

ORIGINAL ARTICLE

Whole Antigenic Lysates of *Ixodes ricinus*, but not Der-p2 Allergen-Like Protein, are Potent Inducers of Basophil Activation in Previously Tick-Exposed Human Hosts

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Keywords:

tick cells; *Ixodes ricinus*; basophils; Der-p2 protein

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Received for publication November 15, 2012

doi:10.1111/tbed.12151

Summary

The clinical suspicion of tick anaphylaxis is based on a history of the bite and occurs often during the warm season. Further arguments are the presence of natural hosts in the immediate environment and, eventually, the identification of the tick. The diagnosis is confirmed when immediate-type sensitization is shown by positive skin prick tests performed with specific tick extracts or the demonstration of specific IgE *in vitro*. In the current study, we hypothesize that hard tick-derived material contains potent inducers being able to promote basophil stimulation, which correlates with a sensitization immunological response following tick bites. To this end, biological material from two hard tick cell lines (IRE11 and IDE8 – derived from *Ixodes ricinus* and *I. scapularis*, respectively) as well as *I. ricinus* salivary gland and body lysates were used in a human basophil activation test (BAT) to analyse binding and cross-linking capacity of membrane-bound IgE, because basophils are one of the main effector cells of allergic reactions. Additionally, Der-p2 allergen-like gene from *I. ricinus* was recombinantly expressed as a 15-kDa histidine-tagged fusion protein, purified and included as a stimulus within the setup. Blood was drawn and submitted to BAT screening from a pool of 36 individuals, both bitten and who served solely as negative controls. We have found that seven subjects (19%), all of whom were at least two times tick-bitten, positively reacted to the aforementioned stimuli, whereas the reactivity level of the ones bearing single bites proved to be within the normal range. Moreover, no significant upregulation of the assessed basophil activation marker was detected in the case of Der-p2, except a faint reaction at high dosages. We conclude that at least two tick bites of the human host must occur in order to induce significant basophil activation.

Introduction

Ticks represent one of the most important groups of arthropods that affect animal and human health. Among the ectoparasites, ticks produce the greatest economic loss in livestock production worldwide (Mulenga et al., 2000). They are haematophagous arthropods capable of parasitizing a wide variety of hosts (mammals, reptiles, amphibians and birds). During their blood meal, ticks secrete bioactive substances in their saliva, including anticoagulants, immunomodulatory and anti-inflammatory compounds capable

of influencing host defence mechanisms at the bite site (Beaufays et al., 2008).

Recent findings suggest that tick bite is associated with the production of IgE antibody and that tick saliva preferentially induces a Th2 response, characterized by the production of high levels of IL-4 and IL-10 and low levels of IFN-gamma and IL-12. Allergic reactions after tick bite have indeed been reported on. Specific IgE antibodies to tick proteins have been demonstrated in patients against hard tick species such as *Ixodes holocyclus*, *I. ricinus* or *I. pacificus* (Acero et al., 2003). Tick bites can trigger

allergic reactions especially during occupational or recreational exposure. It is noteworthy that anaphylaxis caused by tick bite could be more common than suspected. Although not always possible, the correct establishment of allergy to ticks depends on the identification of the tick and appropriate tick extracts (Acero et al., 2003).

The overall clinical picture of an anaphylactic reaction due to a tick bite is characterized by sudden onset of severe muco-cutaneous, respiratory, gastrointestinal and cardiovascular allergic symptoms resulting in loss of consciousness and requiring intensive care in some of the cases (Hilger et al., 2005). An example is human allergic reactions to *I. holocyclus* – the Australian paralysis tick – which are well recognized but poorly defined. Tick-bite reactions were assessed by employing the skin prick tests and radio-immunoassay (RIA), which indicated that all systemic hypersensitivity (class 3) and atypical reactions (class 4) were IgE-mediated. Some 73% of the large local reactions (class 2) and only 1.2–5% of the small local reactions (class 1) were associated with IgE specific for tick allergens (Gauci et al., 1989).

