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

アレルギー性鼻炎診療に対するアンケート調査と鼻汁中のスギ特異的IgE抗体の測定に関する研究

研究分担者　竹内 万彦 三重大学耳鼻咽喉・頭頸部外科・教授
研究協力者　増田佐和子 国立病院機構三重病院耳鼻咽喉科・医長
坂井田 寛 三重大学医学部付属病院・助教
水谷 仁 三重大学大学院医学系研究科皮膚科・教授
山中 恵一 同皮膚科・講師

研究要旨

アレルギー性鼻炎の治療の実態の一端を明らかにする目的で、小中学生を対象として治療の内容とその効果、費用について調査した。小中学校にてアンケートを配布し、鼻アレルギーあるいは花粉症と診断された生徒からアンケートを回収した。回収した322名を解析の対象とした。治療の満足度については、とても満足(4.7%)、やや満足(18.6%)、普通(41.2%)、やや不満(29.9%)、不満 (5.6%)であった。これまでの治療に不満と答えた理由では、治療効果が乏しい(28.7%)、通院が面倒 (23.0%)、眠気などの副作用(17.6%) 、費用がかかる (17.2%)、医師の対応や説明が十分でない (4.5%)であった。

1～78 歳の男性 27 名、女性 27 名の計 54 名に対して 2012 年のスギ花粉飛散期に鼻汁を吸引採取し、CAP-FEIA 法によりスギ特異的 IgE を定量した。 鼻汁中スギ特異的 IgE 抗体価は血清特異的 IgE 抗体価と正相関し、スギ花粉症患者群は未発症群より有意に鼻汁中 IgE が高値であった。スギ花粉症未発症群において感作群は非感作群より有意に鼻汁中 IgE が高値であり、未発症群においても鼻汁中スギ IgE 値は血清 IgE 値と正相関した

A. 研究目的

1)アレルギー性鼻炎の治療は、薬物療法、減感作療法、ステロイド注射治療、民間療法など多岐に渡る。今回小児アレルギー性鼻炎の治療の実態の一端を明らかにする目的で、小中学生を対象としてアレルギー性鼻炎と花粉症に対する治療の内容とその効果、費用について調査した。

2)スギ花粉症患者においては鼻組織に特異的IgEが存在するが、花粉飛散中の鼻汁中のスギ特異的IgE抗体の定量を行った報告は少ない。鼻汁中のスギ特異的IgE抗体価が血清中のそれと相関するか、スギ花粉症未発症者において感作が成立していれば鼻汁中にも特異的IgEがみられるか否かを検討した。

B. 研究方法

1)小中学校にてアンケートを配布し、鼻アレルギーあるいは花粉症と診断された生徒からアンケートを回収した。回収した322名を解析の対象とした。

2)1～78歳の男性27名、女性27名の計54名(スギ花粉症患者27名、未発症者27名、未発症者のうち12名はスギ特異的IgE抗体価クラス2以上)を対象とした。2012年のスギ花粉飛散期に鼻汁を吸引採取し、CAP-FEIA法(Phadia)により上清中のスギ特異的IgEを定量した。

(倫理面への配慮)

三重大学医学部の倫理委員会の承認を得て、ヘルシンキ宣言を遵守した。

C. 研究結果

1)これまでの治療経験(複数回答可)では、病院を受診(85．1%)、薬局で市販薬を購入 (25．8%)、健康食品・民間療法、免疫療法、ステロイド注射の順であった。年間の治療の費用に関しては、全体としては、中央値6000円であり、病院での薬剤と免疫療法の治療費は薬局で購入する市販薬の費用より有意に高かった。治療の満足度については、とても満足

(4.7%)、やや満足(18.6%)、普通(41.2%)、やや不満(29.9%)、不満 (5.6%)であった。これまでの治療に不満と答えた理由では、治療効果が乏しい(28.7%)、通院が面倒 (23.0%)、眠気などの副作用(17.6%) 、費用がかかる (17.2%)、医師の対応や説明が十分でない(4.5%)であった。

2)鼻汁中スギ特異的IgE抗体価は血清特異的IgE抗体価と正相関した(ρ =0.781p<0.0001)。スギ花粉症患者群は未発症群より有意に鼻汁中IgEが高値 であった(p=0.0009)。スギ花粉症未発症群において感作群は非感作群より有意に鼻汁中IgEが高値であった(p=0.0416)。未発症群においても鼻汁中スギIgE値は血清IgE値と正相関した(ρ =0.706 、p=0.023)。

D. 考察

小中学生のアレルギー鼻炎のアンケートは保護者の意識調査という意味合いが大きいが、現在の治療の満足度は高いとはいえず、治療効果の改善などの面で課題が多い。

鼻汁中の総IgEが測定可能であることは過去にも報告があるが、今回スギ特異的IgEが鼻汁中で検出可能であり、未発症者でもスギ特異的IgEがすでに鼻汁にみられていることが明らかになった。採血することなく簡便に鼻汁から特異的IgEが測定可能となれば小児などで利用可能となる可能性がある。

E. 結論

小中学生の保護者の現在の治療の満足度は高いとはいえず、治療効果の改善などの面で課題が多い花粉飛散期の鼻汁中でのスギ特異的IgE抗体が測定可能であった。

G. 研究発表

1. 論文発表

なし

2. 学会発表

1) 竹内万彦、坂井田 寛、増田佐和子、岡本美孝.小中学生を対象としたアレルギー性鼻炎に対する治療の実態と治療に対する満足度調査.第31回日本耳鼻咽喉科免疫アレルギー学会.2013年.倉敷市

2) 坂井田 寛、増田佐和子、竹内万彦. 鼻汁中のスギ特異的IgE抗体測定の試み. 第31回日本耳鼻咽喉科免疫アレルギー学会.2013年.倉敷市

3) 坂井田 寛、岩田賢治、山崎皓亮、増田佐和子、竹内万彦. スギ花粉症未発症者におけるスギ花粉の感作に及ぼす因子の検討. 第62回日本アレルギー学会秋季学術大会.2012年.大阪市

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

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

免疫療法による花粉症予防と免疫療法のガイドライン作成に向けた研究
スギ花粉症に対する舌下免疫療法の経年的効果に関する研究

研究分担者　大久保公裕　日本医科大学大学院医学研究科頭頸部感覚器科学分野　教授

研究要旨
舌下免疫療法（SLIT）は欧州で高い有効性を示し、これを評価した二重盲検比較試験のどれをとってもアナフィラキシーの報告はない。喘息はある程度の確率で生じうるが、重責発作などは小児を含めてもないとされる。我々は1999年に大学倫理委員会の承認を受けて、SLITの臨床研究を開始した。舌下免疫療法は、副作用の発生が極めて少なく、さらに有用性が期待されることが証明された。今年度は複数年の舌下免疫療法の効果の検討を行った。実際にそのQOLの悪化程度などから複数年の方が単年度より花粉症に対し効果がある事が示された。さらに施行年数が長い方がQOLの悪化を抑制できる可能性が示唆された。しかし2012年の検討からは何年続ければ良いかの検討については明らかにすることは出来なかった。SLITの方法論を季節中、季節外の連日投与、あるいは季節前からのパルス的SLITなど詳細に検討してゆくことは、アレルギー治癒に向けて進めてゆくべき検討課題と考えられる。

A. 研究目的

免疫療法は現在、皮下免疫療法が一般的に行われているが、アナフィラキシーショックのような重大な副作用が存在するため、根本的治療法であっても広がりのない治療法となっている。皮下免疫療法ではない根治的治療法開発のためにはいかに安全で有効性の高い方法に改良していくかが課題である。投与エキスは蛋白のままでも投与経路を注射から舌下に変更すれば、副反応の発生頻度は極めて少なくなることが知られており、舌下免疫療法も安全は方法として期待が大きい。１年だけでもスギ花粉症に効果のある事は既に我々をはじめ報告されている。また国際的な論文から舌下免疫療法の経年的効果は単年度での効果を上回ることが示唆されているが、スギ花粉症についてはまだ不確実である。そこで今年度、我々は2012年のスギ花粉症のQOLを舌下免疫療法の施行期間により評価した。

B.方法

投与エキスはトリイ薬品製の標準化スギ花粉エキスを使用し、その舌下免疫療法は維持量まで４週間、複数年の場合には１週間で同じ維持量の2000JAUの1mlに達するようにプロトコールを設定した。初回の舌下投与は単年度では11月より、複数年では毎年12月より開始し、花粉飛散季節終了後まで継続した。効果判定の症状はJRQLQを用いて、3月、4月の鼻眼の症状、健康関連QOL、総括的状況を算出した。

C.結果

2012年のスギ花粉飛散は2011年の大飛散の影響で、東京9地点平均では1867個/1㎝と平年の半分以下であった。くしゃみ、鼻汁、鼻閉などの鼻眼の症状は舌下免疫療法の施行期間の長さに関わらず、低く推移した。健康関連QOL、総括的状況も同様であった。経年的な評価ではそれぞれの年に差が認められなかったが、5年以上と5年未満ではQOLが悪化した（鼻眼の症状は合計5点以下、健康関連QOLは10点以下）症例に差が認められる傾向にあった（表）。しかしそれらも有意差は認められなかった。

D.考察

QOLは5年以上の舌下免疫療法を施行している症例で悪化が少ない傾向にあったが、有意差が認められなかったのは2012年のスギ花粉飛散数が少なく、今回は実薬同士の比較のためと考えられた。しかし、各QOLの推移では5年以上の複数年の舌下免疫療法がそれ以下の経年的な舌下療法より軽度であった。これは今までの報告同様に現状のアレルゲン免疫療法では舌下免疫療法も１年

のみの施行では最大限の効果を示してはいない事を示し、数年以上の施行が望ましいことが考えられた。舌下免疫療法の施行について最適な年限については、明らかにできなかったが、季節前のみパルスのように舌下免疫療法を行う場合には2年などの少ない年限では最大限の効果は得られない事が分かった。

E.結論

舌下免疫療法は、副作用の発生が極めて少なく、さらに有用性が期待される新規の治療法である。今回の検討は昨年の検討と合わせて複数年の舌下免疫療法の方がより花粉症に対し効果がある事が示された。しかし2012年の検討からは何年続ければ良いかの検討については明らかにすることは出来なかった。実際に治験で行われた季節を関係なしに行う舌下免疫療法とは異なるが、パルスのような舌下免疫療法では確実に経年的な施行が求められる。このように疾患を根治させうる免疫療法の方法論を季節中、季節外の連日投与、あるいは季節前からのパルス的舌下免疫療法など詳細に検討してゆくことは、アレルギー治癒に向けて進めてゆくべき検討課題と考えられる。

