初回免疫および2回目の免疫それぞれ2週後に採血して、血清中の OVA 特異的 IgG および IgE 抗体価を ELISA 法により測定した。

6. 倫理面への配慮

動物実験は、国立感染症研究所動物実験委員会の承認（承認番号：108046）、国立感染症研究所動物実験指針にしたがって実施した。

C. 結果

1. パッチ法と dMNA 法の比較

パッチ法では、有意な OVA 特異的 IgG 抗体産生を誘導するためには2回の免疫が必要だったが、dMNA 法では、初回免疫で抗体産生が誘導された。また、パッチ法では抗体の誘導に 100μg の OVA 量が必要だったが、dMNA 法では、10μg の投与でも十分な抗体が誘導された。さらに、パッチ法おいて、アジュバントとしてコレラ毒素を同時に投与した場合でも、アジュバントを使用しない dMNA 法に及ばなかった（図 1）。このように、dMNA 法ではパッチ法よりも効率的に抗体応答が誘導された。

2. dMNA 法と皮内注射の比較

dMNA 法と皮内注射について、それぞれマウスへの OVA 投与量を 0.5μg、2.5μg、10μg に変えて実験を行い、OVA 特異的 IgG 抗体の産生を比較した。皮内注射に比べて dMNA 法では抗体産生量がやや低値となる傾向が認められたものの、大きくは劣らない結果が得られた（図 2）。

3. IgE 抗体産生および IgG サブクラス抗体産生の比較

dMNA 法、パッチ法および皮内注射による OVA 特異的 IgE 抗体産生および IgG サブクラス抗体産生を、2回免疫後2週の段階の血清で比較した。パッチ法に比べて、dMNA 法および皮内注射では、IgE 抗体の産生が低値となる傾向が認められた（図 3）。一方、IgG_1 抗体と IgG_{2a} 抗体の比率には、3種の投与法で違いは認められなかった（図 4）。

D. 考察

dMNA 法は、パッチ法に比べてはるかに効率的に免疫応答を誘導した。その一方で、dMNA は皮膚のごく表層に刺し込まれるだけなので痛みは伴わない。さらに、dMNA 法による経皮免疫では、量的（dose-response）および質的（IgE 抗体の抑制傾向）に、皮内注射と同等の抗体応答が観察された。以上のことより、dMNA 法は皮内注射に替わる低侵襲性のアレルゲン投与法として期待できると思われる。

E. 結論

dMNA 法による経皮免疫法は、皮内注射に替わる低侵襲性の減感作療法投与ルートとして有望である。

F. 研究発表

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2. 学会発表

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G. 知的財産権の出願・登録状況

なし

図1. パッチ法とdMNA法におけるIgG抗体産生の比較

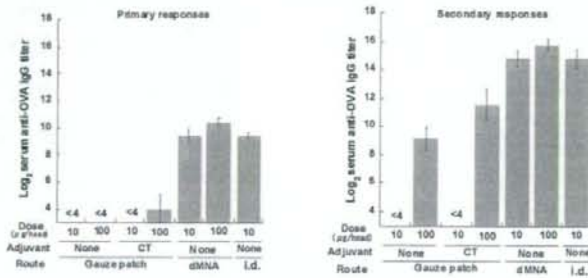


図2. dMNA法と皮内注射におけるIgG抗体産生の比較

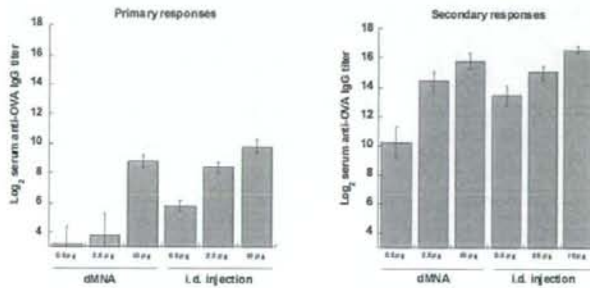


図3. パッチ法、dMNA法、皮内注射によるIgE抗体産生

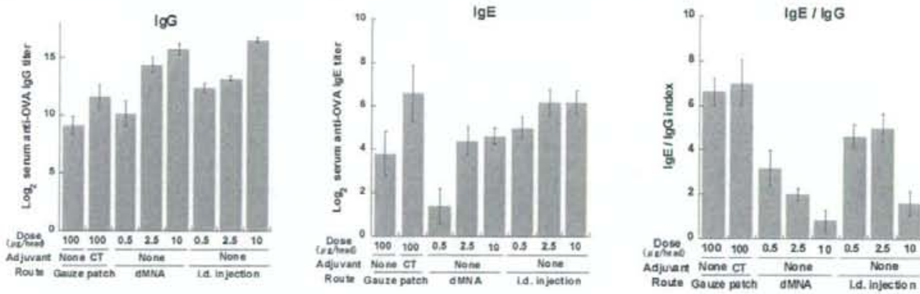
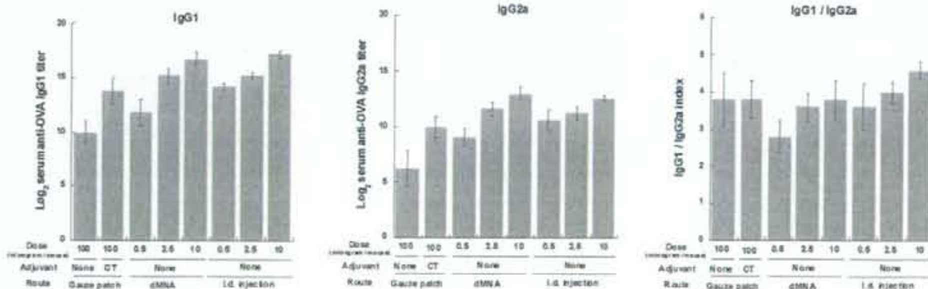