Serious allergic manifestations were also described in the case of the soft European pigeon tick *Argas reflexus*, which is an urban pest parasitizing wild urban pigeons (*Columba livia*). Sometimes this tick may bite humans, causing a wide range of allergic reactions, from local manifestations (itching, oedema or erythema) to severe systemic reactions (e.g. anaphylaxis). Specific IgE to *A. reflexus* has been detected in the serum of patients who experienced allergic responses. Presently, details on the allergens responsible for allergic manifestations are not known (Rolla et al., 2004).

Peripheral blood basophils and tissue mast cells are primary effector cells in IgE-mediated immediate allergic reactions such as rhinitis, asthma and anaphylaxis. Basophils represent less than 0.5% of the total leucocytes in peripheral blood, which makes their purification difficult. As these cells play an important role in immediate allergic reactions, some functional *in vitro* tests have been developed to detect their activation. One of the most reliable tools is considered to be the flow cytometric basophil activation test (BAT), which monitors basophil activation after antigen stimulation using the expression of CD203c membrane surface marker. CD203c antibodies recognize a type II transmembrane protein. The expression of this protein is substantially increased in the cell surface after activation (Sanz et al., 2007). To date, no other cells from human peripheral blood have been reported to express this marker. Its expression on basophils is rapidly upregulated after stimulation with the appropriate allergen in patients sensitized to acarids or hymenoptera or after cross-linking of FcεRI with anti-IgE antibodies (Boumiza et al., 2005). The ectoenzyme CD203c (E-NPP3; pyrophosphatase/phosphodiesterase 3) was found to be expressed on blood basophils, tissue mast

cells and their CD34 + progenitor cells but not on other blood leucocytes. The basophil activation test based on CD203c upregulation has been validated as a reliable tool for the diagnosis of IgE-mediated allergies (Sturm et al., 2010). As it was described that the basophil antigen CD203c is upregulated on IgE receptor cross-linkage (Hauswirth et al., 2002), allergen-induced CD203c upregulation on basophils can be assessed by immunofluorescence.

Based on the availability of two tick cell lines derived from *I. ricinus* and *I. scapularis*, as well as *I. ricinus* salivary gland and body lysates, several experiments have been carried out to analyse the potential allergenic impact of tick allergens on the human host using a range of assays determining basophil activation within a local pool of human blood donors. Considering its homology with several house dust mite allergenic molecules, the recently identified *I. ricinus* Der-p2 allergen-like protein was also taken into account, as it may represent a putative allergen able to trigger basophil activation assessed through flow cytometric measurements.

Materials and Methods

Preparation of tick cell lysates from both IRE11 and IDE8 cell lines

Two cell lines derived from hard ticks, namely IDE8 and IRE11, both courtesy of Dr. Lesley Bell-Sakyi, Pirbright Institute, Wellcome Trust Tick Cell Biobank, were cultured and maintained in 25-cm² tissue culture flasks (PE plug seal cap, Sarstedt, Inc., Newton, USA). Cell culture medium L-15B is used as a nutrient source for IDE8 cells, whereas the L-15B300 was used for IRE11 (Munderloh and Kurtti, 1989). Leibovitz's standard medium (PAA Laboratories GmbH, Pasching, Austria) is supplemented with glutamine, glucose, minerals and vitamins (purchased from Invitrogen GmbH, Darmstadt, Germany and Sigma-Aldrich, Inc., St. Louis, MO, USA). L-15B300 is prepared by adding cell culture-grade sterile water to L-15B in a volume that amounts to 25% of additional water in the diluted medium. All tick cell lines are incubated in an atmosphere of ordinary air, in 25 or 75-cm² sealed flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) in dry incubators at 32°C. Medium was changed once in a week by the removal and replacement of between 50% and 80% of the medium volume. The cells were subcultured at 2- to 3-week intervals by adding an appropriate volume of fresh medium to the parent flask, adherent cells were removed by pipetting, and the appropriate volume of cell suspension was transferred to a new flask, leaving approximately half of the medium in the parent flask.

Following centrifugation using a Heraeus device (Heraeus Sepatech GmbH, Osterode, Germany) and after culture medium was discarded, the cells were washed in PBS (137 mM NaCl, 1.76 mM KH₂PO₄, 10 mM Na₂H-

PO₄x2H₂O, pH 7.2), resuspended in double distilled water and sonicated within 4 cycles using a Branson Sonifier (Gerhard Heinemann, Schwäbisch Gmünd, Germany). Finally, they were centrifuged at 13.000 rpm (3250 g), and the supernatant was collected.

