analyzed by means of one-way ANOVA. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

Results

Highly purified basophils were cultured for 24 h in the presence of mouse IgG2b anti-FcεRI α-chain mAb (CRA-1) and IL-3, and surface CD69 expression was analyzed by flow cytometry. In the absence of IL-3, CD69 was induced weakly by relatively high concentrations of CRA-1 (fig. 1a, b); CD69 levels of basophils cultured with CRA-1 at 100 ng/ml were significantly higher than those of cells cultured with the same concentration of control IgG_{2b} (CD69 levels expressed in MESF units were 4,630 ± 1,730 and 150 ± 70, respectively ($n = 10$); $p < 0.05$). As shown in figure 1a, CD69 expression was obviously enhanced by this mAb in the presence of IL-3. IL-3 at as low as 3 pM, too low to induce CD69 by itself, induced CD69 when the cells were cultured in the presence of CRA-1 mAb. Interestingly, CRA-1 mAb at a relatively low dose (1 ng/ml) showed no less enhancement of IL-3-induced CD69 expression than at a much higher dose (100 ng/ml). We next analyzed the dependency of basophil CD69 expression on CRA-1 mAb in the presence or absence of IL-3. In the presence of IL-3 at 30 pM, CRA-1 at 1 ng/ml or lower concentrations, levels too low to provoke direct histamine release, dose-dependently enhanced surface CD69 expression (fig. 1b). Flow cytometric analysis suggested that CRA-1 mAb at 1 ng/ml demonstrated a low level of binding, only ~5% of the maximal binding capacity on basophils. These results indicate that low doses of CRA-1 mAb can potently affect basophil CD69 expression, for which the presence of IL-3 in the culture medium is essential.

Next, purified basophils from mite-sensitive asthmatics were cultured with recombinant Der f 2 and IL-3, and CD69 expression on basophils was analyzed. The results were essentially similar to those for basophils cultured with CRA-1 mAb; Der f 2 at 10 to 100 pg/ml (note that concentrations of Der f 2 at or above 30 pg/ml are usually necessary to provoke direct degranulation) enhanced CD69 levels in the presence of IL-3, whereas nearly no induction of CD69 was observed with Der f 2 alone (fig. 2).



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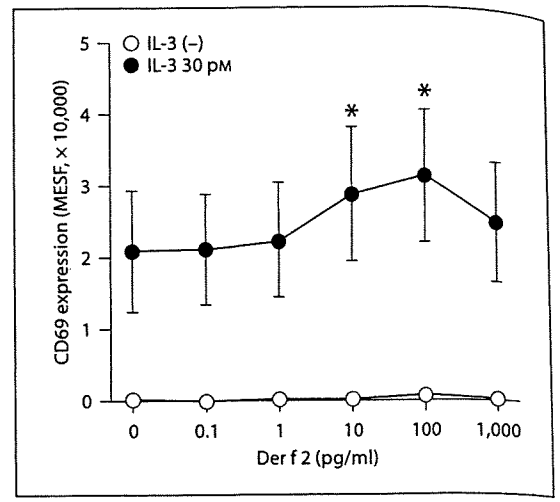

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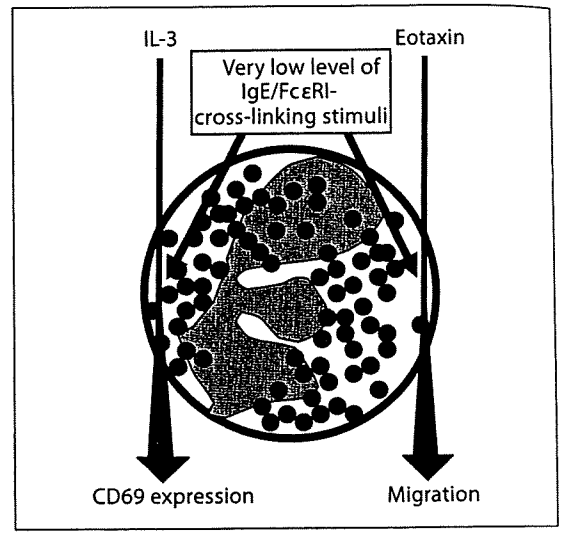

