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7) 秋山晴代, 河又小夏, 中村亮介, 福冨友馬, 甲斐茂美, 松藤 寛, 宮澤眞紀：EXiLE 法を用いた口腔アレルギー症候群の新たな *in vitro* 検査法の検討：第 37 回日本分子生物学会年会 (2014.11, 横浜).

8) 岡本(内田)好海, 中村亮介, 相馬愛実, 石井明子, 最上知子, 川崎ナナ, 川上浩, 手島玲子, 斎藤嘉朗：架橋誘導活性の異なるモノクローナル IgE の抗原認識様式の違いについて. 日本薬学会第 135 年会 (2015.3, 神戸).

9) 秋山晴代, 河又小夏, 政岡智佳, 中村亮介, 福冨友馬, 甲斐茂美, 松藤　寛, 宮澤眞紀：口腔アレルギー症候群における新たな *in vitro* 試験法の検討. 日本薬学会第 135 年会 (2015.3, 神戸).

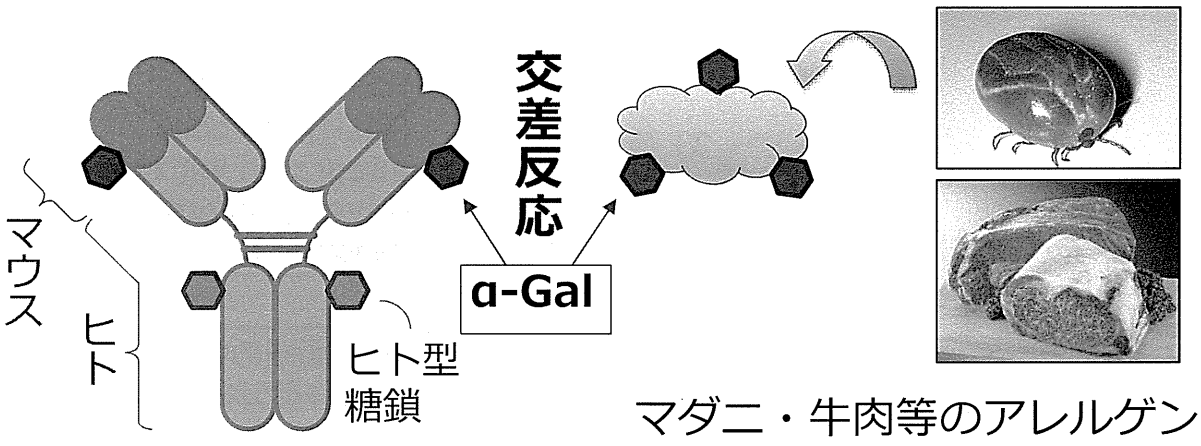
報道発表等

なし

H. 知的財産権の出願・登録状況

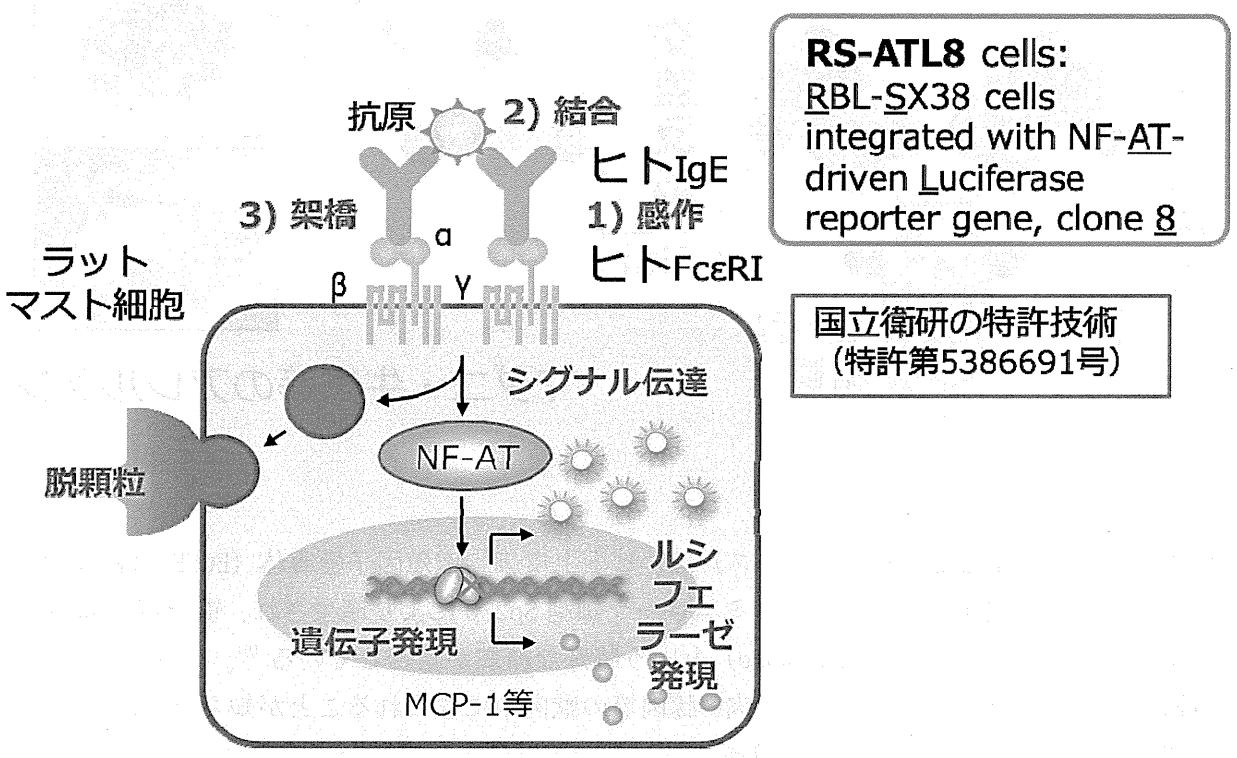
なし

図１　セツキシマブの模式図と糖鎖抗原α-Gal



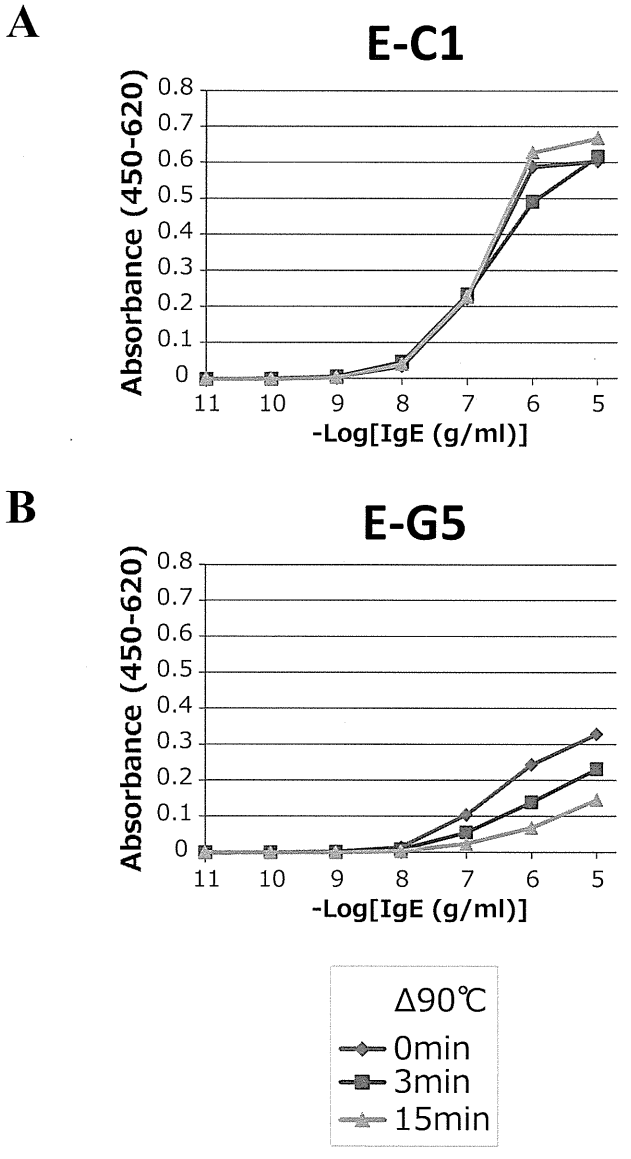
セツキシマブ（商品名：アービタックス）は、ヒト上皮細胞増殖因子受容体（EGFR）特異的なヒトとマウスのキメラ抗体である。マウスミエローマ細胞 Sp2/0 により産生され、重鎖可変部の Asn88 にα-Gal（galactose-α1,3-galactose）という特殊な糖鎖が結合している[18]。α-Gal は、ロッキー山紅斑熱の原因となるマダニや、牛肉・豚肉等の獣肉中に含まれることが知られており、この糖鎖に結合する IgE はこれらすべての抗原に対して交差反応することが示唆されている[5-9]。