Afterwards, the protein concentration of the samples was determined according to the Bradford method (Bradford, 1976). Absorbance was measured using an Expert 96 Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria). The lysates were diluted in PBS to obtain several protein concentrations required for performing the basophil activation test as described below.

Preparation of tick salivary gland and body lysates

Tick material was kindly provided by Dr. Maria Kazimirova, Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia. Several batches of adult *I. ricinus* females were set for feeding on laboratory rabbits for a period of 5 days. Fed ticks were dissected, and salivary glands were extracted and stored separately from the bodies at -20°C. The subsequent processing of this material was performed at Research Center Borstel, Division of Veterinary Infection Biology and Immunology. Salivary gland pools consisting of 15–20 pairs/tube, to which 600 µl sterile ddH₂O was added, were sonicated 4 times on ice, each cycle comprised 1-min sonication and 30-s break in between using a Branson sonifier (Gerhard Heinemann). Then, they were centrifuged at 16000 g for 15 min at 4°C. The pellet was again resuspended in 100 µl sterile ddH₂O and submitted to the same centrifugation step as described above. Supernatants were harvested in 1.5-ml sterile tubes, aliquoted and stored at -20°C, representing the salivary gland extract (SGE), and their protein concentration was measured as described by Bradford, 1976.

Tick bodies were homogenized as follows: a pool of 10 tick bodies were placed into a 2-ml Eppendorf tube (Eppendorf GmbH, Hamburg, Germany) and 3 metal beads were added to each tube. They were deep-frozen in liquid nitrogen for 1 min and then immediately placed into a tissue homogenizer (TissueLyser, Qiagen, Hilden, Germany), which was set to 50 oscillations/s, 5 min/cycle and the procedure was performed twice. The resulting homogenate was resuspended in 1 ml sterile ddH₂O and centrifuged twice for 5 min at 16000 g and 4°C. Protein concentration was measured according to Bradford, 1976.

IRDer-p2 allergen-like protein

IRDer-p2 from the castor bean tick *I. ricinus* belongs to a diverse family of proteins that includes major allergens of house dust mites, human MD-2 or similar proteins from *Drosophila melanogaster* (Horáková et al., 2010).

As the corresponding gene (GenBank: ABL61513.1, >gi|119220845|gb|ABL61513.1| Der-p2-like allergen [*Ixodes ricinus*]) could not be detected in the initial polymerase chain reaction (PCR) trials using the available *I. ricinus* IRE11 DNA and cDNA material, a synthetic gene construct was ordered and purchased from GeneArt AG (Life Technologies – Invitrogen GmbH). The 396-bp cDNA, without the 16 amino acid N-terminal signal peptide, was cloned into *E. coli* strain K12 (dam⁺ dcm⁺), vector backbone pMA-T, flanked by the restriction sites 1–6 BamHI [GGATCC] and 391–396 HindIII [AAGCTT]. This product was subsequently cloned into *E. coli* strain DH5α, followed by plasmid isolation and purification, after which it was inserted for expression into M15 [pREP4] strain of *E. coli* (Qiagen GmbH, Hilden, Germany).