図4. パッチ法、dMNA法、皮内注射によるIgGサブクラス抗体産生



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

免疫療法におけるヒノキ花粉アレルゲンの必要性の検討

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研究要旨

本研究ではスギ花粉アレルゲンを用いた減感作療法がスギ花粉症を根治するための免疫療法として充分かどうか，T細胞エピトープの観点から追及した．スギ花粉アレルゲンの Cry j 1 の主要なT細胞エピトープ部位とそれに相当する Cha o 1 のペプチド部分に対するスギ花粉症患者末梢血単核球の反応性から，それぞれのアレルゲン特異的あるいは共通T細胞エピトープ部位が存在するのか解析した．その結果，pp211-230 は共通エピトープ部位であり，pp61-80, pp91-110, pp161-180, および pp311-330 はそれぞれのアレルゲン特異的T細胞エピトープ部位であることが判明した．解析は充分ではないが，pp231-250 は共通エピトープ部位に，pp151-170 および pp271-280 は Cry j 1 特異的エピトープ部位になることが示唆された．ヒノキ花粉アレルゲン Cha o 1 特異的T細胞エピトープの存在は，スギ花粉アレルゲンエキスを用いた免疫療法を評価する上で重要な情報となり，今後の解析によりヒノキ花粉アレルゲンの必要性が明らかにされると思われた．

A. 研究目的

スギ花粉アレルゲンエキスを用いた減感作療法は，スギ花粉症に対する有効な免疫療法であるが，ヒノキ花粉飛散時期にはその効果が減弱することが知られている．このことは，ヒノキ花粉アレルゲン特異的T細胞エピトープ部位の存在を示唆している．そこで，すでに明らかになっている Cry j 1 のT細胞エピトープ部位とそれぞれに相当する Cha o 1 のペプチド部分に対する花粉症患者末梢血単核球(PBMC)の反応性から，共通および特異的T細胞エピトープの存在を明らかにする．

B．方法

1) Cry j 1あるいはCha o 1特異的T細胞エピトープ部位の存在

最初に，Cry j 1 の主要なT細胞エピトープ部位に相当する Cha o 1 のペプチド部分を合成して，それぞれを抗原として患者末梢血単核球の増殖反応性を調べた（表1）．

2）舌下療法患者の客観的評価

舌下療法の効果を客観的に評価するために、患者ＰＢＭＣの Cry j 1, cry j 2, Cha o 1 および ＰＰＤに対する増殖反応を解析した．

なお，スギ花粉症患者末梢血は千葉大学から提供された．

C. 結果

T細胞エピトープ部位が解析できたのは患者62名であった．その結果，pp211-230は共通エピトープ部位であり，pp61-80, pp91-110, pp161-180,およびpp311-330 はそれぞれのアレルゲン特異的T細胞エピトープ部位であることが示唆された．解析は充分ではないが，pp231-250 は共通エピトープ部位に，pp151-170および pp271-280 はCry j 1特異的エピトープ部位になるが示唆された（図1）．さらに，千葉大学から提供された患者104名について，Cry j 1, Cry j 2, Cha o 1およびPPDに対する増殖反応性を解析した．

D. 考察

ヒノキ花粉アレルゲン Cha o 1 特異的T細胞エピトープの存在は，スギ花粉アレルゲンエキスを用いた免疫療法を評価する上で重要な情報となり，今後の解析によりヒノキ花粉アレルゲンの必要性が明らかにされると思われた．本研究は，Cry j 1 のT細胞エピトープ部位の観点から解析しているので，Cry j 1 のT細胞エピトープ部位とはまったく異なった Cha o 1 特異的T細胞エピトープが存在するのか，網羅的に解析する必要があると思われた．

E. 結語

ヒノキ花粉アレルゲン Cha o 1には，Cry j 1とは異なった Cha o 1 特異的T細胞エピトープのペプチドが存在することが明らかになった．このことは，免疫療法においてヒノキ花粉アレルゲンの必要性を示唆している．

F. 研究発表

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3) Ikeshima-Kataoka H, Shen JS, Eto Y, Saito S, Yuasa S. Alternation of inflammatory cytokine production in the injured central nervous system of tenascin-defeicient mice. In Vivo2008;22(4):409-413.

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5) Ikeshima-Kataoka H, Saito S, Yuasa S. Tenascin-C is required for proliferation of astrocytes in primary culture. In Vivo 2007; 21(4), 629-633.

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2) 飯倉克人，谷野千鶴子，佐藤哲夫，勝沼俊雄，中川秀巳，永田欽也，秋山暢丈，斎藤三郎．喘息患者末梢血単核球の IL-31 産生能．第58回日本アレルギー学会秋季学術大会 2008.

3) 斎藤三郎，秋山暢丈，神野英生，大野裕治．IL-31 は血清 IgE レベルを増強する．　第３８回日本免疫学会総会・学術集会 2008.

G. 知的財産の出願・登録状況

1. 特許取得

特願 2008-149275．斎藤三郎ほか．真核細胞において組換え蛋白質を細胞外に放出させるためのポリヌクレオチド出願中

2. 実用新案登録

なし

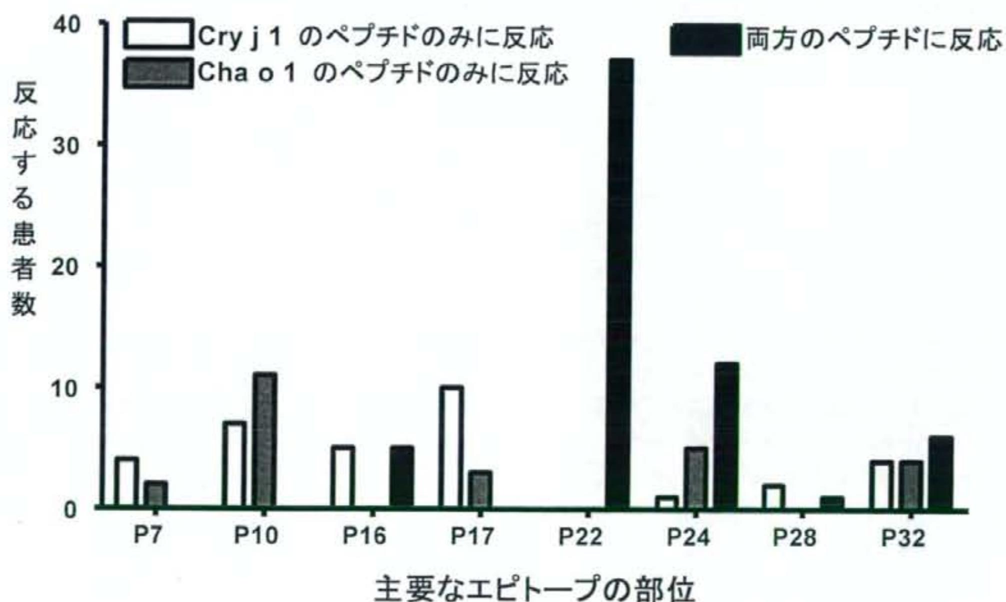
3. その他

斎藤三郎，秋山暢丈．IL-31 の多面的機能．臨床免疫・アレルギー科，50(6) :640-643, 2008.