F.研究発表

1. Ishida A, Ohta K, Suzuki Y, Kakehata S, Okubo K, Ikeda H, Shiraishi H, Izuhara K (2012) Expression of pendrin and periostin in allergic rhinitis and chronic rhinosinusitis. Allergology Int 61 (4Dec): 589-595.
2. Kamio T, Watanabe K, Okubo K (2012) Acoustic stimulation promotes DNA fragmentation in the guinea pig cochlea. J Nippon Med Sch 79(5): 349-356.
3. Bousquet J, Schnemann HJ, Samolinski B, et al 162/226 in collaboration with the World Health Organization Collaborating center for Asthma and Rhinitis(2012) Allergic rhinitis and its impact on asthma (ARIA): Achievements in 10 years and future needs. J Allergy Clin Immunol
4. Hashiguchi K, Okubo K, Wakabayashi K, Tanaka N, Watada Y, Suematsu K, Gotoh M (2012) The assessment of the optimal duration of early intervention with montelukast in the treatment of Japanese cedar pollinosis symptom induced in an artificial exposure chamber. J Drug Assessment 1: 40-47.
5. Mori S, Pawankar R, Ozu C, Nonaka M, Yagi T, Okubo K (2012) Expression and role of MMP-2, MMp-9, MMP-13, TIMP-1 and TIMP-2 in allergic nasal mucosa. Allergy Asthma Immunol Res 4(4):231-239.
6. Igarashi T, Nakazato Y, Kunishige T, Fujita M, Yamada Y, Fujimoto C, Okubo K, Takanhashi H (2012) Mometasone furorate nasal spray releaves the ocular symptoms of seasonal allergic rhinoconjunctivitis. J Nippon Med Sch 79(3): 182-189.
7. Wakabayashi K, Hashiguchi K, Kanzaki S, Fujioka M, Tanaka N, Kawashima K, Suematsu K, Yamamotoya H, Iwasaki M, Gotoh M, Horibe S, Naito K, Okubo K (2012) Puranlukast dry syrup inhibits symptoms of Japanese cedar pollinosis in children using OHIO chamber. Allergy Asthma Proc 33(1): 102-109.
8. 大塚博邦、高梨征雄、大久保公裕：スギ花粉症季節前発症における鼻汁細胞診の解析．アレルギー61(8): 1092-1103, 2012.
9. 秋山一男、長谷川真紀、福田健、平田博国、大久保公裕、後藤穣、榎本雅夫、竹内裕美、出原賢治、川崎誠司、宮本昭正：あらすたっと 3gAllergy の臨床的有効性に関する検討—第1報—多施設共同研究による評価（内科・耳鼻咽喉科における検討）．アレルギー・免疫 19(12):114-128, 2012
10. 大久保公裕：季節性アレルギー性鼻炎に対するフェキソフェナジン塩酸塩と塩酸プソイドエフェドリン配合剤の有効性及び安全性の検討：第Ⅱ相/第Ⅲ相、ランダム化、二重盲検、平行群間比較試験．アレルギー・免疫 19(11): 134-134-146, 2012.
11. 大久保公裕、奥田稔：インターネットを用いたアレルギー性鼻炎患者に対するアンケート調査．アレルギー・免疫 19(1):113-124, 2012

G.知的財産権の出願・登録状況（予定も含む）

なし

健康関連 QOL が 10 点未満の症例(%)

	3月	4月	全季節中
2005 (n=10)	60	70	60
2006 (n=22)	77	68	63
2007 (n=18)	50	67	50
2008 (n=19)	63	63	47
2009 (n=9)	44	56	33
2010 (n=23)	73	70	48
2011 (n=5)	80	100	80

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

小学生におけるアレルギー性鼻炎の実態に関する調査

分担研究者　下条直樹（千葉大学大学院医学研究院小児病態学准教授）
研究協力者　森田慶紀（千葉大学大学院医学研究院小児病態学）

研究要旨
千葉市内の10カ所の小学校（総生徒数8359名）に依頼し医師からアレルギー性鼻炎の診断を受けている小学生とその保護者に2012年10月にアンケート調査を行い、以下の結果を得た。

1. 1517名の有効回答の解析では鼻症状の出現時期は1年前が4%, 2-3年前が34%、4-6年前が44%、7年以上前が23%と大部分が幼稚園から小学校低学年の頃に発症していた。
2. 通年性アレルゲンであるダニよりもむしろ花粉をアレルゲンとする方が多いことが示唆された。
3. およそ85%の児は何らかの治療を必要とした。
4. 使用薬剤の効果に対する評価は、病院受診者の2/3が有効と回答した。代替医療などの効果を感じているのは1/4程度であり、医療機関を受診した児に比較すると明らかに低かった。
5. これまでのアレルギー性鼻炎の治療（主に薬物療法）には半数が大きな不満がないが、半数は不満があると回答していた
6. 今後希望する治療としては、病院受診しての薬物療法がもっとも多く（50%）、免疫療法（減感作療法）は30%弱であった。減感作療法はエビデンスもあり今後の治療として期待されるが認知度はまだ高くなく適切な情報提供が必要である、と考えられた。

A．研究目的

アレルギー性鼻炎の罹患率は近年増加傾向にあり、およそ全国民の1/3が罹患していると推測されている。このようにアレルギー性鼻炎は国民病といっていいほど罹患率が高いのみでなく発症年齢も低年齢化しており、学校生活や学業にも支障を来すことが少なくない。しかしながら、週日は授業や課外活動等で忙しく、また急性発作などがある喘息などと異なり緊急性が乏しいことからアレルギー性鼻炎をもつ生徒は医療機関を必ずしも受診していない可能性がある。このような場合には適切なアレルギー性鼻炎の治療を受けていないことも考えられる。昨年度は中学生におきて調査を行った結果、多くが小学校よりスギ花粉症を含むアレルギー性鼻炎に罹患していることが判明した。そこで本年度は、小学生におけるアレルギー性鼻炎の治療法とその効果とを明らかにすることを目的とした。

B．研究方法

千葉市内の10カ所の小学校（総生徒数8359名）に依頼し、毎年全国の学校で行われている保健調査票にて医師からアレルギー性鼻炎の診断を受けている小学生とその保護者に2012年10月にアンケート調査を行った。調査項目は、性別、鼻症状の出現時期、検査で判明しているアレルゲン、最近1年間の病院・薬局からの薬物療法および健康食品・民間療法の有無、治療薬の内容、免疫療法・代替医療への評価、今までの治療法に対する不満点、今後の治療法への期待、などである。

（倫理面への配慮）

本研究はアンケート調査のみであり、また匿名で個人情報の保護に関しても問題ないものと考えられる。

C．研究結果

1) 1517名（男児848名、女児669名）から解析可能なアンケートを回収した。鼻症状の出現時期は1年前が4%, 2-3年前が34%、4-6年前が44%、7年以上前が23%と大部分が幼稚園から小学校低学年の頃に発症していた。
2) 症状のある時期では、通年性が45%、季節性が54%であり（図1）、検査で陽性になっているアレルゲンについてはダニ単独22%、スギ単独22%、ダニ、スギ両者36%、不明20%であった（図2）。
3) 直近1年間にアレルギー性鼻炎の治療のために病院を受診した児は85%、薬局・市販品などで治療薬を購入した児は10%、未治療が10%であった。複数回答であるために病院受診と薬局で市販薬を購入した児も存在したが、およそ85%の児は何らかの治療を必要としたと考え

られる(図3)。本調査では、健康食品などを利用した児は数%であり、治療の大部分が薬物と思われた。服薬内容としては、病院受診者では80%が内服薬を、50%が点鼻薬を、40%が点眼薬を処方されていた。

4) 使用薬剤の効果に対する評価は、病院受診者の2/3が有効と回答した（図4)。代替医療などの効果を感じているのは1/2弱であり(図5)、医療機関を受診した児に比較すると明らかに低かった。

5) 免疫療法は全体の2%が治療経験があり、38%が治療経験なしと返答した。60%がこの質問に未回答であり、免疫療法の認知度が低いと予測された（図6)。

6) これまでのアレルギー性鼻炎の治療（主に薬物療法）には半数が大きな不満がないが、半数は不満があると回答していた(図7)。不満の理由としては、治療効果が乏しいと答えたのは25.9%であり、費用（25.4%）や通院が面倒（28.3%）といった理由も多かった。一方、眠気などが困ると答えた児は12%であり、およそアレルギー性鼻炎患者の1/10は薬物の副作用により学校生活に支障を来している可能性がある(図8)。

7) 今後希望する治療としては、病院受診しての薬物療法がもっとも多いのは当然だが(50%)、減感作療法がおよそ30%であった(図9)。

D．考察

千葉市のアレルギー性鼻炎を有する小学生を対象としたアンケート調査から、通年性アレルゲンであるダニよりもむしろ花粉をアレルゲンとする鼻炎が多いことが示唆された。また大部分が幼稚園から小学校低学年の頃に発症しており、大部分が薬物療法を必要としていた。今後、発症や重症化の予防を考えると標的となる年代は小学生以下であると考えられる。現在の薬物療法等に満足する児は約半数であり、今後は従来の薬物療法主体の治療のみでなく、薬物療法以外の治療を望む患者が多いことを示している。しかしながら、免疫療法（減感作法）に関する質問からは本治療法の認知がまだ低い可能性も考えられる。根本的な治療法であり、また発症予防にも効果がある可能性が示されている免疫療法の普及を図るべきと考える。

昨年度の中学生を対象としたアンケート調査と比較し、今回のアンケート結果で大きく異なった点は、この 1 年間の AR に対する治療であった（小学生　病院受診 85%、中学生　病院受診 65%)。この違いは中学生になると部活動などのため病院受診ができなくなること、こどもと保護者の関係がかわってくる事などが原因と推測した。

E．結論

千葉市内のアレルギー性鼻炎患者およそ 1000 名のアンケート調査から、1）大部分の児はなんらかの治療を必要としている、2）病院での薬物療法をきちんと受けることが市販の治療薬よりも有効である、3）半数の患者は現在の治療法には満足しておらず、廉価で頻回の通院治療の必要性のない他の治療法を期待している、4）免疫療法はエビデンスもあり今後の治療として期待されるが認知度はまだ高くなく適切な情報提供が必要である、と考えられた。

F．研究発表

1. 論文発表

なし

2. 学会発表

森田慶紀他．千葉市内中学生を対象とするアレルギー性鼻炎の治療法とその効果に関するアンケート調査．第 24 回日本アレルギー学会春季臨床大会　2012 年 5 月 12, 13 日　大阪.

G．知的財産権の出願・登録状況(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

図1. 症状のある時期

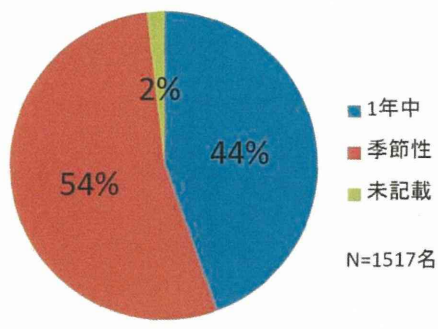


図2. 原因アレルゲン

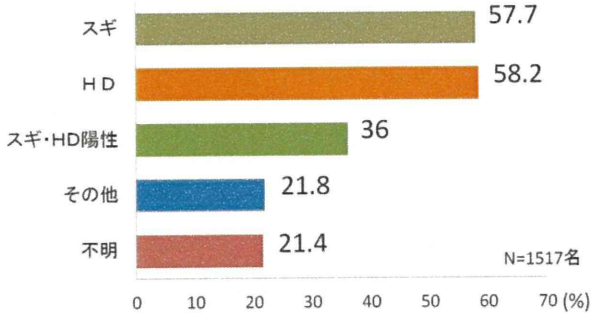
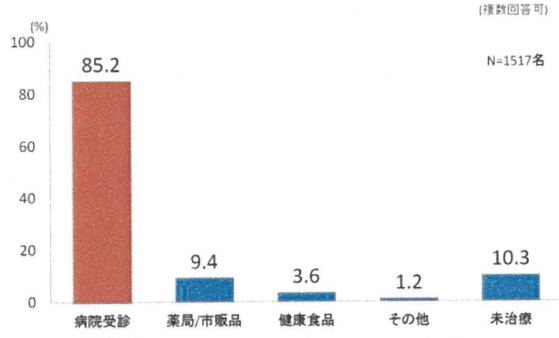
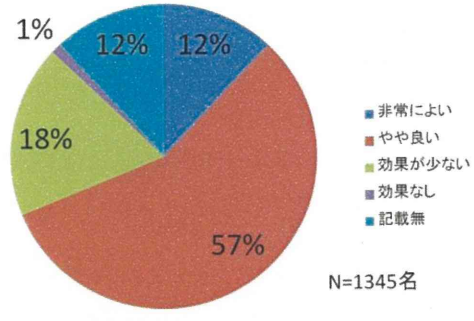