Der-p2 allergen-like protein of *I. ricinus* was then recombinantly expressed as a 15-kDa histidine-tagged fusion protein using the pQE30 expression system (Qiagen). The isolated plasmids, following the instructions of the Analytic Jena kit (innuPREP Plasmid Mini Kit, AJ Innuscreen GmbH, Berlin, Germany), were submitted to double restriction digestion procedures in order to check for the correct clones and thus confirm the polymerase chain reaction (PCR) results. Several clones were shipped for sequencing (Eurofins MWG Operon GmbH, Ebersberg, Germany), and after confirmation of the correct sequence and reading frame of IRDer-p2 insert, a few optimization protocols were carried out in order to establish the suitable expression conditions and solubility status of the recombinant protein. This was found to aggregate within inclusion bodies, and it was subsequently purified under denaturing conditions using Ni-NTA resins (nickel-nitrilotriacetic acid agarose, Qiagen) according to the protocols and instructions of the manufacturer. For the purpose of buffer exchange and protein refolding, several centrifugal filter devices were used, namely Microcon YM-3, 3-kDa cut-off and Amicon Ultra-4, 10-kDa cut-off devices (both Amicon Bioseparations, Millipore, Tullagreen, Ireland). Elution fractions were mixed with PBS buffer supplemented with urea in decreasing concentrations starting from 6 M up to 0,5 M and finally pure PBS in various ratios. The expression and purity level of the recombinant product could be confirmed by Western blot analyses using RGS-His antibody (Qiagen). The samples were run on 15% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gels according to Laemmli, 1970 inside a vertical electrophoresis tank (Mini/Maxi vertical gel chamber, Harnischmacher, Schauenburg, Germany). Each sample was mixed with SDS sample buffer (4 times concentrated buffer: 180 mM Tris/HCl, pH 6.8; 40% (v/v) glycerol; 4% (w/v) SDS; 100 mM DTT, Roth, Karlsruhe, Germany and 0.04% (w/v) Bromophenol blue, Merck, Darmstadt, Germany) and incubated for 5 min at 95°C in

a thermoblock (Eppendorf Thermomixer compact, Eppendorf, Hamburg, Germany). 19 µl sample and 5 µl of molecular mass marker (Thermo Scientific, St. Leon-Rot, Germany) were loaded on 0.75-mm-thick gels (7.5 × 8.5 cm gel size and 0.5-cm-large wells). Protein was separated using the power supply unit EC 3000P Series 90 (E-C Apparatus Corporation, St. Petersburg, Russia) under the following conditions: amperage – 300 mA, 5 min at 50 V, 10 min at 100 V and finally 60 min at 200 V. Afterwards, the in-gel protein detection was carried on using Page Blue™ Protein Staining Solution (Thermo Scientific, Vilnius, Lithuania). The protein was electrophoretically transferred into nitrocellulose membrane (NCM, Protran BA, 0.2 µm, Schleicher & Schüll, Dassel, Germany) using the semi-dry procedure (Kyhse-Andersen, 1984) following the instructions of Millipore (Immobilon-P Transfer Membrane User Guide). A semi-dry blotter (Perfect Blue™ semi-dry Elektrobloetter, Peqlab, Erlangen, Germany) was used for this purpose, and the blotting conditions were as follows: 25 V, 60 mA, 60 min/7.5 cm gel, corresponding to 2.5 mA/cm².

The membranes were subsequently blocked with 5% skimmed milk powder in PBS (Fluka, Buchs, Switzerland) overnight at 4°C. They were next incubated with RGS-His antibody, 1 : 1000 diluted (Qiagen) for 90 min at room temperature. Next, a secondary antibody was applied, namely goat anti-mouse IgG, AP-conjugated (alkaline phosphatase-conjugated, DakoCytomation, Glostrup, Denmark), 1 : 10 000 diluted, for 2 h at room temperature. The stripes were developed with NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt, Thermo Scientific, Germany), and the reaction was stopped with dH₂O.

Basophil activation test (BAT)

All trials were conducted in 5-ml test vials (Falcon tubes, Becton Dickinson, Heidelberg, Germany), containing 70 µl aliquots of heparinized blood drawn from each of the screened donors, which were diluted with 20 µl modified RPMI medium (Biochrom AG, Berlin, Germany, without phenol red and sodium hydrogen carbonate but with 20 mM HEPES) + 10 µl stimulus and incubated in 5-ml falcon tubes for 30 min at 37°C in the presence of different concentrations (PBS dilutions) of the tick-derived stimuli.

PBS (NaCl 154 mM, Na₂HPO₄ × 2H₂O 8.03 mM, KH₂PO₄ 1.98 mM, pH 7.2) was used as a negative control. As a second negative control, recombinant *Theileria annulata* surface protein (rTaSP, 36 kDa), expressed within the above-mentioned bacterial system, was used in the case of Der-p2-stimulated samples. One of the positive controls was FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine, final concentration 1 µM), which is a peptide chain produced by some bacteria and a chemoattractant, having the

capacity to induce the activation of granulocytes (basophils, eosinophils and neutrophils) and monocytes (Calbiochem Merck, Darmstadt, Germany). The other positive control was goat anti-human IgE unconjugated antibody (Invitrogen, Germany) in a final concentration of 0.33 µg/ml.