表1. 解析に用いたCry j 1 の主要なT細胞エピトープ部位と
それに相当するCha o 1のペプチド配列

P7(61-80)	P22(211-230)
GATRDRPLWIIFSGNMNIKL	KSMKVTVAFNQFGPNCGQRM
**** * ****** * ****	*************** ****
GATRERSLWIIFSKNLNIKL	KSMKVTVAFNQFGPNAGQRM
P10(91-110)	P24(231-250)
TFDGRGAQVYIGNGGPCVFI	PRARYGLVHVANNNYDPWTI
* ***** * ******* *	******* ********** *
TIDGRGAEVHIGNGGPCLFM	PRARYGLIHVANNNYDPWSI
P16(151-170)	P28(271-290)
DALTLRTATNIWIDHNSFSN	PNESYKKQVTIRIGCKTSSS
** * * * ****** *	** * ** ** * ** *
DAITMRNVTDVWIDHNSLSD	PNDSDKKEVTRRVGCESPST
P17(161-180)	P32(312-330)
IWIDHNSFSNSSDGLVDVTL	SSGKYEGGNIYTKKEAFNVE
****** * **********	**** ** *** *** **
VWIDHNSLSDSSDGLVDVTL	SSGKNEGTNIYNNNEAFKVE
上段がCry j 1, 下段がCha o 1	

図1. 末梢血単核球の Cry j 1 の主要エピトープ部位とそれに相当するCha o 1 の
ペプチドに対する増殖反応性



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

舌下免疫療法における臨床試験および作用機序の解析に関する研究

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　　　　　　大久保公裕 日本医科大学耳鼻咽喉科　准教授
　　　　　　安枝　浩　国立病院機構相模原病院臨床研究センター　室長
　　　　　　斎藤三郎　東京慈恵会医科大学 DNA 医学研究所　准教授
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　　　　　　大川　徹　千葉大学大学院医学研究院耳鼻咽喉・頭頸部腫瘍学　助教
　　　　　　茶園英明　千葉大学大学院医学研究院耳鼻咽喉・頭頸部外科　助教
　　　　　　藤村孝志　千葉大学大学院医学研究院耳鼻咽喉・頭頸部腫瘍学　G-COE フェロー

研究要旨　舌下免疫療法は、皮下注射型減感作療法に替わるより安全な根治療法として注目されているが、その治療効果および治療機序については明らかになっていない。本研究では小規模キーオープン試験及び大規模2重盲検比較臨床試験により舌下免疫療法の治療効果を評価すると共に、末梢血リンパ球をもちいた培養実験により舌下免疫療法治療バイオマーカーの検索、治療機序の解明を目的とした。キーオープン試験において実薬投与群において活性化T細胞中の $IL10^{+}Foxp3^{+}$ 細胞(iTreg)の割合が無投与群に較べ高かったことから、本細胞集団の治療バイオマーカーとしての可能性を検討した。iTreg の増加群では Th2 サイトカインの産生が減弱し、症状も軽い傾向にあった。また、実薬投与群において症状が軽い集団では iTreg の割合が、症状が重い集団、無投与群に較べ有意に高かった。二重盲検試験においても、iTreg の割合が増加した群において、2年目の花粉飛散期中の症状がより軽い結果が示された。以上より iTreg の臨床症状を反映するバイオマーカーとしての有用性が明らかとなった。

A. 研究目的

抗原特異的免疫療法はアレルギー疾患における唯一有効な根治療法であるが、アナフィラキシー等の重篤な副反応が生じる危険性があるために、一般には普及していない。

近年、副作用の少ない免疫療法として口腔内舌下にアレルゲンエキスを投与する舌下免疫療法が開発され、より安全な免疫療法として注目されている。本研究ではスギ花粉症に対する舌下免疫療法の臨床効果、副反応の出現について調べた。また、平成 19 年度までの結果より、活性化T細胞中の $IL10^{+}Foxp3^{+}$ 制御性T細胞(iTreg)が治療効果・臨床症状を反映するバイオマーカーとして有用であることが示唆された。そこで、iTreg の変動と治療効果との関連について二重盲検試験の結果からより詳細に解析することを目的とした。

B．方法

RAST 値が2以上であるスギ花粉症患者を実薬群、偽薬群に分け、トリイ社の標準化減感作用抗原エキスを舌下投与し、二重盲検比較臨床試験を1年9ヶ月行った。また、舌下免疫療法の治療バイオマーカー探索のため、9ヶ月間のオープン試験を行った。花粉症症状・QOL について比較を行い臨床効果と副反応の差を評価した。分離した末梢血単核球を、スギ花粉抗原で刺激し変動するサイトカイン、細胞発現分子を解析した。また、スギ花粉特異的血清抗体価の変動を解析した。

C. 結果

2006 年9月から 2008 年5月まで、千葉・首都圏のスギ花粉症患者 120 人を対照に二重盲

検比較偽薬対照臨床試験を行った。17 検体が個人的理由によりドロップアウトし、最終的に 103 検体にて終了した。来院や投薬を必要とする副反応は発生しなかった。2 年目の花粉飛散期中の薬剤・症状スコアは実薬群において偽薬群に比べ、7 時点で有意に症状が軽かった。(図 1) オープン試験の結果により、iTreg が症状を反映するバイオマーカーの候補と考えられたことから、実薬群を iTreg の増加群と減少群に階層化し、薬剤・症状スコアを比較した結果、iTreg 増加群では偽薬群と比べ花粉飛散中のほとんどの時点で有意に症状が軽く、iTreg の減少群と比較しても症状が軽い傾向にあった(図 2)。一方で Th2 サイトカイン産生量は実薬群および偽薬群間で有意な差はなく、オープン試験の結果とは異なり、iTreg の増加群においても偽薬群に比べ、有意な差はなかった。一方で、偽薬群においても iTreg の増加群と減少群が存在したが、症状の階層化を行った結果、偽薬群の iTreg 増加群、減少群間で薬剤・症状スコアに違いは見られなかった。