図２　EXiLE 法の模式図



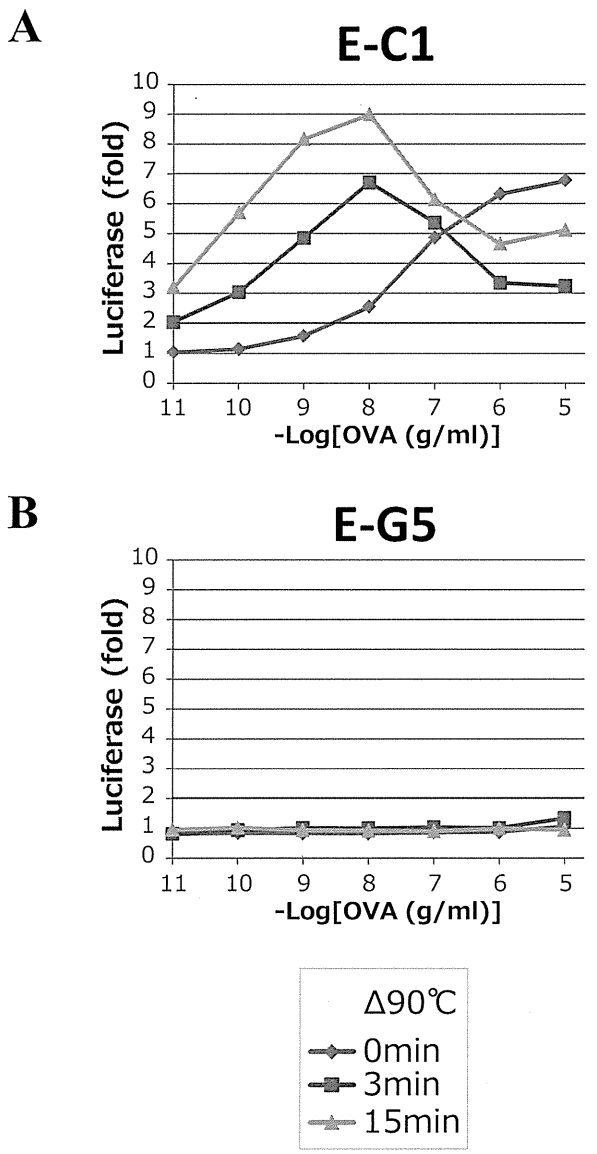
マスト細胞の活性化は、１）抗原特異的 IgE による感作、２）IgE の抗原との結合、３）IgE および高親和性 IgE 受容体（Fc ε RI）の架橋の３段階を経て誘導される。IgE の架橋に伴うマスト細胞の活性化を簡便かつ高感度に検出するため、ヒト化ラットマスト細胞株（RBL-SX38）に転写因子 NF-AT によって転写が誘導されるホタルルシフェラーゼ遺伝子を導入した、RS-ATL8 細胞は、患者血清中の IgE によって感作され、特異抗原により刺激されると、ルシフェラーゼを発現するため、その活性化を感度良く検出することができる。この手法を、「IgE Crosslinking-induced Luciferase Expression（EXiLE）法」と命名した[11]。

図３　ELISA による２種の抗 OVA IgE 抗体の結合性の評価



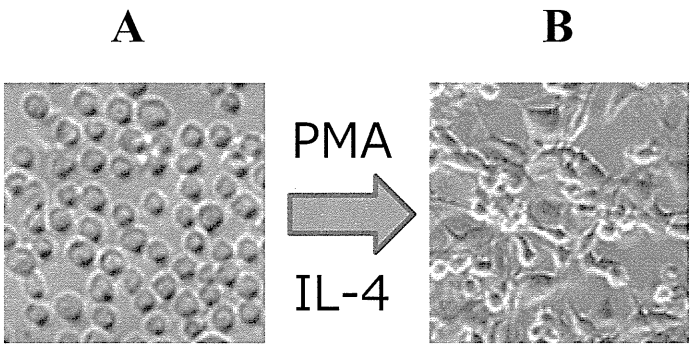
OVA を 90℃で 0，3，15 分間加熱し、100μg/ml の濃度で固相化した。ブロッキング後、E-C1 クローン（A）および E-G5 クローン（B）の OVA 特異的モノクローナル IgE 抗体を各種濃度で作用させ、OVA との結合性を HRP 標識抗マウス IgE 抗体により測定した。

図４　EXiLE 法による２種の抗 OVA IgE 抗体の架橋活性の評価



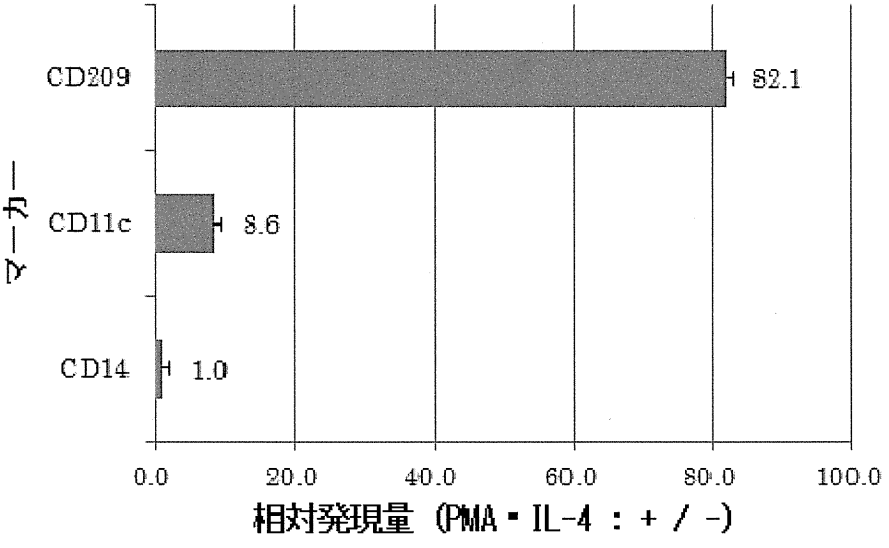
10 ng/ml の E-C1 抗体および E-G5 抗体により RS-ATL8 細胞を一晩感作し、上記の濃度の加熱・非加熱 OVA で３時間刺激を行なった際のルシフェラーゼ発現。無刺激時のルシフェラーゼ発現量を 1.0 とする相対値で表しており、カットオフ値は 2.0 としている。E-C1 抗体は非加熱 OVA を含むすべての抗原に対する応答が認められ、その応答性は加熱時間が長くなるにつれて増大した（A）。E-G5 の応答性は加熱時間にかかわらず認められなかった（B）。