The recombinant birch pollen allergenic protein Betv1 was included as a positive control in the case of one of the blood donors who has a clinically confirmed allergy to this particular birch pollen component.

The test was carried out by adding 5 µl of phycoerythrin-labelled CD203c monoclonal antibody 97A6 (CD203c-PE, Beckman Coulter, Krefeld, Germany) to each tube and incubating for 20 min at room temperature. As a fluorophore conjugate, its fluorescence range is enframed between 488 nm, as excitation wavelength, and 575 nm, emission wavelength. Thereafter, samples were subjected to erythrocyte lysis with 2 ml of quick lysis buffer (Medac GmbH, Hamburg, Germany) for 10 min at room temperature, and the cells were centrifuged for 6 min at 640 g (Eppendorf centrifuge, Eppendorf GmbH). The cell pellets were resuspended in 150 µl PBS and then while vortexing, a volume of 100 µl fixation buffer (3% paraformaldehyde in PBS) was added.

CD203c expression was analysed by means of flow cytometry on a FACS (fluorescence-activated cell sorting) Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) using the CellQuest Pro software (BD Biosciences, Germany). Basophils were detected on the basis of forward and side-scatter characteristics (including the typical regions of lymphocytes and monocytes) and the expression of CD203c. A number of at least 400 basophils were analysed in each sample. In all the analysed samples, a positive response was considered when the mean values of fluorescence intensities (MFI values) exceed two-fold the negative control mean (cut-off value = two-fold mean value of the negative control, marked with a red line on the graphs). Each test can be validated as reliable when at least one of the run positive controls overrides the cut-off value.

Blood from healthy human donors was obtained after getting informed consent, and all personal data, comprising their age, sex and medication status, were anonymized. The appropriate regulations of the World Medical Association Declaration of Helsinki (1975 revised) were followed in full accordance with the ethical principles. Donors were questioned regarding a history of tick bite.

Results

IRDer-p2 expression and purification

Attempts to detect transcripts of the Der-p2 allergen-like gene with reverse transcriptase PCR have not been successful to date, nor was a product amplified from genomic DNA that may represent amplification of the target

gene using the available genetic material derived from the currently cultured IRE11 tick cell line (data not shown). The gene encoding tick Der-p2 allergen-like protein was amplified from cDNA of engorged *I. ricinus* female ticks using the gene-specific primers designed on the basis of partial sequences of related allergen-like genes (Horácková et al., 2010). In ticks, genes encoding proteins belonging to this protein family were identified but their protein products have not been characterized yet. However, the IgE-binding activity of the recombinantly expressed IRDer-p2 was studied by means of ELISA and found to be expressed only within the gut and hemolymph, strongly induced by a blood meal, as shown by RT-PCR (Horácková et al., 2010). The aforementioned authors employed, though, a different expression vector, namely pET100/D-TOPO (Invitrogen, USA), for this purpose.

In our laboratories, the artificial construct was successfully cloned and expressed in the *E. coli* bacterial system, M15 [pREP4] strain. Due to the fact that the expressed protein aggregates within inclusion bodies, it was further purified under denaturing conditions following the protocols according to *The QIAexpressionist*, 06/2003 (Qiagen). Because denaturing buffers contain high urea concentrations of 8 M, eluted Der-p2 fractions were then submitted to several buffer exchange procedures in order to

obtain the soluble and refolded protein which made it suitable for downstream applications. Reasonable amounts of this expressed construct could be purified under these conditions, and refolded protein (PBS stocks) was used for the appropriate downstream applications. The correct size and approximate protein amount were verified routinely by staining with Page Blue™ Protein Staining Solution (Thermo Scientific). The presence and the quality of the his-tagged protein was checked in Western blot assays with anti-histidine antibodies, which confirmed the existence of this expression marker in all elution fractions tested so far (Fig. 1).

Basophil activation test

A number of 36 human blood donors have been tested, 28 of them had a tick-bite record (they recalled to have experienced at least one tick bite at a certain time point). This data are summarized in the table below (Table 1). For each of them, number prefixes were assigned accordingly. A number of 19 donors were bitten at least twice and 6 of them did not suffer any tick contact. The latter ones were adjudged as negative controls. Regarding the age of tested subjects, this varied between 22 and 64 years of age. Gender ratio was 13 male and 23 female (0,56 : 1 male/female sex ratio).