D. 考察

オープン試験において、実薬群で培養上清中の IL5, IL13 産生が低い傾向にあり、制御性 T 細胞割合(iTreg)が高い傾向にあったことから、SLIT における制御性 T 細胞の治療機序への関与が示唆された。一方で、実薬群において制御性 T 細胞割合の違いにより Th2 サイトカインおよび症状に差が見られたことから、SLIT の治療有効性を反映するバイオマーカーとしての有効性が考えられた。

以上の結果を二重盲検試験にて検討した結果、二重盲検比較試験についても同様に iTreg の治療効果を反映するバイオマーカーとしての有効性が示唆された。しかしながら、実薬群ならびに実薬郡中の iTreg 増加群においても Th2 サイトカイン産生に偽薬群と有意な差はなかったことから、治療効果における iTreg、Th2 細胞の役割は明らかとできなかった。

本研究において 2 重盲検試験については、層別化等の更なる解析が必要であるが今回必ずしも末梢の Th 応答と舌下免疫療法、治療効果との関連は明らかとならなかった。一方で、iTreg と臨床症状の軽重に関連が見出された事から、制御性 T 細胞の治療バイオマーカーとしての有用性と治療機序への関与が示唆された。

末梢ではなく、局所における炎症反応の解析も必要であると考えられるが、花粉飛散期中の局所サンプルのサンプリングは困難であるため、花粉飛散室等を利用した検討が今後必要になると考えられる。今回明らかとなった末梢での抑制性 T 細胞応答やマイクロアレイ解析の結果が今後の局所での解析の糸口になると考えられる。

E. 結語

舌下免疫療法に関する 2 つの臨床試験により重篤な副反応はなく、安全性が確認できた。また、2 重盲検試験により臨床症状に対する有効性が確認された。

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G. 知的財産の出願・登録状況

1. 特許取得

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2. 実用新案登録

なし

3. その他

なし

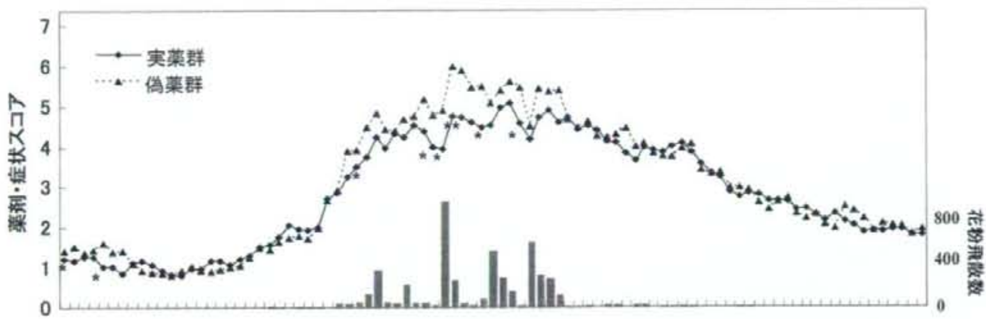

図１　2008 年スギ花粉飛散期中の薬剤・症状スコア (Symptom-medication score)
花粉飛散量を棒グラフで示している。 *: P<0.05 vs 偽薬群

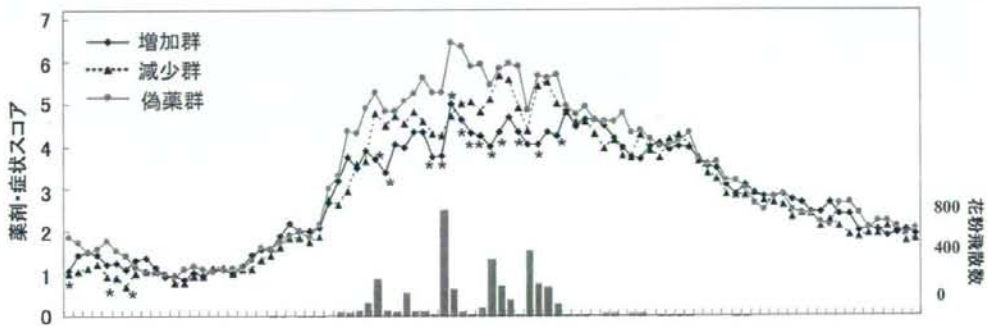

図２　iTreg による階層化後の 2008 年スギ花粉飛散期中の薬剤・症状スコア
花粉飛散量を棒グラフで示している。 *: P<0.05 vs 偽薬群

研究成果の刊行に関する一覧表

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DOI 10.1007/s10453-007-9082-y

ORIGINAL PAPER

Development of electron spin resonance radical immunoassay for measurement of airborne orchard grass (*Dactylis glomerata*) pollen antigens

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Abstract We have developed a highly sensitive method for the measurement of airborne orchard grass (*Dactylis glomerata*: Dac g) pollen antigens using an electron spin resonance (ESR) radical immunoassay. In this immunoassay, the lowest detectable level of Dac g antigen in a sample is 0.1 arbitrary unit; the amount of Dac g antigen in single pollen grains was found to be as 1.84 units. Thus, Dac g antigens can be detected in amounts of 1/20th of that contained in the grain. This immunoassay enables early detection of grass pollen antigens. Such information may be useful for patients with grass pollinosis, especially for those who show symptoms when only low levels of the pollen antigens are present in air. In this study, minor amounts of Dac g antigen (cross-reactive antigens) were detected in late March, after which the levels gradually increased. The levels were detected to be 10 units/m^3 until the middle of May and then increased after blooming of orchard grass. High levels were maintained until the middle of June. Some patients who suffer from grass pollinosis show symptoms in late April and early May, when the airborne Dac g antigen levels were found to be 5–10 units/m^3.

Keywords Airborne pollen · Allergen · ESR radical immunoassay · Orchard grass · Japanese cedar

Abbreviations

BCA	Bicinchoninic acid
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BSA	Bovine serum albumin
CNBr	Cyanogen bromide
Dac g	*Dactylis glomerata*
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal bovine serum
ESR	Electron spin resonance
HRP	Horseradish peroxidase
MONALISA	Monitoring Network of Allergen by Immuno-Sampling

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NHS	*N*-hydroxysuccinimide
PBS	Phosphate-buffered saline
RSI	Relative signal intensity
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPT	Skin prick test

1 Introduction

The number of airborne pollen grains is typically counted morphologically using microscopy. Pollen information is derived from these data. However, because grass pollen shares similar morphological characteristics, it is difficult to discriminate between species of pollen grains using light-microscopic pollen counts. It is known that species of grass pollen antigenicity differ with each species of grass pollen, and the pollen antigens of some grasses are not cross-reactive with those of other grass species (Esch 1999; Martin et al. 1985; Weber and Nelson 1985). Fortunately, most grasses associated with pollinosis in our region are cross-reactive species belonging Poöideae. In addition, submicron-size particles bearing pollen antigens such as starch granules (Spieksma et al. 1991) exist other than as antigens from the pollen itself. Such submicron-size particles have been isolated from pollen grains under wet conditions (Suphioglu et al. 1992) and are also considered to be the causative agents of pollinosis.