図５　PMA・IL4 処理による THP-1 細胞の分化



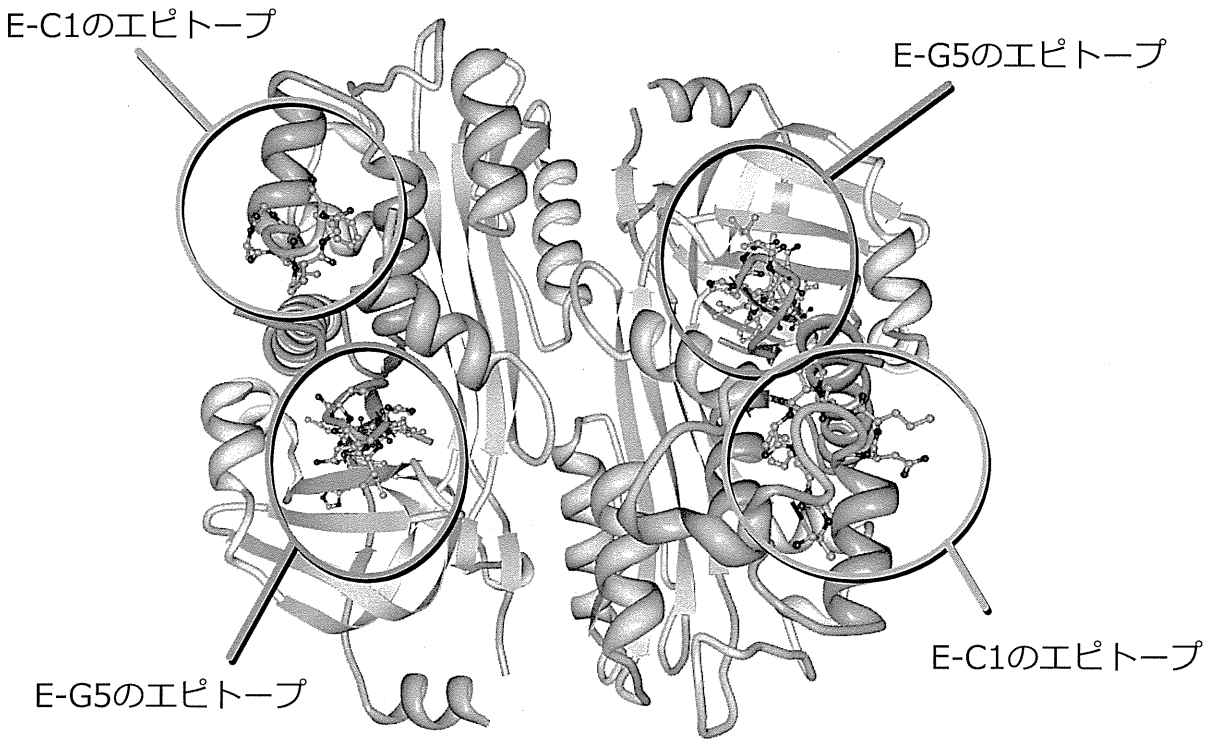
Phorbol 12-myristste 13-acetate（PMA）および Interleukin-4（IL-4）をそれぞれ終濃度 20 ng/ml で THP-1 細胞に曝露し、72 時間培養後の形態を比較した。A，処理前、B，処理後。

図６　THP-1 細胞の PMA・IL4 処理による細胞マーカー分子の発現変動



各マーカー分子が処理後（+）において、処理前（-）と比べて何倍の発現量を示すのかを real time RT-PCR 法（比較定量法）にて解析した。樹状細胞マーカーである CD209（DC-SIGN）および CD11c の発現が増加した。

図７　OVA の結晶構造と推定される２種の IgE のエピトープ



PDB の OVA の結晶構造データ（1OVA)。OVA がホモダイマーを形成している。ここに、Chondrex 社から提供されている E-C1 および E-G5 抗体のエピトープを図示した。E-C1 抗体のエピトープは、OVA の 61-68 残基目に当たる DKLPGFGD、E-G5 のエピトープは 363-374 残基目に当たる HPFLFCIKHIAT であるとされている [16,17]。E-C1 のエピトープは OVA 分子の端に突出しているが、E-G5 のエピトープは、少なくとも上記の構造では分子の内側に隠れている。

厚生労働科学研究委託費（創薬基盤推進研究事業）
委託業務成果報告書（業務項目）

担当研究課題　新規培養細胞株を用いたインフュージョン反応惹起性の評価法開発

担当責任者　森田 栄神　島根大学医学部　皮膚科学教室

アレルギー患者血清および臨床情報の収集

研究要旨　米国ではテネシー州を中心に、マダニ咬傷に起因するα-gal糖鎖への感作から、牛肉アレルギーおよびセツキシマブアナフィラキシーが多発している。今回、島根県在住の牛肉アレルギー患者がセツキシマブと交差反応をするか否かを目的として、血清を用いた免疫ブロット法にて解析した。その結果、牛肉アレルギー10例中8例にセツキシマブ糖鎖に対するIgE抗体が検出された。このことは本邦における牛肉およびセツキシマブへの感作も米国と同様マダニ咬傷に由来する機序であることを示唆しており、今後セツキシマブ投与に際して牛肉アレルギーに関する十分な問診と検査が望まれる。また、大規模な住民調査によりα-gal糖鎖への感作の現状を把握することも必要である。

研究協力者
高橋　仁　島根大学医学部皮膚科学教室助教
千貫祐子　島根大学医学部皮膚科学教室助教

A. 研究目的

2008年、米国のテネシー州を中心とした地域で、セツキシマブによるアナフィラキシー患者が多発したことが報告された。エピトープはセツキシマブに付随するgalactose-alpha-1,3-galactose（α-gal糖鎖）であること、テネシー州周辺の住民の約20%がセツキシマブで治療を受けたことがないにもかかわらずセツキシマブα-gal糖鎖に対するIgE抗体をもっていたことも報告された。同研究グループはこの抗体保有者が牛肉などの獣肉アレルギーを起こすことを明らかにした。つまり、セツキシマブのアナフィラキシーの原因となるα-gal糖鎖は、牛肉、豚肉などの獣肉に含まれるためである。 島根県では牛肉アレルギー患者が多発しており、これらの患者がα-gal糖鎖に対するIgE抗体を保有しているかどうかを明らかにすることはこれらの患者の診療情報として有用である。本研究では、これらの患者血清中のIgEがセツキシマブに反応するか否かを検討することを目的とした。

B. 研究方法

対象：牛肉や豚肉の摂取後にアレルギー症状を示し、血清中牛肉特異的IgEが陽性を示す10名、牛肉アレルギーの既往がなく、血清中牛肉特異的IgEが陰性の健常人4名を対象とした。

方法：患者血清を用いてセツキシマブを抗原としたIgE免疫ブロット法により、セツキシマブ特異的IgEの有無を検討した。さらにセツキシマブを過ヨウ素酸処理して糖鎖を開裂し、患者血清中IgEの結合性の変化を検討した。