図3. この1年間のARに対する治療



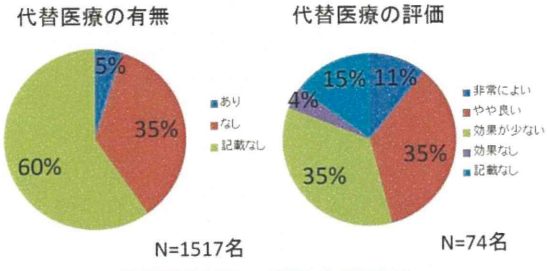
病院受診率は小学生(85%)が中学生(64%)より高い

図4. 病院処方薬の効果への評価



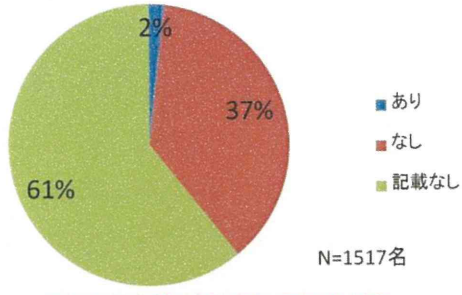
中学生と同様に、病院処方薬には効果に満足している患者が比較的多い

図5. 代替医療治療の効果への評価



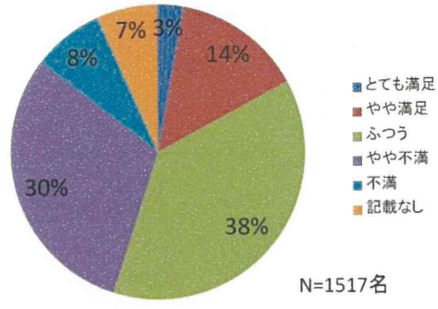
代替医療はほとんど行われておらず、
行われているうちの半数は有効性を評価されていない

図6. 免疫療法の有無



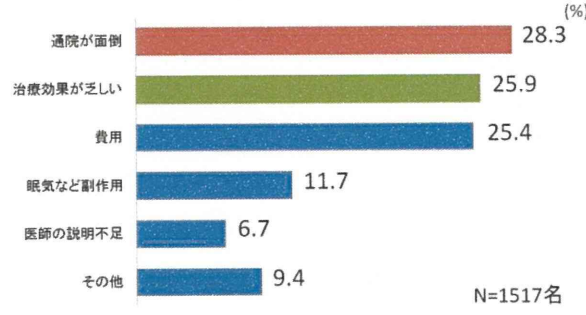
免疫療法の認知度が低い可能性が高い

図7. これまでのAR治療に対する感想



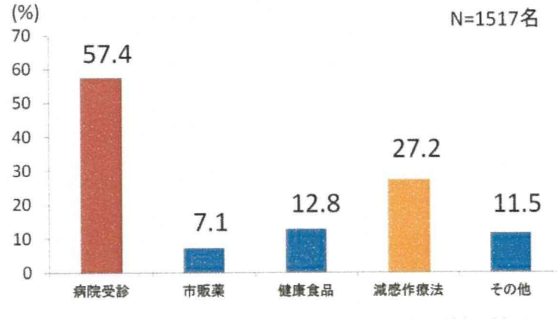
中学生の結果と同様に、既存の治療(薬物療法が主)については半数弱が満足していない

図8. これまでのAR治療の不満点



不満点は多岐にわたるが、自宅で行なえ効果の高い治療法が期待されている

図9. 今後どのような治療を受けたいか



病院受診以外の、これまでとは違う治療を受けたい希望がある

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

発症予防のためのスギ花粉症における最小持続炎症に関する検討

研究分担者　岡　野　光　博　岡山大学大学院医歯薬学総合研究科耳鼻咽喉・頭頸部外科学 准教授
研究協力者　野　山　和　廉　岡山大学大学院医歯薬学総合研究科耳鼻咽喉・頭頸部外科学　医員

研究要旨：
今回我々は、スギ花粉症の発症予防メカニズムを探索するために、スギ花粉症における最小持続炎症の検出を試みた。スギ花粉の非飛散期である 2012 年 8 月に、スギ花粉症患者を対象に、対照ディスクに引き続き低用量スギ花粉エキス付着ディスクによる鼻粘膜誘発反応を連続で行った。1 日目の誘発反応では、20 名中 5 名（25%）が陽性、すなわち発症した。ECP は誘発 1 日目には検出されなかったが、誘発 2 日目の対照ディスクで有意に検出された。トリプターゼは誘発 1 日目より対照ディスクに比較して花粉ディスクから有意に検出された。1 日目誘発の陽性者と陰性者を比較すると、2 日目対照ディスク中の ECP 濃度には 2 群間で差を認めず、また 1 日目誘発陰性者であっても 1 日目対照ディスクと比較して 2 日目対照ディスク中の ECP 濃度は有意に亢進した。トリプターゼについても 1 日目抗原ディスク中の濃度は陽性者と陰性者で有意な差はなく、また誘発陰性者でも 1 日目対照ディスクと比較して 1 日目抗原ディスク中のトリプターゼ濃度は有意に亢進した。以上の結果より、誘発陰性、すなわち未発症であっても鼻汁中 ECP 濃度やトリプターゼ濃度が亢進することが明らかとなり、スギ花粉症においても最小持続炎症が存在することが確認された。

A．研究目的

アレルギー性鼻炎の発症や増悪には鼻粘膜炎症に伴う鼻粘膜過敏性の亢進が重要である。鼻粘膜過敏性亢進のメカニズムとして、最小持続炎症（Minimal Persistent Inflammation）とプライム効果（Priming effect）が知られている。特に最小持続炎症は発症に関与する可能性が指摘されており、発症の予防には最小持続炎症のコントロールが重要であると思われる。しかしながらスギ花粉症における最小持続炎症については不明な点が多い。今回我々は、スギ花粉症の発症予防メカニズムを探索するために、スギ花粉症における最小持続炎症の検出を試みた。

B．研究方法

スギ花粉の非飛散期である 2012 年 8 月に、通年性鼻炎を有さないスギ花粉症患者（n=20）を対象に、対照ディスク（スクラッチ用対照エキス 5μl/ディスク）に引き続き低用量スギ花粉エキス付着ディスク（スクラッチ用スギ花粉エキス 5μl/ディスク）による鼻粘膜誘発反応を 3 日間連続で行った。誘発 5 分後の症状および局所所見を観察し、陽性（発症）と陰性（未発症）の判定を行った。さらにディスクに付着した鼻汁を回収し、抽出液中の ECP、トリプターゼおよび Th2 サイトカイン（IL-5、IL-13、IL-31）濃度を測定した。誘発陽性者と陰性者でのメディエーターやサイトカイン濃度を比較した。

（倫理面への配慮）

被験者に対しては学術的な意義について十

分な説明を行い、同意・協力が得られた上で行った。本研究は学内倫理委員会での承認を得た（受付番号 1436 番）。

C．研究結果

1 日目の誘発反応では、20 名中 5 名（25%）が陽性、すなわち発症した。2 日目では 20 名中 15 名（75%）が陽性、3 日目では 19 名中 13 名（68%）が陽性となった。鼻汁抽出液中の IL-5、IL-13、IL-31 は試験期間を通じて有意に検出されなかった。ECP は誘発 1 日目には検出されなかったが、誘発 2 日目の対照ディスクで有意に検出された。トリプターゼは誘発 1 日目より対照ディスクに比較して花粉ディスクから有意に検出された。1 日目誘発の陽性者と陰性者を比較すると、2 日目対照ディスク中の ECP 濃度には 2 群間で差を認めず、また 1 日目誘発陰性者であっても 1 日目対照ディスクと比較して 2 日目対照ディスク中の ECP 濃度は有意に亢進した。トリプターゼについても 1 日目抗原ディスク中の濃度は陽性者と陰性者で有意な差はなく、また誘発陰性者でも 1 日目対照ディスクと比較して 1 日目抗原ディスク中のトリプターゼ濃度は有意に亢進した。

D．考察

最小持続炎症は、症状を発現しない低レベルの抗原曝露でも鼻粘膜には好酸球や好中球などの細胞浸潤がみられ、さらに上皮細胞における ICAM-1 発現が亢進するなど軽度の炎症が惹起されることを示す（Canonica GW, et al. 2009）。海外では、発症に要する抗原濃度の 1/100 であっても鼻粘膜誘発によって鼻汁中 ECP 濃度が亢進することが報告されている（Roquat A, et al. 1996）。今回の結果では、誘発陰性、すなわち未発症であっても鼻汁中 ECP 濃度やトリプターゼ濃度が亢進することが明らかとなり、スギ花粉症においても最小持続炎症が確認された。一方、Th2 型サイトカインについては鼻汁中では検出されなかった。この結果からは、最小持続炎症においては T 細胞の関与が低い可能性と、Th2 サイトカインは主に粘膜下で作用している可能性などが考えられ、引き続き検討を要すると思われた。

F．健康危険情報

なし

G．研究発表

1. Makihara S, Okano M, Fujiwara T, Kimura M, Higaki T, Haruna T, Noda Y, Kanai K, Kariya S, Nishizaki K. Early interventional treatment with intranasal mometasone furoate in Japanese cedar/cypress pollinosis: a randomized placebo-controlled trial. Allergology International 61; 295-304, 2012.

2. Higaki T, Okano M, Fujiwara T, Makihara S, Kariya S, Noda Y, Haruna T, Nishizaki K. COX/PGE_2 axis critically regulates effects of LPS on eosinophilia-associated cytokine production in nasal polyps. Clinical and Experimental Allergy 42: 1217-1226, 2012.

3. Okano M, Fujiwara T, Higaki T, Makihara S, Haruna T, Nishizaki K. Characterization of Japanese cypress pollinosis and the effect of early interventional treatment for cypress pollinosis. Clinical and Experimental Allergy Reviews 12: 1-9, 2012.

3. Ohta N, Makihara S, Okano M, Kurakami K, Ishida A, Furukawa T, Suzuki Y, Watanabe T, Kakehata S, Aoyagi M. Roles of IL-17, Th1, and Tc1 cells in patients with IgG4-related sclerosing sialadenitis. Laryngoscope 122: 2169-2174, 2012.

4. Okano M, Fujiwara T, Makihara S, Fujiwara R, Higaki T, Kariya S, Noda Y, Haruna T, Nishizaki K. Characterization of IL-18 expression and release in the pathogenesis of chronic rhinosinusitis. International Archives of Allergy and Immunology 160: 275-286, 2012.

5. Higaki T, Okano M, Makihara S, Fujiwara T, Haruna T, Noda Y, Kariya S, Nishizaki K. Early interventional treatment with intranasal corticosteroids is superior to post-onset treatment in pollinosis. Annals of Allergy Asthma and Immunology 109: 458-464, 2012.