To obtain more information about the pollen, it is desirable to measure antigens that are cross-reactive with grass pollen. We applied an immunochemical technique for the quantification of airborne grass pollen antigens. A major problem in this context is that only small amounts of these antigens are present in air, and a highly sensitive analytical technique is required for such measurements. Recently, an electron spin resonance (ESR) radical immunoassay was developed as a highly sensitive method for detecting hepatitis B surface antigen (Matsuo et al. 1998; Aoki et al. 2002). In our previous study, we have shown that the sensitivity of this immunoassay is 10- to 100-fold higher than that of the conventional enzyme-linked immunosorbent assay (ELISA) (Aoyama and Takahashi 2004; Takahashi et al. 2007).

In this study, we used the ESR radical immunoassay to quantify airborne grass pollen antigen. Orchard grass pollen antigen was chosen as the target, because this species is universally distributed throughout our region and is considered to be the most common species contributing to total regional airborne grass pollen antigens.

2 Materials and methods

2.1 Sampling and antigen extract

A cyclone sampler, CM 90 (Burkard Manufacturing, Rickmansworth, UK) was installed at Iwanami in Yamagata City, and airborne pollen antigens were collected in 1-ml tubes. No large community of grasses is present in any direction within 500 m of the sampling site. The tubes were replaced and samples were collected at 6:00 daily. The antigens in the airborne samples were extracted with 100 μl of 0.125 M ammonium bicarbonate in 0.1% bovine serum albumin (BSA) for 2 h at room temperature. The scratch extract of *Dactylis glomerata* pollen (B3SFV2) (Torii Pharmaceutical, Tokyo, Japan) was used for the standard solution, and we defined the concentration of the scratch extract as 100,000 arbitrary units/ml. Dac g content in single pollen grains was determined by extraction of a reference pollen provided from International Biological (Piedmont, OK) with 300 μl of 0.125 M ammonium bicarbonate overnight at 4°C. After centrifugation, the aliquot was used to measure Dac g antigen, and the pellets were used to count the number of Dac g pollen grains.

2.2 Preparation of horseradish peroxidase (HRP)-conjugated antibody

Antibodies against Dac g antigens were prepared with Japanese white rabbits. Two rabbits were immunized subcutaneously with 400 μg (200 μg each rabbit) Dac g antigen from Greer Laboratories (Lenoir, NC) mixed with Freund's complete adjuvant. Boosts were given at 4, 8 and 12 weeks after the first injection and small bleeds were taken a week after each boost to check the antibody response. A large bleed was taken a week after the final boost (Kane and Banks 2000). Antisera were precipitated with 35% saturated ammonium sulfate, and they were dialyzed with 0.125 M NaCl and 0.05 M phosphate-buffered saline (PBS) at

pH 7.0. The ammonium precipitates were then passed through protein A column to purify IgG fraction. A portion of the IgG fraction was further purified with the antigen-combined column, and the purified samples were eluted with 10 mM glycine-hydrochloric acid at pH 2.0. The eluate was immediately replaced with PBS (pH 7.0). The final protein concentration of the refined antibody against Dac g was 0.5 mg/ml as measured using the BCA protein assay kit (Pierce, Rockford, IL). The antigen-combined column was made using Dac g antigen (Funakoshi, Tokyo, Japan) reacted with CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The refined antibody was conjugated with horseradish peroxidase using peroxidase labeling Kit-SH (Dojindo Molecular Technologies, Tokyo, Japan).

2.3 Electron spin resonance (ESR) radical immunoassay for Dac g antigen

Dac g antigen for SPT extract (Torii Pharmaceutical) was diluted 2,000-fold with PBS. The protein concentration of the Dac g antigens measured with BCA protein assay kit (Pierce) was 2.75 mg/ml. One hundred μl of the diluted antigen was put in a 96-well plate (Nunc, Kamstrupvej, Denmark), and the samples were reacted for 6 h at 4°C. After three washes with ultra pure water, 370 μl Stabilguard (SurModics, Eden Prairie, MN) was placed in each well, and the reaction was blocked overnight at 4°C. The plate were washed with ultra-pure water, dried in a desiccator, and kept at 4°C until use. Measurements were carried out as follows. Seven different concentrations of Dac g standard solution (5, 10, 20, 40, 100, 200, and 400 units/ml) were prepared prior for each measurement. One hundred μl of the standard solution or 100 μl of PBS containing 2% BSA was placed in each well of Dac g antigen-coated plate, and then 0.125 M ammonium bicarbonate (30 μl) or an airborne sample (30 μl) was added to each well. Then, 50 μl HRP-conjugated antibody against Dac g diluted with 10% fetal bovine serum (FBS) and 0.1 M PBS was added to each well, and the solutions were mixed thoroughly. Each sample was usually put in a single well, and in some noticeable cases, samples were measured in duplicate. The seven standard solutions were measured each time. The plate was left for 2 h at room temperature or left overnight at 4°C. After several washes in washing solution, 150 μl of 4 mM *p*-acetamidophenol and 0.34 mM 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline 3-oxide and 0.01% hydroperoxide were added to each well and reacted for 1 h at 37°C. The enzyme reaction was stopped with 50 μl sodium azide (100 mM). The amount of nitroxide radical (stable radical) produced as a result of the enzyme reaction was measured with an ESR device (FR30, JEOL, Tokyo, Japan) at a center field of 336.1 ± 5 mT. Details of the ESR measurement technique have been reported elsewhere (Matsuo et al. 1998; Aoki et al. 2002).