＜倫理面への配慮＞

本研究では、匿名化されたアレルギー患者血清を用いることから、研究内容は島根大学医学部医の委員会によって審査され、承認されている（管理番号20090529-1）。患者には、

書面による十分な同意のもと、採血を実施している。

C. 研究結果

セツキシマブを抗原とした免疫ブロット法による解析で、牛肉アレルギー患者10名中8名（P2, P3, P4, P5, P7, P8, P9, P10）にセツキシマブに結合するIgEが検出された（図１）。これらの患者血清中IgEの結合は過ヨウ素酸処理したセツキシマブに対しては著明に減弱した。

D. 考察

今回の検討で、島根県在住の牛肉アレルギー患者においてもセツキシマブと交差反応をする可能性が高いことが示唆された。さらに過ヨウ素酸処理によりIgE抗体の結合は著明に減弱したことからセツキシマブのα-gal糖鎖を認識していると考えられる。米国の研究グループは、患者の臨床的観察からα-gal糖鎖への感作にはマダニ咬傷によるα-gal糖鎖含有タンパク質の刺入が要因と報告している。本研究の対象患者もマダニの生息する地域に在住しており、このことは本邦における感作も米国と同様マダニ咬傷に由来する機序であることを示唆している。これらの患者はセツキシマブ投与の既往はないが、我々は島根県松江市でのセツキシマブによるアナフィラキシー患者を多く経験しており、セツキシマブ投与の際の今後の指導が重要である。また、マダニ咬傷に起因するα-gal糖鎖への感作状況を国内で調査し、セツキシマブ投与に際して啓発することも極めて重要であると思われる。

E. 結論

島根県在住の牛肉アレルギー患者においてもセツキシマブと交差反応をする可能性が高く、セツキシマブ投与に際して牛肉アレルギーに関する十分な問診と検査が望まれる。

F. 健康危機情報

なし

G. 研究発表等

論文発表等

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2) 千貫祐子，伊藤和行，武田真紀子，竹内薫，高橋　仁，森田栄伸．セツキシマブによるアナフィラキシーショックの４例 -α-gal 糖鎖特異的 IgE 検出による回避の可能性-．日皮会誌　124：179-183 (2014).
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報道発表等

H. 知的財産権の出願・登録状況

1. 特許取得

なし

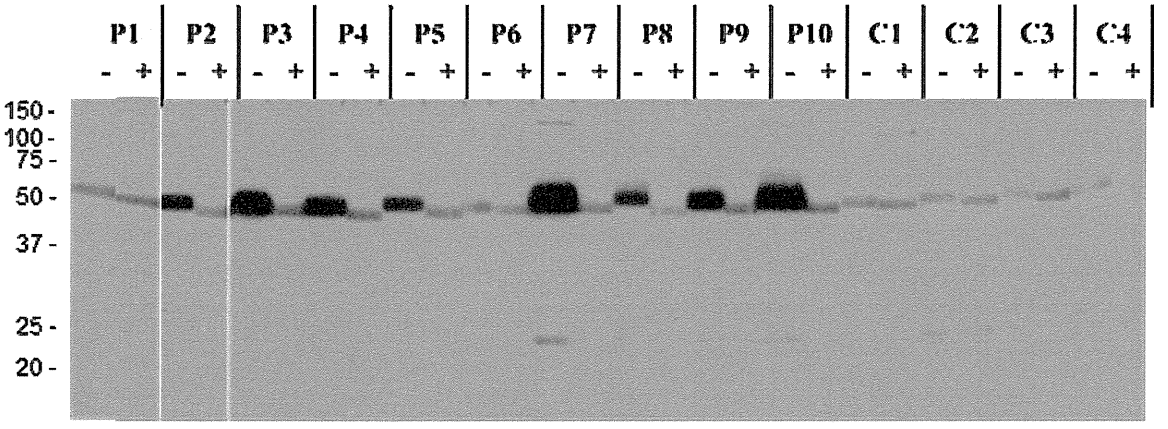
2. 実用新案登録

なし

3. その他

なし

図１　免疫ブロット法によるセツキシマブ特異的 IgE 検査結果



牛肉アレルギー患者（P1～P10）、健常人（C1～C4）（-）：過ヨウ素酸無処理、（+）過ヨウ素酸処理あり。

様式第１９

学 会 等 発 表 実 績

委託業務題目「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」

機関名　国立医薬品食品衛生研究所　中村亮介

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
HLA association with antipyretic analgesics-induced Stevens-Johnson syndrome / toxic epidermal necrolysis with severe ocular surface complications in Japanese patients.（ポスター）	Nakamura R, Kaniwa N, Ueta M, Sotozono C, Sugiyama E, Maekawa K, Yagami A, Matsukura S, Ikezawa Z, Matsunaga K, Tokunaga K, Aihara M, Kinoshita S, Saito Y.	Bern, Switzerland (Drug Hypersensitivity Meeting (DHM) 2014)	2014, 4	国外
Biomarkers for risk of SJS/TEN in Japanese compared to other populations.（口頭）	Nakamura R.	North Bethesda, MD, USA (Drug - Induced Injury of Liver, Heart, Kidney, and Skin: Employing Recent Advanced to Improve Patient Safety and Speed Up the Pipeline)	2014, 5	国外
臍帯血の抗原特異的低親和性IgE検出と，生後6，14ヶ月の高親和性IgEへの変化.（ポスター）	木戸　博，亀村典生，中村亮介，手島玲子，深尾敏幸.	京都（第26回日本アレルギー学会春季臨床大会）	2014, 5	国内
培養細胞を用いるアレルギー試験「EXiLE法」の開発.（口頭）	中村亮介.	神戸（第41回日本毒性学会学術年会）	2014, 7	国内
Experimental assess- ments of the cross-reactivity of IgE from patients sensitized with acid- hydrolysed wheat protein in a cosmetic soap.（ポスター）	Sakai S, Nakamura R, Adachi R, Fukutomi Y, Saito Y, Nishimaki-Mogami T, Teshima R.	Dublin, Ireland (Food Allergy and Anaphylaxis Meeting (FAAM) 2014)	2014, 10	国外
新規蛋白チップによる臍帯血特異的IgEの検出と、離乳完了期までに見られるIgE抗体の低親和性から高親和性への変化.（口頭）	亀村典生，川本典生，中村亮介，手島玲子，下条直樹，深尾敏幸，木戸 博.	四日市（第51回日本小児アレルギー学会）	2014, 11	国内

EXiLE法を用いた口腔アレルギー症候群の新たなin vitro検査法の検討. （ポスター）	秋山晴代，河又小夏，中村亮介，福冨友馬，甲斐茂美，松藤 寛，宮澤眞紀.	横浜 （第37回日本分子生物学会年会）	2014，11	国外
架橋誘導活性の異なるモノクローナルIgEの抗原認識様式の違いについて. （ポスター）	岡本(内田)好海，中村亮介，相馬愛実，石井明子，最上知子，川崎ナナ，川上 浩，手島玲子，斎藤嘉朗.	神戸 （日本薬学会第135年会）	2015，3	国内
口腔アレルギー症候群における新たなin vitro試験法の検討. （ポスター）	秋山晴代，河又小夏，政岡智佳，中村亮介，福冨友馬，甲斐茂美，松藤 寛，宮澤眞紀.	神戸 （日本薬学会第135年会）	2015，3	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所 （学会誌・雑誌等名）	発表した時期	国内・外の別
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Use of Humanized Rat Basophil Leukemia (RBL) Reporter Systems for Detection of Allergen-Specific IgE Sensitization in Human Serum.	Wan D, Wang X, Nakamura R, Alcocer MJC, Falcone FH.	Basophils and Mast Cells : Methods and Protocols, Methods in Molecular Biology. 1192: 177-184 (2014).	2014，6	国外
Use of Humanised Rat Basophilic Leukaemia Cell Line RS-ATL8 for the Assessment of Allergenicity of Schistosoma mansoni Proteins.	Wan D, Ludolf F, Alanine DG, Stretton O, Ali Ali E, Al-Barwary N, Wang X, Doenhoff MJ, Mari A, Fitzsimmons CM, Dunne DW, Nakamura R, Oliveira GC, Alcocer MJ, Falcone FH.	PLoS Negl Trop Dis. 2014;8:e3124.1	2014，9	国外