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Fig. 1. Modulation of basophil CD69 expression by anti-FcεRI α mAb. **a** The indicated concentrations of IL-3 and CRA-1 mAb were included in the medium. After culture for 24 h, CD69 expression on the basophils was analyzed by flow cytometry. The percentages of MESF were calculated based on the MESF of cells cultured with control IgG2b 1 ng/ml plus IL-3 300 pM; the actual MESF level of the control cells was 124,000 ± 9,000. Bars represent the SEM (n = 4). * $p < 0.05$, ** $p < 0.01$ versus corresponding values of cells cultured with control IgG2b. **b** Basophils were incubated with various concentrations of CRA-1 mAb in the presence and absence of IL-3 at 30 pM. Levels of surface CD69 expression are expressed using MESF units. Bars represent the SEM (n = 3). * $p < 0.05$ versus corresponding values of basophils cultured without CRA-1 mAb.

Fig. 2. Modulation of basophil CD69 expression by an antigen. Basophils obtained from mite-sensitive asthmatics were incubated with the indicated concentrations of Der f 2, with or without IL-3 at 30 pM. Data are mean ± SEM (n = 4). * $p < 0.05$ versus corresponding values of basophils cultured without Der f 2.

Fig. 3. Low level of IgE/FcεRI-cross-linking stimuli enhances both IL-3-induced CD69 expression and eotaxin-induced migration in basophils.

Discussion

Activation of basophils and mast cells is a hallmark aspect of IgE-mediated allergic reactions and clinical allergic diseases. And antigen- and IgE-dependent stimulation is thought to play a central role as the trigger of basophil and mast cell activation in allergic reactions. It is widely known that cross-linkage of IgE directly activates many changes in basophils, resulting in not only mediator release but also potently enhanced expression of surface integrin CD11b and other activation markers [6–8]. On the other hand, IL-3 is generally conceived as another

type of basophil-activating substance, and it potentiates the cellular activation profiles induced by IgE-cross-linkage or other stimuli [9, 10]. Our present results revealed a curious situation for regulation of basophil CD69 expression: IL-3 is a central inducer of CD69 expression, while antigen is an enhancing factor acting on basophils cooperatively with IL-3. Interestingly, very low doses of antigen or CRA-1 mAb maximally enhanced basophil CD69 expression in the presence of IL-3; such concentrations of antigen or CRA-1 mAb correspond to threshold or subthreshold doses for triggering degranulation of basophils.

The results of our present study bring to light a new aspect of IgE- and FcεRI-dependent events occurring in basophils. Recently, we demonstrated that similarly low concentrations of CRA-1 mAb (1 ng/ml) significantly enhance basophil migration towards eotaxin [11]. And a previous study by Bochner et al. [12] demonstrated that threshold doses of IgE-cross-linking stimuli can enhance CD11b expression on basophils. However, IgE-dependent weak stimulation does not affect all events in basophils; our preliminary experiments using pure basophils indicate that threshold doses of CRA-1 mAb do not suppress apoptosis or enhance the anti-apoptotic effect of IL-3 [13]. These results suggest that antigens may influence some, if not all, basophil functions even when the levels of the antigens are too low to provoke direct degranulation (fig. 3). The findings of the present study and our previous report collectively suggest that upregulated CD69 expression on locally accumulated basophils in bronchial asthma may be attributed at least in part to a combination of local cytokines, especially IL-3, plus IgE-cross-linking allergens. It thus may be important to elucidate the delicate actions of low-dose allergens on basophils, and probably on mast cells as well, in order to fully understand the pathogenesis of chronic asthma associated with continual exposure to low levels of environmental allergens.

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