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The pollen protein samples were analyzed by SDS-PAGE (12.5% acryl amide concentration; Bio-Rad, Hercules, CA). Prestained protein molecular weight standards-high (Life Technologies, Tokyo, Japan) was used as a molecular weight standard. Electrophoresis was performed at a constant voltage of 12 V for 1 h. After electrophoresis, the gel was placed on a nitrocellulose membrane, and the antigens were transferred onto the membrane. The gels were stained with Coomassie-blue G-250. The membrane was blocked with PBS in 5% BSA overnight at 4°C, then the samples were reacted with 500-fold diluted biotinylated antibody against Dac g for 2 h. Antibody against Dac g was biotinized with long-arm NHS biotin (Vector Laboratories, Burlingame, CA) (Abdul-Ahad and Brett 2000). After three washes with PBS, the samples were reacted with 500-fold diluted alkaline phosphatase conjugated streptavidine (Vector Laboratories). Finally, the Dac g antigen bands were visualized with BCIP/NBT substrate (KPL, Washington, DC).

3 Results

3.1 Dac g reacts with the antibody against Dac g

The Dac g antigen from Greer laboratory that was used for immunization showed five clear bands in SDS-PAGE, with approximate molecular weights of

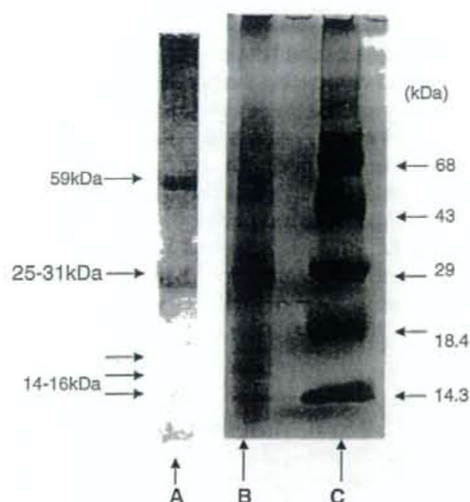

Fig. 1 Determination of molecular components of Dac g extract analyzed by SDS-PAGE and antibody against Dac g specificity by Western blotting. A: Antibody against Dac g analyzed by Western blotting. B: Molecular components of the Dac g extract analyzed by SDS-PAGE. C: Molecular weight markers: lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), and albumin (68 kDa)

59, 25–31, 16, 15, and 14 kDa. The 25–31 kDa band was broad and dense. The 59 and 25–31 kDa bands reacted with the antibody against Dac g. Three bands of low molecular weights, 14, 15, and 16 kDa, did not react with the antibody against Dac g (Fig. 1). Therefore, the antibody against Dac g contained antibodies against 59 and 25–31 kDa antigens.

3.2 Standard curve of ESR radical immunoassay

A standard curve was obtained using ESR radical immunoassay, and the results are shown in Fig. 2. The vertical axis indicates the relative signal intensity (RSI) (%); the horizontal axis indicates Dac g concentration expressed as unit/ml. The RSI (%) was calculated from the following equation.

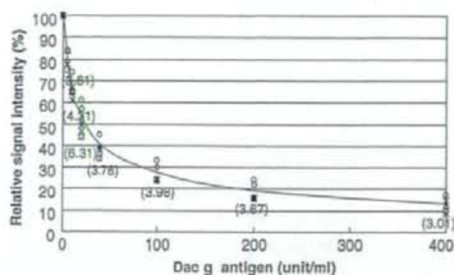

Fig. 2 Relationship between Dac g levels and relative signal intensity determined by ESR radical immunoassay. Seven concentrations (5, 10, 20, 40, 100, 200, and 400 units/ml) of Dac g were measured five times, and the RSI (%) was plotted on the vertical axis. A regression curve is derived from the mean value of the five measurements. Standard errors are shown in parentheses

The standard error of RSI (%) was in the range of 3.0–6.3% (mean 4.1%, $n = 6$) (Fig. 2), and therefore we defined a 90% intercept value of RSI to the zero standard signal intensity as the detection limit in this study. A zero standard means maximum binding capacity of the system in this case. The 90% intercept value of the signal intensity was approximately 3.5 units/ml. On the other hand, the amount of Dac g in single pollen grains was found to be 1.84 units/grain (1.38–2.24 units/grain, $n = 8$) based on a reference pollen obtained commercially.

3.3 Measurement of airborne Dac g antigen

The level of airborne Dac g antigens was examined during the period of 22 March and 30 June in 2006 (Fig. 3). Minor amounts of cross-reactive antigens to Dac g were already detected in late March, and the levels gradually increased thereafter. The levels were found to be 10 units/m^3 until the middle of May. The Dac g levels increased after the blooming of orchard grass, and a high level of Dac g antigen was maintained until the middle of June. Symptoms of some grass pollinosis patients had already begun in

$$\mathrm{RSI}(\%) = \frac{\text{Signal intensity of a standard Dac g}}{\text{Signal intensity of a zero standard (without Dac g)}} \times 100$$

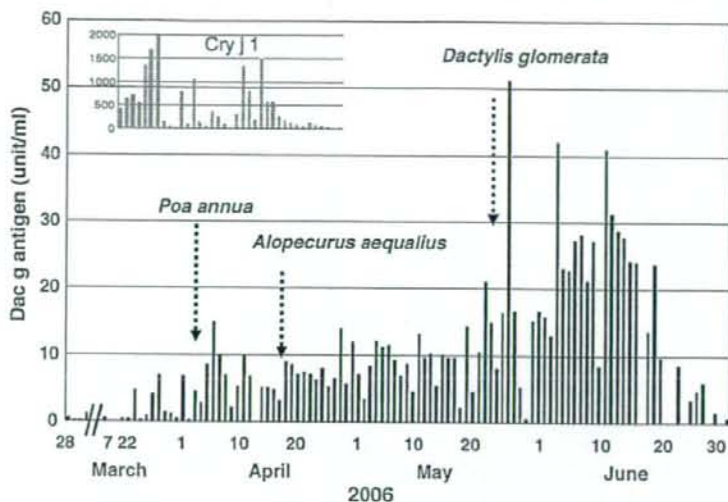

Fig. 3 Daily fluctuation of airborne Dac g measured by ESR radical immunoassay during the period from 7 March to 30 June 2006. Arrows indicate the flowering time of relevant grasses near the sampling site. Daily fluctuation of airborne Cry j 1 level is shown. The horizontal scale is adjusted for comparison

late April, when the airborne Dac g levels were fluctuating between 5 and 10 units/m^3.

The relationship between the daily amount of airborne Cry j 1 (a major pollen allergen from Japanese cedar pollen) and that of Dac g was examined, and no relationship between the two values was observed (between 22 March and 10 May, $r = -0.1828$, $n = 50$).