6. Kariya S, Okano M, Higaki T, Makihara S, Haruna T, Eguchi M, Nishizaki K. Neutralizing antibody against granulocyte/macrophage colony-stimulating factor inhibits inflammatory response in experimental otitis media. Laryngoscope 2012 (doi: 10.1002/lary.23795).

7. 岡野光博. 代替医療として有効なものに何があるか？ JOHNS 28: 85-88, 2012.

8. 岡野光博. ヒノキ花粉症についての知見. アレルギー・免疫 19; 28-35, 2012.

9. 岡野光博. こどものアレルギー性鼻炎. 耳鼻咽喉・頭頸部外科 84: 23-28, 2012.

10. 岡野光博. Q29.アレルギー性鼻炎の診断. 小児科学レクチャー 2: 643-649, 2012.

11. 岡野光博. 抗 PGD2・TXA2 薬. アレルギーの臨床 32: 319-324, 2012.

12. 岡野光博. アレルギー性鼻炎. 日本胸部臨床 71: S112-8, 2012.

13. 岡野光博. 序～真菌とアレルギー・炎症～. アレルギー・免疫 19; 1041-1042, 2012.

14. 岡野光博. 慢性副鼻腔炎における真菌の関与. アレルギー・免疫 19; 1080-1087, 2012.

15. 岡野光博. アレルギー性鼻炎における治験の問題点. アレルギー・免疫 19: 1693-1700, 2012.

16. 岡野光博. スギ・ヒノキ花粉症に対する免疫療法. 臨床免疫・アレルギー科 58: 689-696, 2012

17. 岡野光博. 好酸球性副鼻腔炎の病態と治療. 日医雑誌 141: 2191-2194, 2013.

18. 岡野光博. 鼻噴霧用ステロイド薬の新たな位置付け. アレルギーの臨床 33: 37-41, 2013

19. 岡野光博. 鼻噴霧用ステロイド薬の初期治療としての可能性. 医薬ジャーナル 49: 75-82, 2013.

２．学会発表

1. 岡野光博. スギ・ヒノキ花粉症に対する免疫療法. 第 24 回日本アレルギー学会春季臨床大会. 大阪. 2012 年(シンポジウム).

2. 岡野光博. アレルギー性鼻炎における鼻噴霧用ステロイド薬の位置づけ-早期介入の意義-.第 62 回日本アレルギー学会秋季学術大会. 大阪. 2012 年.

H. 知的財産権の出願・登録状況(予定を含む)

１．特許取得

なし

２．実用新案登録

なし

３．その他

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Uekusa Y, Inamine A, Yonekura S, Horiguchi S, Fujimura T, Sakurai D,Yamamoto H, Hanazawa T, Okamoto Y	Immunological parameters with the development of allergic rhinitis: A preliminary prospective study.	*American Journal of Rhinology and Allergy*	26	92-96	2012
Inoue H, Mashimo Y, Funamizu M, Yonekura S, Shigtoshi H, Shimojo N, Kohno Y, Okamoto Y, Hata A, Suzuki Y	Association of the MMP9 gene with childhood cedar pollen sensitization and pollinosis.	*J Hum Genet on line publication*		1-8	2012
Yonekura S, Okamoto Y, Shimojo N, Yamamoto H, Sakurai D, Horiguchi S, Hanazawa T, Inoue Y, Arima T, Tomiita M, Kohno Y.	The onset of allergic rhini-tis in Japanese atopic children: A preliminary prospective study.	*Acta Otolaryngol*	132	981-987	2012
Yonekura S, Okamoto Y, Horiguchi S, Sakurai D, Chazono H, Hanazawa T, Okawa T, Aoki S, Konno A	Effects of aging on the natural history of seasonal allergic rhinitis in middle-aged subjects in South chiba, Japan	*Int Arch Allergy Immunol*	157	73-80	2012
Inamine A, Sakurai D, Horiguchi S, Yonekura S, Hanazawa T, Hosokawa H, Matuura-Suzuki A, Nakayama T, Okamoto Y.	Sublingual administration of Lactobacillus paracasei KW3110 inhibits Th2-dependent allergic respon-ses via upregulation of PD-L2 on dendritic cells.	*Clinical Immunology*	143	170-179	2012
Yamamoto H, Yonekura S, Sakurai D, Inamine A, Sakurai T, Iinuma T, Horiguchi S, Okamoto Y .	Comparison of nasal steroid with anti-histamine in prophylactic treatment against pollinosis using an environmental challenge chamber.	*Allergy and Asthma Proceeding*	33	397-403	2012
Matsumoto Y, Noguchi E, Imoto Y, Nanatsue K, Takeshita K, Shibasaki M, Arinami T, Fujieda S	Upregulation of IL17RB during natural allergen exposure in patients with seasonal allergic rhinitis.	*Allergol Int.*	60	87-92	2011
Haenuki Y, Matsushita K, Futatsugi-Yumikura S, Ishii KJ, Kawagoe T, Imoto Y, Fujieda S, Yasuda M, Hisa Y, Akira S, Nakanishi K, Yoshimoto T.	A critical role of IL-33 in experimental allergic rh-initis.	*J Allergy Clin Immunol.*	130	184-194	2012
Higaki T, Okano M, Fujiwara T, Makihara S, Kariya S, Noda Y, Haruna T, Nishizaki K	COX/PGE_2 axis critically regulates effects of LPS on eosinophilia-associated cytokine production in nasal polyps.	*Clinical and Experimental Allergy*	42	1217-1226	2012
Goyoh M, Okubo K.	Sublingual immunotherapy for Japanese cedar polinosisi:current status in Japan.	*Clinical and Experimental Allergy*	12	36-40	2012

Immunological parameters associated with the development of allergic rhinitis: A preliminary prospective study

Yasuhiro Uekusa, M.D., D.D.S., Ph.D.,[1,2] Ayako Inamine, Ph.D.,[1] Syuji Yonekura, M.D., Ph.D.,[1] Shigetoshi Horiguchi, M.D., Ph.D.,[1] Takashi Fujimura, Ph.D.,[1,3] Daijyu Sakurai, M.D., Ph.D.,[1] Heizaburo Yamamoto, M.D., Ph.D.,[1] Homare Suzuki, M.D., Ph.D.,[1] Toyoyuki Hanazawa, M.D., Ph.D.,[1] and Yoshitaka Okamoto, M.D., Ph.D.[1]

ABSTRACT

Background: *Many subjects are sensitized to Japanese cedar pollen but do not develop allergic rhinitis (AR). The aim of this study was to examine the immunologic parameters related to the development of AR in sensitized subjects.*

Methods: *The subjects were 33 adults who were sensitized to Japanese cedar pollen, but had not developed as of 2007. Cedar pollen–specific IgE (sIgE) and total IgE (tIgE) in serum, cedar pollen antigen (Cry j 1) Cry j–specific memory Th2 cell clone size, and the Cry j–specific induced regulatory T cell (iTreg) level were examined before and after the season in 2008.*

Results: *Eight of the 33 subjects developed cedar pollinosis. The sIgE titers before the season in these eight subjects did not differ from those in the subjects who did not develop pollinosis, but the titers after the season were significantly higher in the group that developed pollinosis. The sIgE/tIgE ratio increased in almost all subjects, but the ratio was significantly higher before the season in the subjects who developed pollinosis. Cry j–specific Th2 cells were detected in all subjects, but the clone size only increased in those that developed pollinosis. The Cry j–specific iTreg population did not differ between the two groups.*

Conclusion: *A high sIgE/tIgE ratio before the season may be predictive of development of pollinosis, and an increase in the allergen-specific Th2 clone size during the pollen season could be a biomarker for pollinosis. The role of allergen-specific iTreg cells in the development of pollinosis could not be clarified in this preliminary study.*

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There has been a recent increase in the prevalence of allergic rhinitis (AR), with the prevalence rate exceeding 30% in many countries.[1,2] Along with the burden of the disease and decrease in quality of life associated with AR, there are high costs related to the disorder.[3–5] Genetic and environmental factors are involved in the onset of AR, as with many other diseases. The relevance of genetic factors can not be ignored, but environmental factors may have played a major role in the recent increase in allergic diseases.[6–11] Factors that change the predisposition to produce IgE are thought to have contributed to the recent increase of AR.

Many people have high sensitization rates to a variety of allergens, but some do not develop AR. A higher specific IgE (sIgE) titer is correlated with a higher incidence of AR,[12] but a significant number of subjects with a high sIgE titer do not have AR.[13,14] The incidence rate also varies based on allergens, age, and gender.[15–21] Subjects who are sensitized but do not develop AR might be thought of as being preconditioned to develop AR, and recent studies have also suggested that regulatory T cells (Treg) might play an important role in the development of allergic diseases.[22]

Early intervention strategies are important for management of AR[23–25] and clarification of the underlying mechanisms is required to develop an optimal strategy for secondary intervention. However, the mechanisms underlying development of AR are not well understood. In this preliminary study, we prospectively examined the immunologic parameters that may influence the development of AR.

From the [1]Departments of Otolaryngology, Head and Neck Surgery and [2]Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan, and [3]Research Center for Allergy and Immunology, Yokohama Institute, The Institute of Physical and Chemical Research (RIKEN), Yokohama, Kanagawa, Japan

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Address correspondence and reprint requests to Yoshitaka Okamoto, M.D., Ph.D., Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8670, Japan

E-mail address: yokamoto@faculty.chiba-u.jp

MATERIALS AND METHODS

Inclusion Criteria

The study population consisted of subjects who complained of transient nasal symptoms and were diagnosed with an acute upper airway infection at our hospital in 2007. All subjects met the following inclusion criteria: a serum cedar pollen sIgE score of ≥2 on a CAP-radioallergosorbent test (CAP-RAST; SRL, Tokyo, Japan) performed at the end of 2007 (before the cedar pollen season in 2008), and no history of perennial AR or other pollinosis. Subjects with a history of bronchial asthma were also excluded from the study.

Diagnosis of Japanese Cedar Pollinosis

Diagnosis of cedar pollinosis was based on the following the criteria: symptoms of pollinosis, such as paroxysmal sneezing, runny nose, nasal congestion, nasal itching, and eye itching that persisted for >2 weeks during cedar pollen season (beginning in February and lasting until the middle of April), and positive identification of eosinophils in a nasal smear obtained during the peak of pollen dispersal. The symptoms and presence of eosinophils in the nasal smear disappeared after the pollen season.

Measurement of Parameters

Blood samples were collected before (December 2007) and after (April 2008) the cedar pollen season. Peripheral blood mononuclear cells (PBMCs) were obtained by the Ficoll-Hypaque method and stored in liquid nitrogen until analysis. Total IgE (tIgE) and sIgE titers for Japanese cedar, house-dust mites, and orchard grass were evaluated by the CAP-RAST method (Phadia, Uppsala, Sweden).

Analysis of the Cry j–Specific Memory Th2 Clone Size

The number of IL-4–, IL-5–, or IL-13-producing cells after stimulation with Cry j 1 was determined by an enzyme-linked immunosorbent spot assay, as previously described.[26] Briefly, anti-human IL-4, IL-5, and IL-13 monoclonal antibodies were diluted to a concentration of 15 μg/mL in sterile, filtered (0.45 μm) PBS (pH 7.2), and added to nitrocellulose plates (Millititer; Millipore Corp., Bedford, MA) at 100

μL/well. The plates were incubated overnight at 4°C and unbound antibodies were washed away with filtered PBS. After the last wash, the PBS was sucked through the membrane under a vacuum (Millipore Corp.). A prestimulated cell suspension (100 μL) was added to each well in duplicate and the plates were incubated for 10 hours at 37°C. The cells were washed before addition of 100 μL of biotinylated monoclonal antibodies (1 μg/mL) and incubation for 2 hours at room temperature. The plates were then washed and incubated for 90 minutes at room temperature with 100 μL of streptavidin alkaline phosphatase (Mabtech, Stockholm, Sweden) at a dilution of 1:1000. The unbound conjugate was removed by another series of rinses before 100 μL of 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride substrate solution (BioRad, Richmond, CA) was added, and the plates were incubated at room temperature until dark spots emerged (1 hour). Color development was stopped by repeated rinsing with tap water. After drying, the spots were captured electronically and counted by computer analysis to avoid any visual bias, using an Auto Counter (ImmunoScan; CTL, Gmünd, Germany).