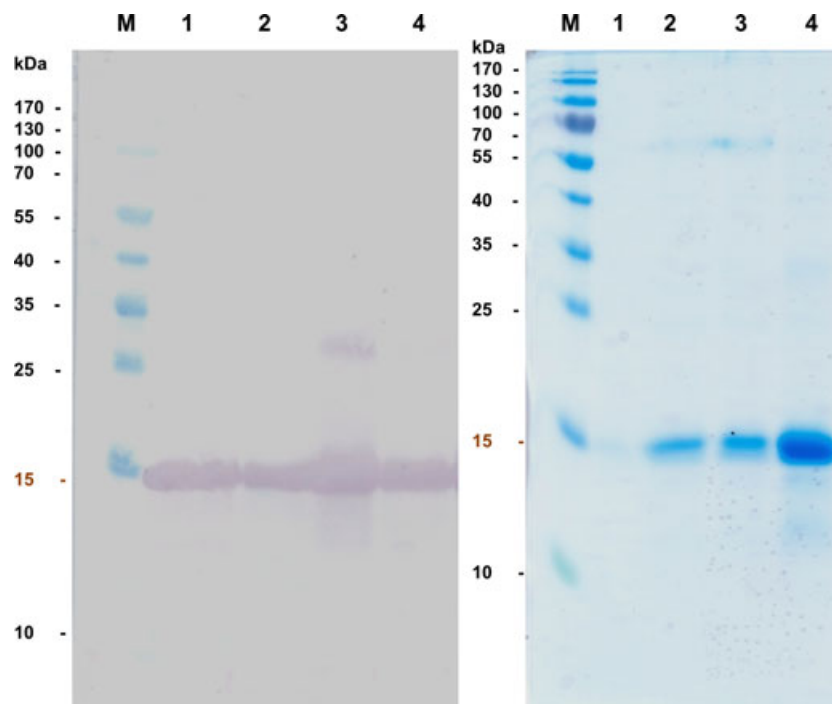


Fig. 1. Confirmation of IRDer-p2 expression through immunoblotting and Coomassie staining. IRDer-p2 expression was confirmed by immunoblotting with RGS-His antibody (Qiagen) – left and in-gel protein detection with Page Blue™ Protein Staining Solution (Thermo Scientific) – right. M – molecular mass marker (Thermo Scientific, St. Leon-Rot, Germany); 1–4 elution fractions after purification on Ni-NTA columns (IRDer-p2 size: ~15 kDa)

Table 1. Tick-bite history of the human blood donors included in the study

Blood donor (BD)	Gender		Tick bites	Reactions following the bite	Known allergies
36	Male	Female	Ranging from <1 year up to more than 20 years ago	Itching, redness of the skin bite site as well as swelling of the surrounding area in a few cases	Some of the subjects reported common allergies, mostly to grass pollen, house dust mites and insects, animal dander and nuts
	13	23			

Several stimuli concentrations along with the respective controls were used in order to evaluate their ability to induce basophil activation upon cross-linking of IgE on the basophil surface. The procedure of detecting CD203c expression on basophils by flow cytometry is depicted exemplarily in Fig. 2. All tested samples incubated with PBS as the negative control showed no significant upregulation of CD203c expression on basophils (Fig. 2a) compared to stimulation with the positive controls (such as FMLP) and upregulation of CD203c expression on basophils (Fig. 2b). Similarly, a non-bitten donor showing no reactivity to tick cell lysate is shown in Fig. 2c in comparison with a donor showing stimulation of basophils as assessed by upregulation of CD203c expression measured by an MFI significantly above the threshold (Fig. 2d). Both samples (c and d) were stimulated with 10 µg/ml cell lysate from the *I. ricinus*-derived cell line IRE11 (IR10).