4 Discussion

We have developed a highly sensitive method for measuring airborne Dac g antigens using ESR radical immunoassay. The 90% intercept value was calculated as 3.5 units/ml as described in Sect. 3. Each measurement needs 30 μl extract; therefore, a level of Dac g in excess of 0.1 unit in the sample is detectable. The Dac g content in single pollen grains of *D. glomerata* was determined as 1.84 units. Thus, the amount of Dac g that could be detected was as low as 1/20th of that contained in single grains. Using this method, the Dac g antigen was detectable during the early stage of the grass pollen season. This approach is expected to provide useful information for grass pollinosis patients, especially for those who show symptoms at times when only a low level of antigen is present in air. Data are available 2½ h after sampling, namely, 30 min extraction (Takahashi et al. 2001), 60 min antibody reaction and 60 min radical reaction. We could use the above treatment times without sensitivity loss on the occasion of the pollen allergen information. It is possible to supply the pollen allergen information to local residence through mass media until evening time on the same day.

Also, we have been providing information about Cry j 1 antigen in airborne pollen during the pollen season since 2005. It is well known that some patients displayed symptoms several weeks before airborne Japanese cedar pollen has been detected by microscopy. No airborne Cry j 1 has been detected using the conventional ELISA during this times. The development of the ESR radical immunoassay has now made it possible to conduct such measurements, and it has been reported that some patients show their symptoms during a period in which airborne Cry j 1 levels fluctuating between 1 and 3 pg/m^3. Such information could be useful for the patients whose symptoms begin early in the flowering season (Takahashi et al. 2007). Now we have been supplying airborne Cry j 1 information through internet and local TV at the pollen season.

Low levels of Dac g were present during the latter half of the Japanese cedar pollen season. Japanese cedar pollinosis is the most common in Japan, and more than 10% of Japanese suffer from it. Some patients who suffer from the pollinosis during this

season think that Japanese cedar pollen is their causative pollen, but it is clarified from this study that Dac g pollen was also detected in air at that time. It has been reported that some grass pollinosis patients show symptoms in late April and early May in our region (Takahashi et al. 1987), and this time corresponds to the period when early flowering species of grasses start to bloom. It is likely that the symptoms of these patients are provoked not only by Japanese cedar pollen, but also by grass pollen. Interestingly, no positive correlation was observed between the amounts of Cry j 1 and Dac g antigen in air, samples of which were collected during Japanese cedar pollen season. Dac g and Cry j 1 do not have any cross-reactive antigens. The comparison was made because we want to examine whether they appear in air simultaneously or independently controlled under different meteorological conditions, and further whether there are more patient symptoms in connection with the appearance of these antigens.

Airborne samples were taken using Burkard Cyclone sampler in this research. The manufacturer of the sampler announced that the collection efficiency of the sampler is 90% in 1-μl range particles; that is to say, allergens existed as minute particles under 1 μl, and gas-shaped particles could not be sampled. In Europe, the MONALISA (Monitoring Network of Allergen by Immuno-Sampling) project was initiated in 2005. The aim of the project is to characterize pollen allergens such as Poaceae, *Betula, Ambrosia, Artemisia, Cupressus, Parietaria*, and *Olea* pollens for the benefit of pollinosis patients. Several sampling methods were examined in the MONALISA project. According to Rantio-Lehtimäki of Turk University, samplings into the liquid were diluted, and ELISA results were not reliable (personal communication). The advantage of the Cyclone sampler is that allergens in large volumes of air could be collected in a very small quantity of extraction medium (at least 50 μl). Therefore, we chose the cyclone sampler for the study.

In this research, we used an antibody against Dac g antigen, because *D. glomerata* is the most widely distributed species in our region and is considered to be the main species contributing to the total airborne grass pollen antigens in this region. A number of studies have been conducted to analyze *D. glomerata* pollen antigens (Esch and Klapper 1989; Cuerin-Marchand et al. 1996; Roberts et al. 1993; Leduc-Brodard et al. 1996; van Oort et al. 2001). The extent of cross-antigenicity among grass antigens remains difficult to estimate. The antibody used in this study recognized antigens with molecular weights of 59 and 27–35 kDa. It is likely that 59 kDa antigen is Dac g 4, and 27–35 kDa antigen is Dac g 1 and/or Dac g 5. According to Esch (1999), Dac g 5 has a wide molecular weight distribution and is cross-reactive within pollen antigens from Poöideae. Therefore, Dac g 5 was considered to be one of the suitably defined antigens for our purpose.

As for antibody, several categories of antibodies are available for the airborne antigen measurements, e.g., antibodies created from crude antigen extracts, antibodies created from defined antigens, monoclonal antibodies, and polyclonal antibodies. There are no commercial products for antibodies against grass pollen antigens in Japan. First of all, we must prepare antibodies against grass pollen antigens. Monoclonal antibodies are easy to manage concerning quality control as indicated by the example that the MONALISA project has selected them. However, we prepared polyclonal antibodies because we want to use antibodies that have wide specificities related to local grass pollinosis, and it can be made easily. There is, certainly, a problem with quality control among preparations. However, we do not worry about this matter concerning the standpoint of pollen allergen information, because outstanding sensitivity of the ESR method made it possible to dilute the HRP-conjugated antibody to 10,000-fold. So, we can use the same preparation for many years. Addition to this, we think suitable antibody will differ from place to place as discussed in the next paragraph.

Some patients show symptoms before the flowering of *D. glomerata*. The amounts of airborne allergens causing grass pollinosis can be roughly estimated to quantify the airborne Dac g levels, because the antibody used in the present study reacts not only to Dac g pollen antigens, but also to grass pollen antigens cross-reactive with Dac g from other plants of the Poöideae subfamily. Thus, the airborne grass pollen antigens observed before *D. glomerata* flowering season that bloom until late May (Takahashi et al. 1993) may not have been Dac g itself, but may have been other species having cross-reactive antigens with Dac g antigen. Two early blooming species of grasses in our region are *Alopecurus aequalis* and *Poa annua*. However, early blooming species are not

limited to the above-mentioned species; for example, *Anthoxanthum odoratum* blooms prior to the major grass pollen season in some areas in Japan (Sudo et al. 2005). It would be desirable to investigate antibodies with a broad range of specificity against major allergens related to local grass pollinosis. Moreover, it would be helpful to examine antibodies in immunization with a mixture of grass pollen antigens from different species. Antibodies suitable for such studies will be those against the most important species in a particular region, and we think the suitable antibody will differ from place to place. Further research is still needed to clarify the differences among applied antibodies and to identify the appropriate antibodies for the measurement.