Flow Cytometric Analysis

The levels of Cry j 1–induced Treg (iTreg) and IL-10-Tr1 were analyzed by flow cytometry, as described previously.[27] Briefly, for intracellular staining of Foxp3 and IL-10, PBMCs were cultured with or without Cry j 1 for 3 days, followed by culture with 10 ng/mL of phorbol 12-myristate 13-acetate, 1 μM of ionomycin, and 2 μM of monensin for 6 hours. The PBMCs were stained with phycoerythrin-anti-CD25 (eBioscience, San Diego, CA) and phycoerythrin-Cy7-anti-CD4 (BD Biosciences, San Diego, CA) antibodies in PBS containing 1% FCS and 0.1% sodium azide for 20 minutes at 4°C. After surface staining, the PBMCs were stained with FITC-anti-Foxp3 (clone: PCH101; eBioscience) and allophycocyanin-anti-IL-10 (BD Biosciences) antibodies for 30 minutes at 4°C using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions.

To detect the Treg population, the first gate was set for mononuclear cells based on the profiles of their cell size, and then set a quadro gate to separate negative and positive populations for CD4 and CD25. The threshold of intensity for surface staining to separate the positive and negative populations was based on the intensity of the valley between the positive and negative peaks on each histogram plot for CD4 and CD25 staining. The threshold of intracellular staining was determined by considering the staining profiles of Cry j 1^- healthy subjects without antigen stimulation (negative controls). The intensity of staining control was confirmed to be weaker than the threshold of intracellular staining of the samples. The percentage of IL-10^+/Foxp3^+ cells and that of IL-10^+ cells among the CD4^+/CD25^+ cells were calculated.

Pollen Counts

Cedar pollen dispersion in Chiba City was measured using a gravimetric method with a Durham sampler (Nishizaki Co., Ltd., Funabashi, Japan).

Statistical Analysis

Data were analyzed using two-tailed tests at a significance level of 5% or by chi-square test and Mann-Whitney *U* test.

Ethical Considerations

The study received prior approval from the Ethics Committee of Chiba University (Chiba, Japan). Written, witnessed, informed consent was obtained from all subjects.

RESULTS

Development of Japanese Cedar Pollinosis

The subjects were 33 adults (20 male and 13 female subjects) who were sensitized to Japanese cedar pollen but who had not developed cedar pollinosis as of December 2007. Age ranged from 18 to 41 years, with an average of 26.0 years. No subjects had a history of allergy (bronchial asthma, atopic dermatitis, or perennial AR), 25% had a family history of pollinosis, and 2% had a family history of bronchial asthma.

In 2008, cedar pollen dispersal started on February 20th and ended on April 10th, and the annual cedar pollen count was 4665/cm^2. Of the 33 subjects, 8 (4 male and 4 female subjects; 24%) developed cedar pollinosis in 2008. The ages of these 8 subjects ranged from 18 to 41 years (average, 25.1 years). In these subjects, cedar pollen sIgE titers before the cedar pollen season ranged from 2.62 to 79.5 UA/mL, tIgE titers ranged from 54.7 to 886 IU/mL, and 6 of the subjects (75%) were sensitized to dust mites. The 25 subjects (16 male and 9 female subjects) who did not develop pollinosis ranged in age from 19 to 37 years (average, 26.4 years), had cedar pollen sIgE titers before the cedar pollen season from 0.77 to 57.7 UA/mL, and tIgE titers from 7.3 to 1590 IU/mL, with 72% sensitized to dust mites. There were no significant differences in pollen-specific titers, tIgE titers, and sensitization to dust mites between the two groups (Table 1).

Table 1 **Baseline characteristics of the subjects**

	AR$^+$	AR$^-$
Subjects	8	25
Sex (M/F)	4/4	16/9
Age, yr		
Mean	25.1 ± 7.7	26.3 ± 5.7
Range	18–41	19–37
sIgE titer (UA/mL)		
Mean	30.9 ± 28.8	10.7 ± 14.1
Range	2.62–79.5	0.77–57.7
tIgE titer (IU/mL)		
Mean	350.3 ± 284.0	390.48 ± 411.6
Range	54.7–886	7.3–1590
Sensitization to house-dust mites	6 (75%)	18 (72%)

sIgE and tIgE titers were measured at the end of 2007.
AR = allergic rhinitis; sIgE = cedar pollen–specific IgE; tIgE = total IgE.

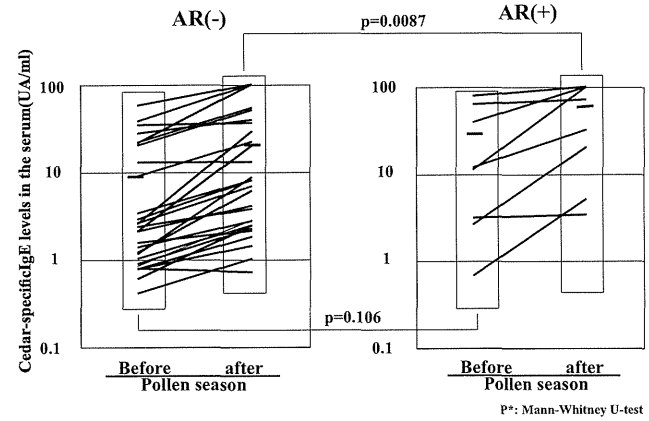


Figure 1. Cedar pollen–specific IgE levels in serum before and after pollen dispersal in 2008. The specific IgE (sIgE) titers before the pollen season did not differ significantly between subjects who did and not develop pollinosis. During the pollen season, the levels increased in almost all subjects. The titers after the pollen season were significantly higher in subjects who developed pollinosis (p = 0.0087).

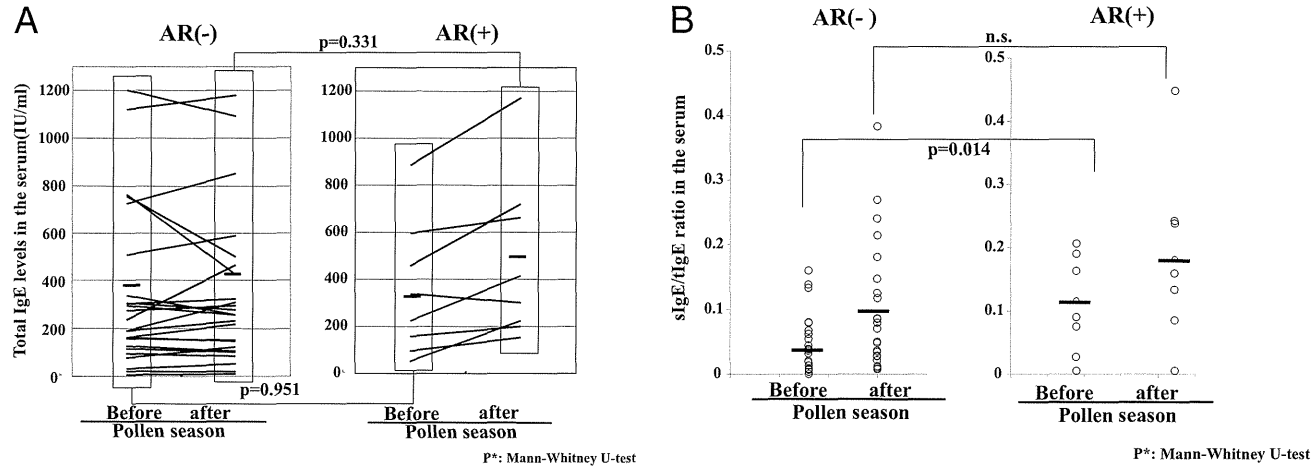


Figure 2. *(A) Total IgE (tIgE) levels in serum before and after pollen dispersal in 2008. The tIgE titers did not differ significantly between subjects who did and not develop pollinosis. During the pollen season, titers increased in almost all subjects and were not significantly different in the two groups. (B) The sIgE/tIgE ratios before and after pollen dispersal in 2008. The average ratio of Japanese cedar pollen sIgE/tIgE in serum before the pollen season was significantly higher in the AR^+ group compared with the AR^- group (0.105 ± 0.074 versus 0.037 ± 0.042; p = 0.022).*