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

様式第１９

学　会　等　発　表　実　績

委託業務題目「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」

機関名　島根大学医学部皮膚科学講座　森田栄伸

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Galactose-α-1,3-galactose (α-gal)-specific IgE test is highly useful for predicting cetuximab-induced anaphylaxis.（ポスター）	Morita E, Chinuki Y, Takahashi H, Takeda M, Takeuchi K, Ito K.	Bern, Switzerland (Drug Hypersensitivity Meeting (DHM) 2014)	2014, 4	国外

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Laminin γ-1 and collagen α-1 (VI) chain are galactose-α-1,3-galactose -bound allergens in beef.	Takahashi H, Chinuki Y, Tanaka A, Morita E.	Pediatr Blood Cancer. 61: 1160-1161 (2014).	2014, 7	国外
セツキシマブによるアナフィラキシーショックの4例-α-gal糖鎖特異的IgE検出による回避の可能性-.	千貫祐子，伊藤和行，武田真紀子，竹内薫，高橋　仁，森田栄伸.	日皮会誌　124：179-183 (2014).	2014	国内
蕁麻疹～牛肉による蕁麻疹はセツキシマブのアナフィラキシーを予知している～.	森田栄伸，千貫祐子，高橋　仁.	アレルギー・免疫　21：81-85 (2014)	2014	国内
重症薬疹としてのアナフィラキシー.	森田栄伸，千貫祐子，高橋　仁.	アレルギー・免疫　21：66-71 (2014).	2014	国内

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

（注２）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

LETTER TO THE EDITOR
Anaphylactic Transfusion Reaction in Homozygous Haptoglobin Deficiency Detected by CD203c Expression on Basophils

To the Editor: It has been reported that the incidence of anhaptoglobinemia who are homozygous for Hp^{del} is very rare in Africans or European-Africans. However, it is relatively high incidence in East Asian; 1/4,000 in Japan, 1/1,500 in Korea, and 1/1,000 in China [1]. Some patients with anhaptoglobinemia develop anaphylactic or allergic transfusion reactions related to antihaptoglobin (Hp) antibodies [2]. However, if anti-Hp IgE antibody is negative, it is difficult to elucidate an anaphylactic reaction. Flow cytometric basophil activating test (BAT) based on upregulation of the cell degradation/activation marker CD203c has been developed for monitoring the clinical course of patients with allergic diseases [3,4]. Here, we describe that BAT was a useful tool for diagnosing anaphylactic transfusion reaction associated with anti-Hp antibody.

This case was an 8-year-old Japanese boy with acute lymphoblastic leukemia receiving intensive chemotherapy required transfusion. He was transfused with 14 units of leukocyte-reduced red cell concentrates (RCCs) and 150 units of leukocyte-reduced platelet concentrates for chemotherapy-induced anemia and thrombocytopenia without any transfusion-related reactions. However, immediately after starting the next infusion of RCCs, he developed dyspnea, urticaria, and hypotension. Screening tests for nonhemolytic transfusion reactions (NHTRs) were performed at the Japanese Red Cross. Serum analysis by nephelometry revealed Hp deficiency and their PCR method [1] showed complete absence of the Hp gene. He was diagnosed as having anhaptoglobinemia due to homozygous allelic Hp^{del}. Although anti-Hp IgG antibody was detected by ELISA and Western blotting, IgE antibody against Hp was not detected by ELISA or the IgE crosslinking-induced luciferase expression (EXiLE) test [5].

To address the mechanism of this anaphylactic reaction, we performed BAT. As a control, a healthy adult and five age-matched children with severe egg allergy who had histories of food-induced anaphylaxis were examined. A commercial kit (Allergenicity Kit; Beckman Coulter, Fullerton, CA) was used for quantifying basophil CD203c expression [4]. Upregulation of CD203c on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells and expressed as CD203c high % [6]. CD203c high % was 23% for the patient and 4.38 ± 0.53% for the control, respectively (Table I). Haptoglobin induced enhancement of CD203c expression in the patient, but not in the control.

Since basophils express high-affinity IgE receptors, it is reasonable to assume that the haptoblobin-induced basophil activation observed in the patient was mediated by haptoglobin-specific IgE bound to basophils. We did not detect antihaptoglobin IgE by the ELISA or EXiLE methods, but it is possible that the serum IgE concentration was too low to be detected, whereas the amount of cell-bound IgE was sufficient to induce anaphylaxis.

Another possibility is that haptoglobin-specific IgG induced basophil activation. An IgG-mediated systemic anaphylactic reaction involving FcγRs, basophils, and platelet-activating factor was reported in a murine system [7], although it remains unclear whether such an IgG-mediated pathway also exists in humans.

TABLE I. The Basophil Activation Test (BAT) Using Flow Cytometry Based on CD203c Up-Regulated by Haptoglobin

	CD203c high % on basophils		
	Negative control	Positive control	Target
Subject	No stimulation	Anti-IgE	Haptoglobin 1:100
Index patient	3.6	80.5	23.0
Control person	4.9	91.9	5.0
Food allergy Pt 1	3.9	93.1	8.5
Food allergy Pt 2	4.9	30.5	6.8
Food allergy Pt 3	4.0	60.9	2.0
Food allergy Pt 4	3.8	97.0	5.1
Food allergy Pt 5	4.8	55.2	4.6

Flow cytometric analysis of haptoglobin-induced CD203c expression on basophils of a patient with haptoglobin deficiency and from age-matched children with food allergy and health adult donor as a control. EDTA-containing whole blood was incubated with haptoglobin at 1:100 dilution for 15 minutes. Anti-IgE antibody as a positive control and PBS as a negative control were used as stimulators. Basophils were detected on the basis of forward and side scatter characteristics and as the CD3-negative, Chemoattractant Receptor-homologous molecule expressed on Th2 cells (CRTH2)-positive population. Upregulation of CD203c was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control) and expressed as CD203c high %.

Our findings indicate that BAT may be useful for assessing the cause of NHTRs, especially anaphylactic reactions via high-affinity IgE receptors on basophils, leading to development of type I hypersensitivity response when no IgE can be detected in serum.

ACKNOWLEDGMENTS

The authors thank Yumi Tanaka, MSc (Blood Transfusion Service, Mie University Hospital) for kindly blood supplies. This work was supported by a Research Grant for Allergic Disease and Immunology (H20-015) from the Japanese Ministry of Health, Labor, and Welfare.

Grant sponsor: The Japanese Ministry of Health, Labor, and Welfare; Grant number: H20-015

Conflict of interest: Nothing to declare.

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Received 7 December 2013; Accepted 3 January 2014

DOI 10.1002/pbc.24965
Published online in Wiley Online Library (wileyonlinelibrary.com).