The initial experiments were performed with cellular lysates from both IRE11 and IDE8 cell lines. These seemed to induce strong basophil activation, starting from a concentration of 1 µg/ml in one of the cases (BD8), but mostly at 5 and 10 µg/ml (Fig. 3a – BD8 and b – BD24). Still, one of the positively reacting subjects (BD21, single tick bite) showed no basophil reactivity towards these cell lysates (data not shown). *I. ricinus* salivary gland and whole-body lysates proved to be very potent inducers of basophilic granulocytes activation, applied in concentrations beginning from 0.5 µg/ml up to 20 µg/ml. They were able to stimulate this cell population in the case of four positively responding subjects (Fig. 3).

On the other hand, whole-blood incubation with the above-mentioned antigenic preparations did not stimulate basophils with respect to human individuals who experienced a single tick exposure (Fig. 4a). Similar results were observed for the negative control blood donors, all of

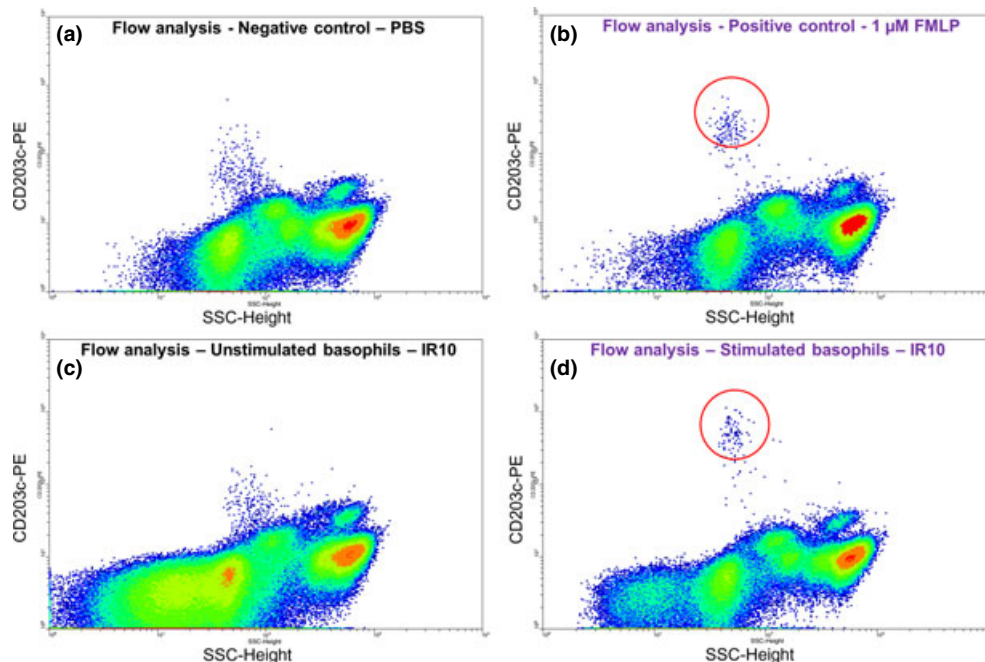


Fig. 2. CD203c expression in whole blood in the case of unstimulated cell population as well as following basophil activation upon incubation with the indicated stimuli. Flow cytometric analysis of basophil activation by assessing expression of membrane surface marker CD203c; CD203c-PE – phycoerythrin-labelled CD203c monoclonal antibody 97A6 (Beckman Coulter, Germany); SSC-Height: side-scatter; IR10– IRE11 cell line lysate, 10 µg/ml; activated basophil population was circumscribed in red – b, d. Left histograms depict resting cells, with basophils expressing low levels of CD203c. Right histograms depict activated cells, which are easily recognized on the basis of their high CD203c expression.