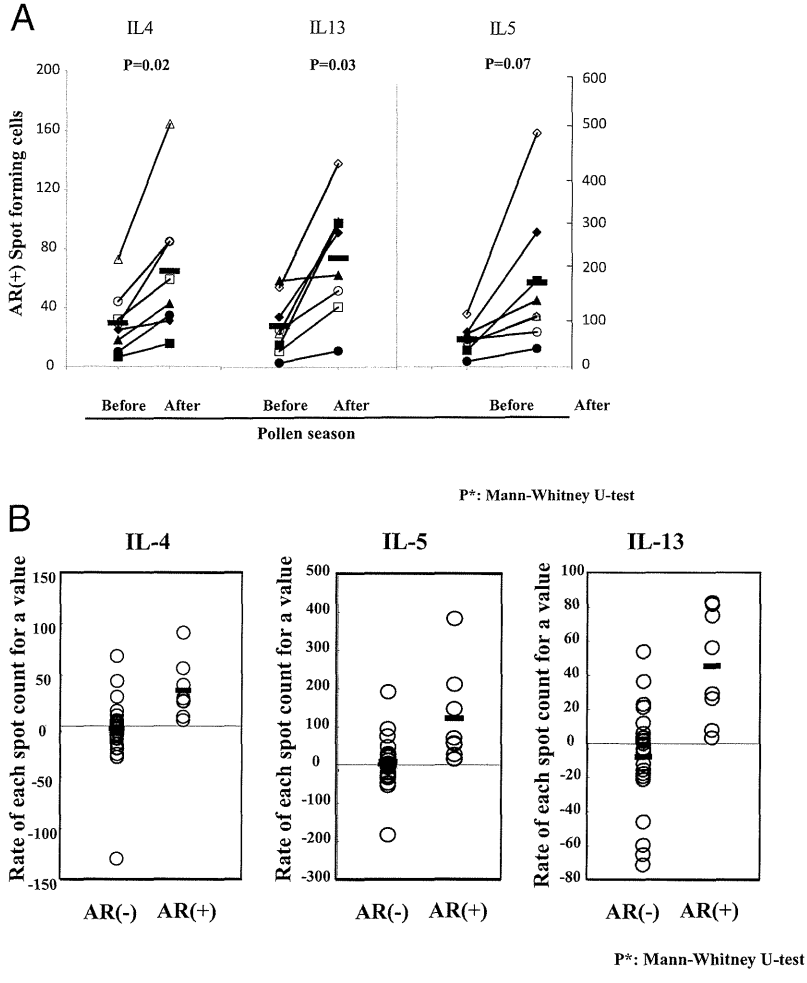


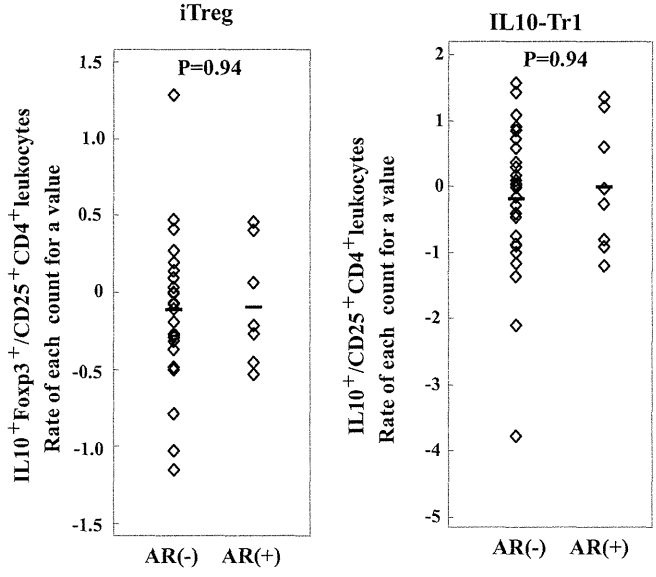
Figure 3. *Cry j 1 peptide–specific IL-4–, IL-5–, and IL-13–producing cells were counted by enzyme-linked immunosorbent spot (ELISPOT) assay before and after the pollen season in 2008. Samples at each time point were analyzed simultaneously. (A) IL-4, IL-5, and IL-13 spots after the pollen season in subjects who did and did not develop cedar pollinosis, compared with those before the season (p < 0.05). (B) Relative change of Cry j–specific IL-4, IL-5, and IL-13 clone sizes after the pollen season, compared with those before the season (p < 0.05).*

Cry j Pollen sIgE Levels in Serum

Serum sIgE levels are shown in Fig. 1. Cedar pollen sIgE titers before the pollen season did not differ significantly between subjects who did and did not develop pollinosis. Titers increased during the pollen season in almost all subjects, but after the pollen season the titers were significantly higher in subjects who developed pollinosis.

tIgE Levels in Serum

Total serum IgE levels are shown in Fig. 2 *A*. The tIgE titers did not differ significantly between subjects who did and did not develop pollinosis. Titers increased during the pollen season in almost all subjects and were not significantly different in the two groups.



P*: Mann-Whitney U-test

Figure 4. Detection of IL-10$^+$ Foxp3$^+$/CD25$^+$CD4$^+$ T cells and IL-10$^+$/CD25$^+$CD4$^+$ T cells in flow cytometric analysis of peripheral blood mononuclear cells (PBMCs). The relative changes from before to after the pollen season are shown for subjects who did and did not develop cedar pollinosis.

sIgE/tIgE Ratio

The average ratio of Japanese cedar pollen sIgE to tIgE in serum (sIgE/tIgE) before the pollen season was significantly higher in subjects who developed pollinosis (0.105 ± 0.074 versus 0.037 ± 0.042; p = 0.022 by Mann-Whitney U test; Fig. 2 *B*).

Cry j-Specific Memory Th2 Cell Clone Sizes

The numbers of antigen-specific IL-4, IL-5, and IL-13 spots are shown in Fig. 3 *A*. The number of IL-4 spots before the cedar pollen season was similar for subjects who did and did not develop pollinosis, but a significant increase in IL-4 spots after the pollen season occurred only for those that developed pollinosis. The same trend was obtained for IL-5 and IL-13 spots. Changes in Th2 clone sizes are shown in Fig. 3 *B*. The Th2 clone size in the group that developed pollinosis showed a significant increase after the pollen season.

Level of Cry j–Specific iTregs

We analyzed the population of IL-10$^+$Foxp3$^+$ cells and IL10$^+$ cells in CD25$^+$CD4$^+$ leukocytes as potential markers for iTreg and IL10$^+$Tr1 cells after stimulation with or without Cry j 1 before and after the pollen season in 2008. Flow cytometric analysis of the IL-10$^+$ Foxp3$^+$/CD25$^+$CD4$^+$ T cells and IL-10$^+$/CD25$^+$CD4$^+$ T cells among PBMCs is shown in Fig. 4. There were few Cry j-specific iTreg (0.2–1.9%) and Tr1 cells (1.1–2.9%) among the CD25$^+$CD4$^+$ T cells in all subjects before the pollen season. The number of Cry j–specific Tregs did not increase on pollen exposure and did not differ between the two groups of subjects or from before to after the cedar pollen season.

DISCUSSION

Eight of the 33 subjects who were sensitized to Japanese cedar pollen but who had not developed cedar pollinosis by the end 2007 developed cedar pollinosis in 2008. In this context, it is of note that cedar pollen counts in 2008 were higher than the average over the last 15 years (3000/cm^2). In our prospective study, the serum sIgE titer increased in almost all subjects during the pollen season and was significantly higher after the pollen season in the group that developed pollinosis; however, no significant difference between the groups was observed before the pollen season. Subjects with a higher sIgE titer in serum are known to have a higher incidence of AR,[11,13] but a high sIgE titer may not always predict development of AR. Thus, some subjects with a CAP-RAST score of 5 or 6 do not have AR.[16]

The sIgE/tIgE ratio in serum was significantly higher in subjects who developed pollinosis, even before the pollen season. In immunotherapy for grass pollinosis, the sIgE/tIgE ratio is significantly higher in responders than in nonresponders.[28] However, in this study, a high sIgE/tIgE ratio was found to correlate with development of cedar pollinosis. This may reflect the amount of surface IgE on effector cells such as mast cells and basophils, and a low level of sIgE might cause these cells to be less likely to be activated by antigen cross-linking. Our results suggest that the sIgE/tIgE ratio is a more sensitive marker for prediction of onset of Japanese cedar pollinosis, compared with the serum sIgE titer, and this ratio might be a useful predictive marker for development of AR.

The profiles of allergen-specific Th cells differed between subjects who did and did not develop pollinosis. Cedar-specific IL-4–, IL-5–, and IL-13–producing memory T cells in peripheral blood were examined by enzyme-linked immunosorbent spot assay using Japanese cedar pollen–specific peptides. The number of cedar peptide–specific Th2 cells was low, but all subjects examined exhibited 5–100 spots/10^5 of PBMCs. The size of the cedar pollen–specific Th2 cell clones did not differ between the two groups before the pollen season. However, the Cry j–specific Th2 clone sizes increased by ~1.5-fold during the cedar pollen season in the group that developed pollinosis. This increase did not occur in subjects who did not develop pollinosis. The change in clone size may correlate with the allergen sIgE level and was more sensitive to this level compared with the change in serum sIgE as reported in our previous study.[26] The increased pollen-specific Th2 clone size is more susceptible to change and results in up-regulation of Th2-mediated immune responses by pollen exposure.

The immunologic mechanisms underlying the development of AR are complicated and depend on factors including the allergen, allergen exposure, age, and gender. However, elucidation of these mechanisms is necessary for promotion of early intervention. Recent studies have suggested the significance of Tregs[21,29,30] and higher IL-10 levels in the off-season in AR patients without bronchial hyperresponsiveness, compared with those with bronchial hyperresponsiveness.[31] In our study, there were few Tregs in subjects who were sensitized to Japanese cedar pollen but who had not developed pol-

linosis, and these Tregs did not increase after pollen exposure. The suppression of allergen-specific Th2 clones observed in this study may have been induced through development of Tregs, although no significant contribution of specific iTreg and IL-10^+ Tr1 cells was observed. Therefore, the precise composition of the various types of T cells requires clarification in further studies.

In summary, this preliminary study suggests that development of cedar pollinosis is associated with a high sIgE/tIgE ratio before pollen exposure and with an increased specific Th2 lymphocyte clone size induced by exposure to cedar pollen. The precise role of the sIgE/tIgE ratio and the contribution of T-cell subsets need to be examined in a large cohort study.

REFERENCES

1. Bousquet J, Khaltaev N, and Cruz AA; Aria Workshop Group. World Health Organization: Allergic rhinitis and its impact on asthma (ARIA) 2008 update. Allergy 63(suppl 86):8–160, 2008.
2. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet 351:1225–1232, 1998.
3. Michael S, and Blaiss MD, Allergic rhinitis: Direct and indirect costs. Allergy Asthma Proc 31:375–380, 2010.
4. Eli OM, Robert N, Jennifer D, et al. Sleep, quality of life, and productivity impact of nasal symptoms in the United States: Findings from the Burden of Rhinitis in America survey. Allergy Asthma Proc 30:244–254, 2009.
5. So YK, Seok-Jun Y, Min-Woo J, et al. Economic burden of allergic rhinitis in Korea. Am J Rhinol Allergy 24:e110–e113, 2010.
6. Kurz T, Altmueller J, Strauch K, et al. A genome-wide screen on the genetics of atopy in a multiethnic European population reveals a major atopy locus on chromosome 3q21.3. Allergy 60:192–199, 2005.
7. Barnes KC, and Marsh DG. The genetics and complexity of allergy and asthma. Immunol Today 19:325–332, 1998.
8. Kompauer I, Henrich J, Wolfman G, and Linseisen J. Association of carotenoids, tocopherols and vitamin C in plasma with allergic sensitization in adults. Public Health Nutr 9:472–479, 2006.
9. Am JS, Swartz J, Lilja G, et al. Atopy in children of families with an anthroposophic lifestyle. Lancet 353:1485–1488, 1999.
10. Lee YL, Shaw CK, Su HJ, et al. Climate, traffic-related air pollutions and allergic rhinitis prevalence in middle-school children in Taiwan. Eur Respir J 21:964–970, 2003.
11. Strachan DP. Hay fever, hygiene, and household size. BMJ 299:1259–1260, 1989.
12. Bodtger U, Poulsen LK, and Linneberg A. Rhinitis symptoms and IgE sensitization as risk factors for development of later allergic rhinitis in adults. Allergy 61:712–716, 2006.
13. Pastorello EA, Incorvaia C, Ortolani C, et al. Studies on the relationship between the level of specific IgE antibodies and the clinical expression of allergy: I. Definition of levels distinguishing patients with symptomatic from patients with asymptomatic allergy to common aeroallergens. J Allergy Clin Immunol 96:580–587, 1995.
14. Niemeijer NR, Fluks AF, and de Monchy JG. Optimization of skin testing. II. Evaluation of concentration and cutoff values, as compared with RAST and clinical history, in a multicenter study. Allergy 48:498–503, 1993.
15. Mabry RL. Rhinitis of pregnancy. South Med J 79:965–971, 1986.
16. Okamoto Y, Horiguchi S, Yamamoto H, et al. Present situation of cedar pollinosis in Japan and its immune responses. Allergol Int 58:155–162, 2009
17. Okawa T, Konno A, Ymakoshi T, et al. Analysis of natural history of Japanese cedar pollinosis. Int Arch Allergy Immunol 131:39–45, 2003.
18. Schoefer Y, Scäfer T, Meisinger C, et al. Predictivity of allergic sensitization (RAST) for the onset of allergic disease in adults. Allergy 63:81–86, 2008.
19. Fagan JK, Scheff PA, Hryhorczuk D, et al. Prevalence of asthma and other allergic disease in an adolescent population: Association with gender and race. Ann Allergy Asthma Immunol 86:177–184, 2001.
20. Lee JH, Haselkorn T, Chipps BE, et al. Gender difference in IgE-mediated allergic asthma in the epidemiology and natural history of asthma: Outcomes and Treatment Regimens (TENOR) study. J Asthma 43:179–184, 2006.
21. Bertelsen RJ, Instanes C, Granum B, et al. Gender differences in indoor allergen exposure and association with current rhinitis. Clin Exp Allergy 40:1388–1397, 2010.
22. Han D, Wang C, Lou W, et al. Allergen-specific IL-10-secreting type 1 T regulatory cells, But not $CD4^+CD25^+Foxp3^+$ T cells, are decreased in peripheral blood of patients with persistent allergic rhinitis. Clin Immunol 136:292–301, 2010.
23. Wahn U, Lau S, Bergmann R, et al. Indoor allergen exposure is a risk factor for sensitization during the first three years of life. J Allergy Clin Immunol 99:763–769, 1997.
24. Brozek JL, Bousquet J, Baena-Cagnani CE, et al. Allergic Rhinitis and Its Impact on Asthma (ARIA) guidelines: 2010 Revision. J Allergy Clin Immunol 126:466–476, 2010.
25. Holt PG, and Sly PD. Prevention of allergy respiratory disease in infants: Current aspects and future perspectives. Curr Opin Allergy Clin Immunol 7:547–555, 2007.
26. Horiguchi S, Tanaka Y, Uchida T, et al. Seasonal changes in antigen-specific Th clone sizes in patients with Japanese cedar pollinosis: A 2-year study. Clin Exp Allergy 38:405–412, 2008.
27. Fujimura T, Yonekura S, Taniguchi Y, et al. The induced regulatory T-cell level, defined as the proportion of $IL10^+Foxp3^+$ cells among $CD25^+CD4^+$ leukocytes, is a potential therapeutic biomarker for sublingual immunotherapy: A preliminary report. Int Arch Allergy Immunol 153:378–387, 2010.
28. Di Lorenzo G, Mansueto P, and Pacor ML, Evaluation of serum s-IgE/total IgE ratio in predicting clinical response to allergen-specific immunotherapy. J Allergy Clin Immunol 123:1103–1110, 2009.
29. Fujimura T, and Okamoto Y. Antigen-specific Immunotherapy against allergic rhinitis: The state of art. Allergol Inter 59:21–31, 2010.
30. Palomares O, Yaman G, Azkur AK, et al. Role of Treg in immune regulation of allergic disease. Eur J Immunol 40:1232–1240, 2010.
31. Emel K, Ayse A, Zafer G, et al. The effects of natural pollen exposure on inflammatory cytokines and their relationship with nonspecific bronchial hyperresponsiveness in seasonal allergic rhinitis. Allergy Asthma Proc 31:126–131, 2010. □