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PLOS | NEGLECTED TROPICAL DISEASES

Use of Humanised Rat Basophilic Leukaemia Cell Line RS-ATL8 for the Assessment of Allergenicity of *Schistosoma mansoni* Proteins

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Abstract

Background: Parasite-specific IgE is thought to correlate with protection against *Schistosoma mansoni* infection or re-infection. Only a few molecular targets of the IgE response in *S. mansoni* infection have been characterised. A better insight into the basic mechanisms of anti-parasite immunity could be gained from a genome-wide characterisation of such *S. mansoni* allergens. This would have repercussions on our understanding of allergy and the development of safe and efficacious vaccinations against helminthic parasites.

Methodology/Principal Findings: A complete medium- to high-throughput amenable workflow, including important quality controls, is described, which enables the rapid translation of *S. mansoni* proteins using wheat germ lysate and subsequent assessment of potential allergenicity with a humanised Rat Basophilic Leukemia (RBL) reporter cell line. Cell-free translation is completed within 90 minutes, generating sufficient amounts of parasitic protein for rapid screening of allergenicity without any need for purification. Antigenic integrity is demonstrated using Western Blotting. After overnight incubation with infected individuals' serum, the RS-ATL8 reporter cell line is challenged with the complete wheat germ translation mixture and Luciferase activity measured, reporting cellular activation by the suspected allergen. The suitability of this system for characterization of novel *S. mansoni* allergens is demonstrated using well characterised plant and parasitic allergens such as Par j 2, SmTAL-1 and the IgE binding factor IPSE/alpha-1, expressed in wheat germ lysates and/or *E. coli*. SmTAL-1, but not SmTAL2 (used as a negative control), was able to activate the basophil reporter cell line.

Conclusion/Significance: This method offers an accessible way for assessment of potential allergenicity of anti-helminthic vaccine candidates and is suitable for medium- to high-throughput studies using infected individual sera. It is also suitable for the study of the basis of allergenicity of helminthic proteins.

Citation: Wan D, Ludolf F, Alanine DGW, Stretton O, Ali Ali E, et al. (2014) Use of Humanised Rat Basophilic Leukaemia Cell Line RS-ATL8 for the Assessment of Allergenicity of *Schistosoma mansoni* Proteins. PLoS Negl Trop Dis 8(9): e3124. doi:10.1371/journal.pntd.0003124

Editor: Cinzia Cantacessi, University of Cambridge, United Kingdom

Received May 7, 2014; **Accepted** July 17, 2014; **Published** September 25, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data have been included in this submission.

Funding: The work was supported by the following fellowships: CNPq (FL), FAPEMIG (FL), Boehringer Ingelheim Fonds (FL), ISID/ESCMID (FL). We also wish to acknowledge the support of the School of Pharmacy's Strategic Research Committee to FHF. EAA received a bursary from the Gordon Memorial College Trust Fund for this work, which is gratefully acknowledged here. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Helminthic parasites are well known to induce a strong Th2-biased response in their hosts, characterised by elevated levels of total and parasite-specific IgE, IL-4, IL-5 and IL-13, with concomitant expansion and mobilization of specific effector cells [1,2]. This IgE response is widely believed to have evolved to protect against ectoparasites and parasitic helminths, and *Schistosoma* in particular [3], although this widespread view has been recently challenged [4].

Human infection with the trematode *Schistosoma mansoni* is well known to correlate with a progressive increase of serum IgE levels [5]. *S. mansoni* infection usually peaks in early adolescence and declines in adulthood, a pattern that suggests that individuals

Author Summary

Infection with parasitic helminths is characterised by a marked elevation of total and parasite-specific Immunoglobulin E (IgE). It is widely believed that this IgE response has evolved to protect hosts against large metazoan parasites. Such a protective function has been well characterised in particular against members of the genus *Schistosoma*. However, with a few notable exceptions, the molecular targets of the IgE response and the downstream immunological mechanisms leading to host protection are not well understood. The molecular targets of a specific IgE response are by definition called allergens. While almost 3,000 different allergens, contained in e.g. plant pollen or seeds, moulds or animal materials, have been characterised at the molecular level, and are listed and described in databases such as the Allergome database (www.allergome.org), only a few dozen allergens have been characterised in parasitic helminths. A more detailed understanding of the molecular targets of the anti-helminth IgE response can not only be expected to further our basic understanding of protective immune responses and allergy in general–such knowledge can also be expected to have important repercussions on the production of safe and effective anti-helminthic vaccines. This research describes a novel approach suitable for genome-wide functional identification of allergens in *S. mansoni* and other parasites, paving the way for the identification of the *Schistosoma* allergome.

in endemic areas can gradually acquire an age-related resistance to reinfection [6,7]. Progressive acquisition of anti-schistosome immunity coincides with natural death of worms (averaging 10–15 years of life), an event during which the parasites release and expose previously inaccessible antigens to the immune system [8]. Similarly, repeated treatment with praziquantel can speed up the process of immunity, resulting (in some individuals) in so-called post-treatment resistance to infection [7,9–11]. A Th1-type or mixed Th-1/Th2-type response is associated with putative natural resistance in 'endemic normal' individuals [12]. However, post-treatment resistance is associated with a stronger Th2-type response dominated by IgE and IgG4 [5], with the higher IgE/IgG4 ratio, rather than their absolute levels, best predicting resistance [13–15]. A group of antigens related to the different infection status of endemic area residents in Brazil was recently identified by a serological proteomic analysis which may be related to susceptibility or resistance to infection [14]. However, despite recent progress and decades of research, the targets of this protective antibody response and the basis of its 'inefficient' acquisition are still unknown.

The occurrence of natural and post-treatment resistance suggests that immunity could be conferred by appropriately formulated vaccines, possibly using mixtures of antigens. Strategies used for vaccine development have changed as the genomic data for schistosomes have become increasingly available and post-genomic technologies have matured [15]. The traditional approach has been to identify immunogenic antigens using immunological screening (i.e. Western Blots), followed by cloning, expression and case-by-case testing for protection in murine or other animal models. To date, even the best vaccine candidates have achieved protection levels below 70% in animal models, with higher protection only achieved by using high doses of irradiated cercariae [16,17]. The most promising vaccine candidate (SmTSP-2) achieved 57% and 64% reduction (adult worm and egg burden, respectively) and importantly was recognized by IgG1 and IgG3, but not IgE, in sera of naturally resistant, but not uninfected or chronically infected individuals [18].

There is however a major unsolved conundrum specific to the development of anti-helminthic vaccines. While the bulk of the evidence points to a major protective role of the parasite-specific IgE response against the parasite, vaccinating with an allergen bears the inherent risk of potentially inducing hazardous allergic reactions in sensitised individuals, as recently reported during clinical trials for an anti-hookworm vaccine using Na-ASP-2, where adult volunteers experienced generalized urticarial reactions immediately after vaccination [19]. It could be shown that individuals who displayed urticarial reactions possessed high levels of IgE against Na-ASP-2. This led to testing of specific IgE levels for other candidate vaccine antigens such as *Necator americanus* GST1 and APR1 using sera from individuals resident in helminth-endemic areas [20]. Thus it would be beneficial to identify such allergens at an early stage during vaccine development.

We have previously shown that human basophils are sensitised within 6 weeks of a single, low-dose infection with *N. americanus* infective stage larvae [21]. Basophil activation could be detected by flow cytometry in the absence of measurable parasite-specific IgE levels in the serum. This suggests that basophils may offer a sensitive way of measuring the presence of parasite antigen-specific IgE in infected individuals, and, perhaps more importantly in the context of vaccination, to demonstrate the ability of allergens to induce basophil or mast cell activation, in contrast to measuring allergen binding by specific IgE alone. Therefore, we recently developed a new detection system for antigen-specific IgE based on the NFAT-dependent luciferase expression in a humanised rat basophilic leukaemia cell line (RS-ATL8) [22,23]. When sensitised with egg white-allergic patient's serum, this cell line detected at least 1 fg/mL of egg white extract proteins as a luciferase expression [23].