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Identification of *c-kit* mutations-independent neoplastic cell proliferation of canine mast cells

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Abstract

Gain-of-function mutations in the proto-oncogene *c-kit* have been considered the molecular mechanism of neoplastic proliferation of mast cells. However, the importance of *c-kit* gene mutations is not well evaluated in canine mast cell tumors (MCTs). In the present study, we established and characterized a mast cell line, HRMC, derived from a dog with MCT. We also examined *c-kit* mutations in HRMC cells and assessed an inhibitory effect of a tyrosine kinase inhibitor, STI571, on HRMC cells. HRMC cells had cytoplasmic metachromatic granules, chymase and tryptase, and expressed both KIT and FcεRI on the cell surface. HRMC cells contained histamine and released β-hexosaminidase through FcεRI cross-linking and calcium ionophore stimulation. Nucleotide sequence analysis demonstrated no mutations in an open reading frame of *c-kit* cDNA and genomic DNA of the juxtamembrane domain of *c-kit* in HRMC cells. STI571 did not show any inhibitory effects on the proliferation of HRMC cells. These findings clearly demonstrated the existence of *c-kit* mutations-independent neoplastic canine mast cell proliferation. The growth factor-independent mast cell line established in this study might be valuable to explore novel mechanisms of *c-kit* mutations-independent neoplastic proliferation of mast cells in dogs.

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Keywords: *c-kit*; Mutations; Mast cell tumor; Dog

1. Introduction

Mast cell tumors (MCTs) are one of the common tumors in dogs, accounting for approximately 20% of cutaneous canine tumors (London and Seguin, 2003). Most of MCTs develop in the dermis and subcutaneous tissue of dogs, while visceral MCTs occasionally occur in the spleen, liver, gastrointestinal organs, and bone marrow (London and Seguin, 2003). MCTs in dogs show various biological behaviors, ranging from benign

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to malignant progress with metastasis and eventual death (London and Seguin, 2003). Surgical excision has been indicated for the treatment of all MCTs in dogs. Additional treatments such as radiation therapy and chemotherapy are required for dogs with aggressive MCTs (London and Seguin, 2003).

The proto-oncogene *c-kit* encodes the transmembrane KIT receptor that belongs to a type III receptor tyrosine kinase subfamily (Yarden et al., 1987). KIT consists of three major domains: an extracellular domain with five immunoglobulin-like loops, a transmembrane domain, and an intracellular domain. The intracellular domain is divided into a juxtamembrane domain and a kinase domain that is further split into an adenosine triphosphate binding region and a phosphotranspherase region by a kinase insert (Roskoski, 2005b). KIT is expressed on various cells including mast cells, hematopoietic stem cells, melanocytes, germ cells, and intestinal cells of Cajal (Akin and Metcalfe, 2004). Stem cell factor (SCF), a ligand of KIT, binds to KIT and leads to its receptor dimerization, resulting in autophophorylation of tyrosine residues in the kinase domain of KIT which initiate the intracellular signaling cascade (Roskoski, 2005a). The signaling pathway initiated by KIT is essential for differentiation, proliferation, and survival of mast cells (Akin and Metcalfe, 2004).

Previous studies identified mutations in the juxtamembrane domain of *c-kit* in canine MCT cell lines and dogs with spontaneous MCTs (London et al., 1999; Ma et al., 1999). These mutations consisted of novel internal tandem duplication (ITD), deletions, and point mutations at exon 11, intron 11 and exon 12 of *c-kit*. The ITD and point mutations in the juxtamembrane domain of *c-kit* were shown to induce constitutive phosphorylation and activation of KIT in the absence of SCF binding (London et al., 1999; Ma et al., 1999). Therefore, activating mutations in the juxtamembrane domain of *c-kit* have been considered the molecular mechanism for the tumorigenesis of MCTs in dogs.

The incidence of mutations in the juxtamembrane domain of *c-kit* was reported to be between 13.6% and 33% in dogs with spontaneous MCTs (Downing et al., 2002; Zemke et al., 2002). However, since these studies determined the sequence of *c-kit* only in the juxtamembrane domain, the presence or absence of mutations in other domains of *c-kit* has not been elucidated. It is also possible that there may be a population of canine MCTs devoid of *c-kit* mutations. Thus, the importance of *c-kit* mutations in the pathogenesis of canine MCTs still remains unclear.

In the present study, we established a growth factor-independent canine MCT cell line, HRMC, which did not have any mutations in *c-kit*. In addition, STI571, imatinib mesylate, did not show any inhibitory effects on the growth of the cell line at concentrations up to 10 μM. These findings indicate that neoplastic proliferation of HRMC cells was not mediated by *c-kit* mutations, and provide the evidence that novel mechanisms except *c-kit* mutations are capable of regulating neoplastic proliferation of canine mast cells.

2. Materials and methods

2.1. Cell culture

The tumor tissue isolated from a dog with MCT was minced finely and treated with collagenase (Sigma–Aldrich, St. Louis, MO), followed by filtration through a 40-μm mesh. After washing with phosphate-buffered saline (PBS), tumor cells were cultured in serum-free AIM-V medium (Gibco-BRL, Gaithersburg, MD) at 37 °C in a humidified atmosphere with 5% CO_2. To maintain the culture condition, half of the medium was changed every 10–14 days. After apparent cell growth was observed, the cells were cultured in serum-free AIM-V, and were passaged once a week by dilution of 1:5.

2.2. Ultrastructural analysis

For transmission electron microscopic analysis, the cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4 °C overnight. After washing, the cells were postfixed in 1% O_5O_4 in 0.05 M phosphate buffer for 2 h at 4 °C. The cells were then dehydrated in graded ethanol and embedded in Spurr resin. Ultrathin sections (80–100 nm) were cut and stained with uranly acetate and lead citrate. Microstructures were observed with a transmission microscope (JEM-1200EX; JEOL, Tokyo, Japan).

2.3. Cytochemical staining

For identification of cytoplasmic metachromatic granules, cytospin preparations were fixed with Carnoy's solution for 10 min at room temperature (RT), and then stained with 0.05% toluidine blue (pH 7.0) for 30 min at RT. Chymase was detected with naphthol-AS-D-chloroacetate as a substrate and Fast Red Violet LB salt as dye by using a commercially available kit (Sigma–Aldrich).