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ORIGINAL ARTICLE

Association of the *MMP9* gene with childhood cedar pollen sensitization and pollinosis

Hiroki Inoue[1], Yoichi Mashimo[1], Makiko Funamizu[1], Shuji Yonekura[2], Shigetoshi Horiguchi[2], Naoki Shimojo[3], Yoichi Kohno[3], Yoshitaka Okamoto[2], Akira Hata[1] and Yoichi Suzuki[1]

Matrix metalloproteinase 9 (*MMP9*) gene has been shown to be involved in the pathogenesis of allergic rhinitis (AR) and asthma. Previous studies suggested that single-nucleotide polymorphisms (SNPs) of the *MMP9* gene conferred a risk for childhood asthma. However, whether the SNPs confer a risk for AR has not been previously investigated. The objective of this study was to investigate whether SNPs of the *MMP9* gene are associated with risk of seasonal AR (pollinosis), perennial AR and allergen sensitization. A total of 670 school children were recruited in Japan and genotyped for functional polymorphism in the promoter (−1590C/T: rs3918242) and three amino-acid substitutions (R297Q: rs17576; P574R: rs2250889; R668Q: rs17577). Serum levels of total and specific IgE were determined. Disease status and other clinical characteristics of the subjects were investigated using a questionnaire. Associations between the *MMP9* SNPs and both AR and serum IgE levels were evaluated. −1590C/T showed significant association with cedar pollinosis (corrected *P* (*Pcor*)=0.039). R668Q was in strong linkage disequilibrium (LD) with −1590C/T and showed significant association with cedar pollinosis (*Pcor*=0.023) and serum cedar pollen-specific IgE level (*Pcor*=0.022). A haplotype associated with −1590T and 668Q showed a significant association with cedar pollinosis, orchard grass pollinosis and cedar pollen-specific IgE (*Pcor*=0.0012, *Pcor*=0.0059 and *Pcor*=0.0041, respectively). R297Q and P574R were in weak LD with the rest of the SNPs and did not show significant association with disease. Compared with wild-type MMP9 protein (279R–574P–668R), a variant enzyme (279R–574P–668Q) that showed association with pollinosis had lower activity. However, lower enzyme activity was not associated with disease risk because another variant (279Q–574R–668R) showed lower enzyme activity but was not associated with pollinosis. The −1590T allele and its corresponding haplotype was associated with higher promoter activity and with pollen-specific IgE levels and pollinosis, suggesting that −1590C/T may have more impact on sensitization and disease development than R668Q. Our results suggest that the *MMP9* gene confers susceptibility to cedar pollinosis in Japanese children. The *MMP9* gene may be associated with pollinosis through sensitization processes.
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INTRODUCTION

The main symptoms of allergic rhinitis (AR) are nasal congestion caused by mucosal edema; runny nose caused by hypersecretion; and repetitive sneezing. Pollinosis is a seasonal type of AR caused by an allergic reaction to pollen. Japanese cedar pollen is the most common causative allergen for pollinosis in Japan. According to a recent study, the prevalence rates of AR and cedar pollinosis in 2006 were 27.2 and 8.0%, respectively; both of which were higher than the rates in 1996.[1]

Matrix metalloproteinases (MMPs) are a family of enzymes that not only degrade the extracellular matrix but also mediate activation of other proteases and secretion of cytokines, thereby affecting inflammatory processes.[2,3] MMP9, also known as gelatinase B, was shown to be an important mediator of inflammation in a murine model of asthma[4,5] and in immune complex-mediated lung injury.[6] In a mouse asthma model, loss of the *MMP9* gene was found to inhibit the development of allergic inflammation by impairing the recruitment of dendritic cells (DCs) into the alveoli and the local production of proallergic chemokines by DCs.[7] MMP9 levels in bronchoalveolar lavage and in the plasma are positively associated with allergen challenge[8] and severity of disease[9,10] in asthmatic patients, suggesting the involvement of MMP9 in asthma pathogenesis in humans. Compared with what is known about the role of MMP9 in asthma, knowledge regarding AR is limited. It has been demonstrated that nasal provocation with allergen induces release of MMP9 during the late-phase inflammatory response.[11] Lim *et al.*[12] reported that airway remodeling associated with long-term allergen challenge can occur in the nasal mucosa and the lung, and that expressions of MMP9 and tissue inhibitors of metalloproteinase 1 (Timp-1) were increased in

[1]Department of Public Health, Graduate School of Medicine, Chiba University, Chiba, Japan; [2]Department of Otolaryngology, Graduate School of Medicine, Chiba University, Chiba, Japan and [3]Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba, Japan
Correspondence: Professor Y Suzuki, Department of Public Health, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
E-mail: ysuzuki@faculty.chiba-u.jp
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subepithelial regions. Shimizu *et al.*[13] reported that the attenuating effect of tranilast on MMP9 production from nasal fibroblasts induced by inflammatory stimulation may underlie the therapeutic mode of action of this agent in patients with allergic diseases including AR.

The *MMP9* gene is located at chromosome 20q,[14] where linkage to bronchial hyperresponsiveness[15] and specific sensitization[15,16] has been reported. In a previous association study of the *MMP9* gene with asthma in the Japanese population,[17] single-nucleotide polymorphisms (SNPs) 2127G/A (rs2274755) and 5546G/A (R668Q; rs17577), which were in strong linkage disequilibrium (LD), were shown to be significantly associated with atopic childhood asthma. The SNP 2127G/A was in complete LD with a promoter SNP (−1590C/T, rs3918242) in which the T allele showed higher promoter activity than the C allele in a promoter assay in a bronchial epithelial cell line.[17] There are two more SNPs that change the amino-acid sequence of MMP9: R279Q (rs17576) and P574R (rs2250889). R279Q was shown to be associated with aortic pulse wave velocity and serum MMP9 level.[18] However, the effects of these three amino-acid changes on enzyme activity or function at the molecular level have not been reported previously. To our knowledge, no studies have been conducted to investigate whether the *MMP9* SNPs that showed association with asthma confer a risk for AR or allergic sensitization (atopy).

To investigate the association between the *MMP9* gene and both AR and sensitization to common aero-allergens, we genotyped the functional promoter SNP and three potentially functional coding SNPs, and evaluated the symptoms of AR and serum total/specific IgE levels in Japanese school children. We also evaluated the effect of the amino-acid changes on MMP9 enzyme activity.

MATERIALS AND METHODS

Subjects

Japanese elementary school children in Chiba and Yamanashi prefectures were recruited for this study. A total of 473 school children aged 6–12 years were enrolled in Chiba city in Chiba prefecture, details of which were described previously,[19] and 260 school children within the same age range were enrolled in Hokoto city in Yamanashi prefecture. Blood samples were collected for serum IgE measurement and DNA preparation from 410 children in Chiba and 260 children in Yamanashi in July and August 2006.

Total and specific serum IgE levels were assayed using the CAP-radioallergosorbent test (Pharmacia Diagnostics, Uppsala, Sweden). Eight specific IgEs were measured house dust mite (*Dermatophagouides pteronyssinus*), dog (*Canis familiaris*) dander, cat (*Felis domesticus*) dander, black mold (*Alternaria alternata*), cedar (*Cryptomeria japonica*) pollen, orchard grass (*Dactylis glomerata*), egg white, and golden, black bellied, or djungarian hamsters (*Mesocricetus auratus/Cricetus cricetus/Phodopus sungorus*). Atopy was defined as the presence of ($\geqslant$0.35 IU ml^{-1}) specific IgE positive against at least one of the assessed allergens. To assess the status of allergic diseases, questionnaires based on the International Study of Asthma and Allergies in Childhood[20] were used. Subjects with symptoms of AR in any month from February to May and positive serum IgE to cedar pollen (class 1 and higher) were defined as having cedar pollinosis. Subjects with symptoms of AR in any month from May to July and positive serum IgE to orchard grass pollen were defined as having orchard grass pollinosis. Mite-positive perennial AR was diagnosed in children who had symptoms throughout the duration of a year and positive serum mite-specific IgE. Children who were negative for IgE specific to any assessed allergen and had no allergic diseases were assigned to the non-atopic control group. This study was approved by the ethics committee of Chiba University Graduate School of Medicine.

SNP selection and genotyping

In a previous association study by Nakashima *et al.*,[17] 2127G/A (rs2274755) and 5546G/A (R668Q: rs17577) showed significant association with asthma in Japanese children. We selected these as candidate SNPs for AR. In the same study, −1590C/T (rs3918242) was shown to be a functional SNP and in complete LD with 2127G/A in 24 individuals. This SNP, however, was not genotyped in all samples. In light of the importance of this SNP, we genotyped −1590C/T in the present study. We also included two non-synonymous SNPs, R279Q (rs17576) and P574R (rs2250889), because of their possible effect on enzyme activity and susceptibility to AR.

Genomic DNA was prepared from whole blood samples using a standard protocol. Whole genome amplification was performed using the illustra GenomiPhi V2 amplification kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's standard protocol.