The sensitivity of this detection method makes it possible to study the potential allergenicity of a protein using only minute amounts of protein. The lack of requirement for a high yield allows the use of a fast and easy cell-free expression system such as wheat germ lysate, which allows for expression of microgram amounts of protein [24] in less than two hours. This in turn makes it possible to produce many correctly folded antigens of interest in short time [25].

Here, we demonstrate proof-of-principle of how the RS-ATL8 cell line, in combination with a cell-free *in vitro* translation system and a set of stringent quality controls, can be used for assessment of allergenicity of *S. mansoni* antigens. This technology paves the way for high-throughput, genome-wide assessment of *S. mansoni* antigen allergenicity - the *Schistosoma* allergome.

Materials and Methods

Human sera

Samples of schistosomiasis patients were from a Ugandan study. All EDTA plasma samples were obtained from a male cohort from the village of Musoli on Lake Victoria. Samples used in this study are from a subgroup of infected people described in Fitzsimmons *et al.* [26]. The blood samples were selected based on their known content of SmTAL1-specific IgE as measured by isotype specific ELISA, as described by Naus and co-authors [27]. Details of the plasma samples are summarised in Table S1 in the Supplementary data.

Ethical clearance was obtained from the Uganda National Council of Science and Technology (ethics committee for Vector Control Division, Ugandan Ministry of Health) who approved the

age of consent as 15 y at the time of sample collection (2004/2005). Consent forms were translated into the local language and informed written consent was obtained from all adults and from the parents/legal guardians of all children. Parental consent was not sought for individuals 15–18 y old.

Sera from patients allergic to *Parietaria judaica* (commonly known as spreading pellitory in the Mediterranean area, and sticky weed or asthma weed in Australia) were collected after informed consent from the patients, and under a study protocol approved by the institutional ethical committee to establish the sera bank. Institutional Review Board of IDI-IRCCS, Rome, Italy (n. 106-CE-2005). The sera obtained from nine patients were pooled in equal amounts and the levels of specific IgE, IgG4, and total IgG against 104 103 different allergens measured by ImmunoCAP ISAC multiplexing analysis (ThermoScientific) following the protocol previously described [28]. The pooled sera showed high levels of specific IgE to Par j 2 (55 U/ml). The complete ISAC characterisation of the pooled sera is shown in the Supplementary data (Table S2).

Viral inactivation of plasma or serum. To reduce biohazard of the blood samples, which otherwise would require the work to be performed in laboratories with increased biological safety levels, all acellular blood samples were subjected to a detergent treatment known to inactivate enveloped viruses in serum or plasma samples [29] and has been used for IgE testing by Poulsen and Sørensen [30]. Sera were incubated with a mixture of 0.3% (v/v) TNBP (Tri-N-butylphosphate) and 1% (v/v) Tween-80 (Polyoxyethyleneorbitan) under rotation at room temperature for 1 hr, aliquoted and frozen until further use.

Polymerase chain reaction (PCR)

Fifteen chosen genes representing proteins from a diverse families such as major egg allergens, troponin, fatty acid binding protein (complete list described in supplementary data in Table S3) were amplified from relevant cDNA libraries (adult worm λZAP cDNA library generous donation by K. Hancock, CDC Atlanta, USA; egg stage λZAP cDNA library kindly contributed by Helmut Haas and Gabi Schramm, Research Centre Borstel, Germany), or available cDNA clones (kindly donated by Alan Wilson, University of York, UK) using 25 μL JumpStart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich), 2.5 μL of each forward and reverse custom made gene specific primer (Sigma Aldrich, final concentration 0.5 μM), 2 μL of *S. mansoni* cDNA library in 50 μL final volume. For longer sequences, Q-5 polymerase (New England Biolabs) was used to take advantage of its high proofreading activity. Forward primers were constructed by adding a SgfI restriction site and a start ATG (where not available, i.e. when expressing the mature protein sequence after leader peptide cleavage at the 5′ end), and reverse primers by adding a His_6-tag followed by a valine (for facilitation of subcloning into other expression vectors) and a stop codon, followed by a PmeI restriction site at the 3′ end. The complete primer sequences are listed in Table S3 in Supplementary materials.

Temperature gradients were run initially to optimise the annealing temperature for the different genes; however an annealing temperature of 54°C worked for most of the genes. Successful PCR was confirmed by 1% agarose gel electrophoresis, using 100 bp Tridye DNA ladder (New England Biolabs) for reference. The final products were purified using a Promega Wizard SV Gel and PCR Clean-Up System, as described by the manufacturer.

Cloning and transformation

The concentration of the amplified genes was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The purified PCR products were inserted into pF3A WG (BYDV) Flexi Vector (Promega) using the Flexi Vector System (Promega). The ligations were heated at 65°C for 5 min for T4 DNA ligase (HC) inactivation, before transformation, which substantially increased the number of colonies obtained. Transformation was achieved by employing electro-competent DH5α *Escherichia coli* cells using a BioRad Micropulser electroporation apparatus, following standard molecular biology procedures. Plasmids were purified from the transformed cells using a QIAGEN QIAprep Spin Miniprep Kit, as per the manufacturer's protocol. All purified plasmids were verified by DNA sequencing (Source Bioscience, Nottingham, UK).

Coupled *in vitro* transcription-translation

Plasmids were used to produce proteins through coupled *in vitro* transcription-translation, using TnT SP6 High-Yield Wheat Germ Protein Expression System (Promega), as per the manufacturer's instructions. Protein synthesis was initiated by mixing the appropriate DNA template (2–3 μg), 30 μL of the TnT SP6 High-Yield Wheat Germ Master Mix and water for a 50 μL final volume, and then incubating the reaction at 25°C for 2 hours. Protein expression was analyzed by the incorporation of labelled lysine residues (FluoroTect GreenLys, Promega) in a 10 μl aliquot of the plasmid/wheat germ lysate mixture (WGL) as directed in the instructions. Samples were heated for 3 minutes at 70°C, run on 4–20% SDS-PAGE gradient gels (BioRad, UK), under reducing conditions and imaged with a laser-based fluorescent gel scanner (Fujifilm LAS-4000 319 Imaging System). Molecular weights (MW were estimated using the Kaleidoscope Precision plus marker (BioRad, UK) which contains several fluorescently labelled components.

Recombinant bacterial expression of Sm-TALs

SmTAL1 and Sm-TAL2 were expressed in E. coli as described previously [31].

Western blots

IPSE/alpha-1, SmTAL1, and SmTAL2 proteins produced in the wheat germ lysate system were transferred to a 0.45 μm nitrocellulose membrane (NCM) (Sigma-Aldrich) and sections of membrane were incubated with mouse anti-IPSE/alpha-1 monoclonal antibody (1:2000) or rabbit anti-sera (1:500) against SmTAL1 or SmTAL2, using the method described by Burnette [32]. The anti-IPSE/alpha-1 monoclonal antibody used is from clone 74 2G4 [33,34]which recognises both monomeric and dimeric IPSE/alpha-1. Both antibodies were diluted in TBS with 3% Tween, 30% Wheat Germ Extract and 5% skimmed milk powder.

The mouse or rabbit primary antibodies were detected with an HRP-conjugated secondary antibody using chemiluminescence (ECL Plus Western Blotting Detection System, GE Healthcare) diluted 1:5000 and visualised using a Fujifilm LAS-4000 imaging system.

Cell culture and measurement of basophil activation

RS-ATL8 cells were cultured in 75 cm^2 flasks, with 0.2 μm vent caps (Corning, USA), in an incubator set at 37°C with 5% carbon dioxide with a humidified atmosphere [22,23]. The flasks contained 10 mL MEM (GIBCO, USA), supplemented with 5% v/v heat-inactivated FCS (GIBCO, USA), 100 U/mL penicillin,

100 μg/mL streptomycin (Sigma, UK) and 2 mM L-glutamine (Sigma, UK), with medium change every 2–3 days. Cells were detached by washing the flasks twice with calcium/magnesium-free DPBS, followed by incubation with 2 mL trypsin-EDTA (GIBCO, USA) for 10 minutes. Alternatively, cells were scraped using cell scrapers (TPP, Switzerland). 1 mg/mL G418 (Fisher ThermoScientific, UK) and 600 μg/mL hygromycin B (Invitrogen, Paisley, UK) were used to maintain expression of human FcεRI genes and NFAT-luciferase, respectively. Prior to testing, cells were incubated overnight in culture medium with various dilutions of pooled serum from *Parietaria judaica* patients or *S. mansoni* infected individuals, and washed once prior to addition of the stimulus (recombinant Par j 2 from Bial, Zamudio, Spain, accession: R-17). The following positive control was used for all RS-ATL8 experiments: sensitization with 1 μg/mL of human IgE (AbD Serotec) followed by stimulation with 1 μg/mL of goat anti-human IgE polyclonal IgG (Vector Labs).

ONE-Glo Luciferase Assay System (Promega, UK) was used for all luciferase assays, following the manufacturer's instructions. Half volume (50 μL) reactions were used. Chemiluminescence was measured on an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) not later than 30 minutes after the addition of the luciferase substrate.

Statistical analysis

One-way ANOVA followed by Dunnett's or Tukey's post hoc test was performed using GraphPad Prism 6 software for Figures 3, 4, 6, 7. Spearman's rank correlation test was performed to compare IgE titres with the luminescence response in activated RS-ATL8 cells (Figure 8).

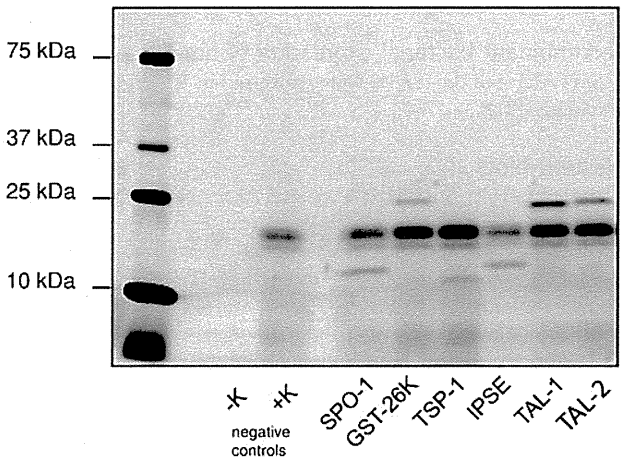

Figure 1. In gel detection of five different *S. mansoni* antigens expressed in vitro using WGL. SPO-1: Smp_113760; GST-26k: Smp_102070; TSP-1, extracellular loop 2: Smp_095630; IPSE/alpha-1: Smp_112110; SmTAL1: Smp_045200; SmTAL2: Smp_086480. Details of sequences and expected molecular weights are given in the Supplementary Data in Table S3. Success of translation was monitored by incorporation of BODIPY-labelled fluorescent Lysine in separate aliquots during translation. Samples were run on 4–20% SDS-PAGE gradient gels under reducing conditions and imaged in a Fujifilm LAS-4000. The left lanes show the wheat germ lysate control without template DNA, either without (−K) or with Lysine incorporation (+K), indicating fluorescent components produced during *in vitro* translation from endogenous mRNA.
doi:10.1371/journal.pntd.0003124.g001

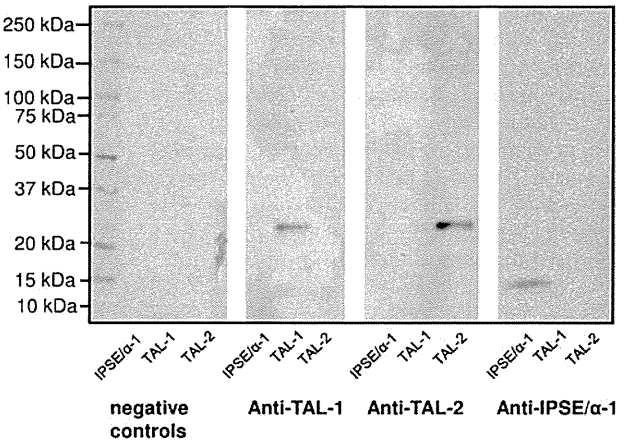

Figure 2. Western blot demonstrating antigenicity of the *S. mansoni* proteins expressed in the WGL system. Non-purified wheat germ extracts containing *in vitro* translated IPSE/alpha-1, SmTAL1 or SmTAL2 were separated in a 4–20% SDS-PAGE gel and blotted onto NCM. Separate strips of NCM were treated with anti-TAL1 rabbit serum, anti-TAL2 rabbit serum or anti-IPSE/alpha-1 mouse monoclonal antibody. The negative control (neg. control) was incubated without primary antibody/serum, but with secondary antibody. Membranes were imaged using chemiluminescence and a Fujifilm LAS-4000.
doi:10.1371/journal.pntd.0003124.g002

Results

All the genes described herein were successfully amplified and ligated into the p3FA (BYDV) WG vector. The annealing temperature of 54°C was efficient for all the genes and DNA sequences were confirmed for the clones before *in vitro* expression. All fifteen reported parasitic genes reported herein were then successfully translated *in vitro* using the coupled transcription/translation WGL mixture and an aliquot of the translation mix was used for monitoring of translation by incorporation of fluorescently-tagged lysine. An example of 5 genes obtained by this method is shown in Figure 1. As seen in the negative control, protein translation with WGL results in two endogenous fluorescent components in the 15–20 kDa range (lane three in Fig. 1) which have the potential to interfere with detection of the translated parasitic protein if of this size. More examples can be seen in Supplementary data Figure S1.

In order to assess whether cell-free translation in wheat germ lysates results in proteins with unaltered antigenic properties, three unlabelled *S. mansoni* genes (IPSE/alpha-1, SmTAL1, SmTAL2), for which either polyclonal antisera or monoclonal antibodies were available, were expressed using wheat germ lysates and tested by immunoblotting. As shown in Figure 2, all three antigens were specifically recognised by the corresponding antibodies, demonstrating that the method chosen for expression does not appear to alter antigenicity of the parasitic proteins.

Initial experiments using cells sensitised with human monoclonal myeloma IgE (with unknown specificity) overnight and stimulated with an anti-human IgE antibody had demonstrated a high sensitivity of 10 pg IgE per assay (Supplementary Figure S2). To determine the sensitivity of the assay using polyclonal IgE in serum, a dose response curve of RS-ATL8 cell sensitised overnight with pooled sera diluted 1:50 obtained from *Parietaria judaica* allergic patients, stimulated with the matching allergen Par j 2, was performed. These experiments showed that activation of basophils sensitised with this pooled serum