Genotyping was performed using the SNaPshot method (Applied Biosystems, Foster City, CA, USA). Multiplex PCR amplification was performed in a 10-μl aliquot of reaction mixture containing 5 ng amplified template DNA, 0.025 U TaKaRa ExTaq HS (TaKaRa Bio Inc., Otsu, Japan), 1 μl 10× Ex buffer, 200 μM of each dNTP and 0.5 μM each of the PCR primer pairs shown in Supplementary Table 1. Amplification was carried out using a GeneAmp PCR System 9700 (Applied Biosystems) according to the following program: initial denaturation at 95 °C for 3 min followed by 40 cycles at 95 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min, with final extension at 72 °C for 5 min. Post-PCR treatment to remove primers and unincorporated dNTPs was performed with SAP (shrimp alkaline phosphatase; TaKaRa Bio Inc.) and *ExoI* (New England Biolabs, Ipswich, MA, USA). PCR products were incubated with 0.5 U SAP and 1 U *ExoI* for 1 h at 37 °C, followed by incubation for 15 min at 80 °C to induce enzyme inactivation. The SNaPshot reaction was performed in 10 μl reaction mixture containing 0.5 μl SNaPshot Ready Reaction Mix, 2 μl SAP/*ExoI*-treated PCR products and 0.1 μl SNaPshot primers, as shown in Supplementary Table 1. SNaPshot primers were designed to anneal adjacent to the SNP of interest and to contain an additional sequence with several (incomplete) repeats of the "acgt" sequence (indicated by lower case) at the 5′-end to obtain a convenient length to discriminate it from other SNaPshot products. The reaction mixture was subjected to an initial step of 96 °C for 1 min to activate the enzyme, followed by 35 single-base extension cycles of denaturation at 96 °C for 10 s and annealing and extension at 60 °C for 30 s. Post-extension treatment to remove the 5′-phosphoryl group of the ddNTPs was performed with CIAP (calf intestine alkaline phosphatase; TaKaRa Bio Inc.). The final mixture (6 μl) was treated with 1 U CIAP for 60 min at 37 °C, followed by 15 min at 80 °C for enzyme inactivation. The SNaPshot products (1 μl) were mixed with 10 μl HiDi formamide and 0.05 μl GeneScan-120 LIZ size standard (Applied Biosystems) and electrophoresed using a 50-cm length capillary with Performance Optimum Polymer 6 (POP6) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The resulting data were analyzed with GeneMapper v3.5 software (Applied Biosystems).

Expression vector construction

A cDNA fragment encoding human *MMP9* was obtained from normal human small airway epithelial cells using reverse transcriptase PCR and the following primers: forward, 5′-CCC AAG CTG GCT AGC GAC ACC TCT GCC CTC ACC ATG A-3′; reverse, 5′-CCC TCT AGA CTC GAG GTT GGT CCC AGT GGG GAT TTA-3′ (both primers include the 15-bp homology extension for In-Fusion cloning into vector pcDNA3.1). The cDNA fragment was cloned into pcDNA3.1 (+) (Life Technologies, Carlsbad, CA, USA) and digested with *NheI*–*XhoI* using the In-Fusion Advantage PCR Cloning Kit (TaKaRa Bio Inc.). The Q279R, R574P and R668Q mutations were introduced by PCR-based site-directed mutagenesis using PrimeSTAR MAX polymerase (TaKaRa Bio Inc.). We constructed four MMP9 expression vectors containing four different haplotypes of *MMP9*: pcDNA3.1–MMP9-H1 (279R–574P–668R: type 1), pcDNA3.1–MMP9-H2 (279Q–574R–668R: type 2), pcDNA3.1–MMP9-H3 (279R–574P–668Q: type 3) and pcDNA3.1–MMP9-H4 (279Q–574R–668Q: type 4). The nucleotide sequences of *MMP9* in these constructs were confirmed by DNA sequencing before transfection of cells.

Stable transformants

HEK293 (human embryonic kidney cell line) cells were cultured in minimum essential medium supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% fetal bovine serum and antibiotics. Empty vector pcDNA3.1 or MMP9 expression vectors pcDNA3.1–MMP9 were transfected into the cells

using FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Stable transformants were obtained after 2–3 weeks by selection with 600 μg ml^{-1} Geneticin (G-418).

Enzyme activity

Stable transformant cell lines were maintained with 10% fetal calf serum in minimum essential medium. For the enzyme assay, 5×10^6 cells were seeded in a 25-cm^2 flask with minimum essential medium without fetal calf serum. After 24 h of incubation at 37 °C, medium was recovered and stored at −20 °C until use. MMP9 proenzyme secreted into the culture medium was activated with 1 mM 4-aminophenylmercuric acetate before adding to the assay mixture. Protease activity of MMP9 in the conditioned medium was evaluated with synthetic fluorescence peptide as a substrate using SensoLyte 520 MMP9 Assay Kit *fluorimetric* (AanSpec Inc., Fremont, CA, USA). Fluorescence of 5-carboxyfluorescein was monitored at excitation/emission wavelengths of 490 and 520 nm using infinite F200 (TECAN, Männedorf, Switzerland). BioPlex 200 (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure MMP9 protein concentrations in the medium with Fluorokine MAP assay kit (R&D Systems, Inc., Minneapolis, MN, USA). Standard proteins included in the kit were used to calculate MMP9 protein concentration.

Statistical analysis

We used r^2 as an estimator of the strength of pairwise LD of SNPs.[21] A 2×2 contingency χ^2 test of independence was performed to test an association between genotypes and the disease in a dominant model. Haplotype inference was performed with an expectation–maximization algorithm implemented in SNPAlyze ver.4.1 (DYNACOM, Mobara, Japan). In single SNP association studies, significant values were corrected for number of SNPs and phenotypes tested (Bonferroni's correction). In the haplotype-wise test, significant values were corrected for multiple comparisons by multiplying the *P*-value by the number of haplotypes and phenotypes. The effects of genotypes on $\log_{10}$-transformed total serum IgE levels were evaluated using analysis of variance. Statistical analysis was performed with SPSS software (version 15.0J; SPSS Japan, Tokyo, Japan). Because the number of specific IgE values out of the detection limit (0.34–100.0 U ml^{-1}) was not negligible, we conducted tobit regression analyses using the AER add-on package in R (http://www.r-project.org/) to evaluate the effect of SNPs on allergen-specific IgE values. Tobit regression analysis allows for modeling a continuous variable in which censored values at a specific value were not negligible.[22] A corrected *P* (*Pcor*)-value <0.05 was considered statistically significant.

RESULTS

Association between polymorphisms of the *MMP9* gene and AR

Basic characteristics of the children in this study are shown in Table 1. We measured total and specific serum IgE levels in 670 schoolchildren in Chiba and Yamanashi prefectures. Cedar pollinosis, orchard grass pollinosis and mite-positive perennial AR were diagnosed according to symptoms and serum IgE levels as described above. Of 54 children with orchard grass pollinosis, 48 also met the criteria for cedar pollinosis. A total of 104 children were found to have cedar-only pollinosis and six had orchard grass-only pollinosis. Children who showed no symptoms of AR, asthma, atopic dermatitis or food allergy and were negative for all measured specific IgE were included in the non-atopic control group. There was no difference in age and sex ratio between children with seasonal or perennial AR and the control group.

We genotyped five *MMP9* SNPs in all subjects. The location of these SNPs is shown in Figure 1, with SNP and LD data from the HapMap database.[23] Three SNPs, −1590C/T (SNP1; rs3918242), 2127G/A (SNP2; rs2274755) and 5546G/A (SNP5; rs17577), were in strong LD and had previously been shown to be associated with childhood asthma.[17] SNP5 is an amino-acid substitution, R668Q. SNP3 and SNP4 are also amino-acid substitutions (R297Q (SNP3; rs17576) and P574R (SNP4; rs2250889)). LD between SNP3 and SNP4 was strong (r^2=0.711); however, these two SNPs had minimal LD with any of the other SNPs. All SNPs were in Hardy–Weinberg equilibrium in the case and control groups. Table 2 shows the genotype frequency of each polymorphism and association test results for the patients with AR and the non-atopic controls. Because SNP1 and SNP2 were in almost complete LD, the genotype results were almost the same. Both SNPs showed significant association with cedar and orchard grass pollinosis. In a dominant model (CC vs CT+TT), the odds ratio (OR) of SNP1 was 0.436 (95% confidence interval (CI)=0.252–0.752, *P*=0.0026, *Pcor*=0.039) for cedar pollinosis, and 0.234 (95% CI=0.097–0.566, *P*=0.00071, *Pcor*=0.011) for orchard grass pollinosis. ORs of SNP2 (GG vs GT+TT) were 0.419 (95% CI=0.242–0.726, *Pcor*=0.025) for cedar pollinosis and 0.234 (95% CI=0.097–0.566, *Pcor*=0.011) for orchard grass pollinosis. The ORs of SNP5 (GG vs GA+AA) for cedar pollinosis and orchard grass pollinosis were 0.430 (95% CI=0.255–0.726, *Pcor*=0.023) and 0.241 (95% CI=0.107–0.541, *Pcor*=0.0049), respectively. Neither SNP3 nor SNP4 showed significant association with the two types of pollinosis.

ORs of SNP1 and SNP5 for the 48 patients with cedar and orchard grass pollinosis were 0.183 (95% CI=0.067–0.499, *Pcor*=0.0051) and 0.206 (95% CI=0.085–0.499, *Pcor*=0.0033), respectively. Diagnoses of cedar pollinosis and orchard grass pollinosis were significantly associated (χ^2 test, $P=1.29\times10^{-7}$).

The results of the association test between SNPs and mite-positive perennial AR are also shown in Table 2. Although raw *P*-values for SNP1 and SNP5 were <0.05, none of the SNPs showed significant association with the disease after correction for multiple testing. ORs of SNP1 and SNP5 for this type of AR were 0.484 (95% CI=0.228–1.028, *P*=0.056) and 0.458 (95% CI=0.221–0.938, *P*=0.031), respectively. Among the 51 mite-positive perennial AR patients, 31 (61%) also met the diagnostic criteria for cedar pollinosis. ORs of SNP1 and SNP5 for this group were 0.302 (95% CI=0.108–0.848, *P*=0.018) and 0.351 (95% CI=0.140–0.884, *P*=0.023), respectively. ORs of SNP1 and SNP5 for mite-positive perennial AR-only patients were 0.848 (95% CI=0.312–2.29, *P*=0.742) and 0.648 (95% CI=0.240–1.75, *P*=0.390), respectively. Diagnoses of cedar pollinosis and mite-positive perennial AR were not significantly associated (χ^2 test, *P*=0.303).

Table 1 Characteristics of the study population

	Whole study population	*Cedar pollinosis*	*Orchard grass pollinosis*	*Mite-positive perennial AR*	*Non-atopic control*	*Atopic*[a]
Number	670	152	54	51	108	495
Age, mean (years)	9.37	9.42	9.44	9.29	9.23	9.36
Age, range (years)	6–12	6–12	6–12	6–12	6–12	6–12
Sex (male:female)	1.06:1.0	1.41:1.0	2.6:1.0	1.83:1.0	1.0:1.30	1.0:0.86
Mean total IgE (log(IU ml^{-1}))	2.10	2.53	2.67	2.51	1.31	2.36

Abbreviation: AR, allergic rhinitis.
[a]Positive for at least one specific IgE, regardless of allergic disease status.