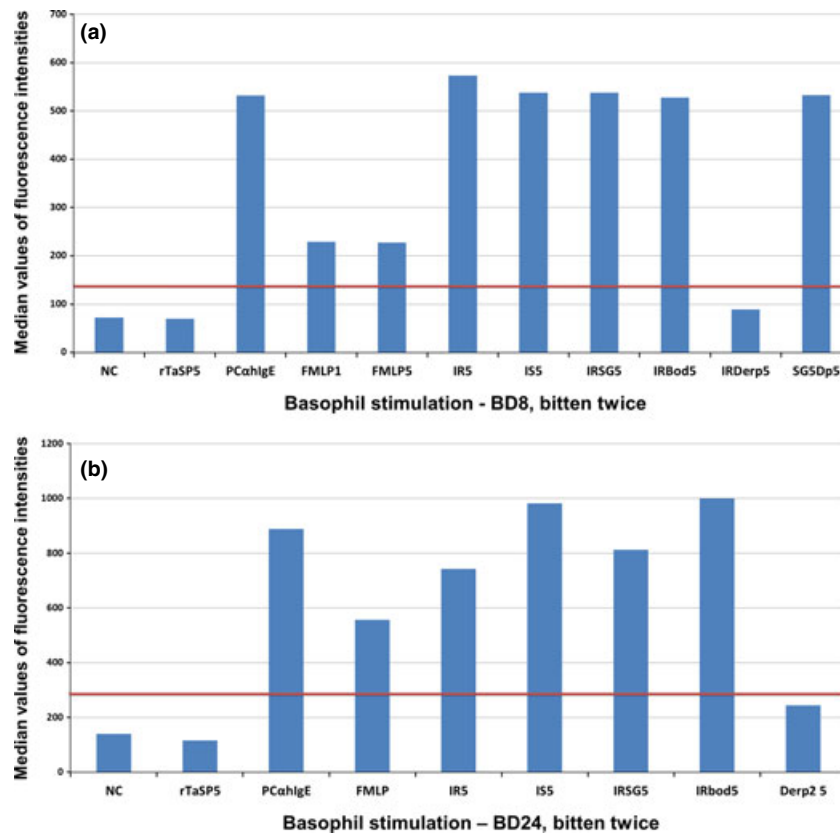


Fig. 3. Subjects being twice tick-bitten show high basophil responsiveness, as illustrated by the mean values of fluorescence intensities (MFI), after whole-blood incubation with tick antigenic lysates. Basophil activation of two blood donors (BD), twice tick-bitten, following whole-blood incubation with several stimuli: NC – negative control, PBS; recombinant *Theileria annulata* surface protein, 5 µg/ml; PCαhlGE – positive control, goat anti-human IgE antibody (Invitrogen, Germany), unconjugated, 0.33 µg/ml; FMLP1, 5 – Formyl-methionyl-leucyl-phenylalanine, 1 and 11.4 µM, respectively; IRS – IRE11 cell line lysate, 5 µg/ml; ISS – IDE8 cell line lysate, 5 µg/ml; IRSG5 – *I. ricinus* salivary gland extract, 5 µg/ml; IRBod5 – *I. ricinus* body lysate, 5 µg/ml; (IR)Der-p5 – IRDer-p2 allergen-like protein, 5 µg/ml; SG5Dp5 – *I. ricinus* salivary gland extract, 5 µg/ml + IRDer-p2, 5 µg/ml, respectively; horizontal line marks the cut-off value

whom have reported not to have contact with ticks (Fig. 4b). Nevertheless, one of the negative control individuals has showed a high basophil responsiveness following BAT procedure, maybe due to the reasons which are discussed below.

Samples incubated with recombinant IRDer-p2 alone or in combination with salivary gland extracts revealed no significant reactivity by any of the analysed human donors. The positively reacting subjects, however, mounted a dissimilar response to this stimulus. Applied in lower concentrations, such as 1 or 5 µg/ml, no CD203c upregulation could be detected, as displayed through MFI values (Fig. 3 a, b). When the concentration of this recombinant protein was increased to 20 µg/ml, a faint increment of the MFI values, slightly surpassing the set threshold, was detected for BD2, BD23 and BD24 (data not shown). Notwithstanding, double stimulation of basophils with SG and several IRDer-p2 concentrations revealed no augmentation of the assessed parameter,

irrespective of the latter's rate, as it might be deduced from Fig. 3a.

Concerning the single tick-bitten as well as the negative control donors, there was no indication of a CD203c consistent upregulation, as a result of IRDer-p2 basophil exposure, independent of its concentration (Fig. 4a, b). Mixtures of IRDer-p2 and SG, revealed, as already mentioned above, no increased stimulatory potential referring to SG alone.

It can be mentioned that rTaSP, as a recombinantly expressed protein within the same bacterial system as the studied protein, evidenced no capacity to induce upregulation of the marker taken into study.

Discussion

The study described in this article aimed to investigate whether a tick bite may induce allergic reactions in humans. Current standard diagnostic procedure of allergy

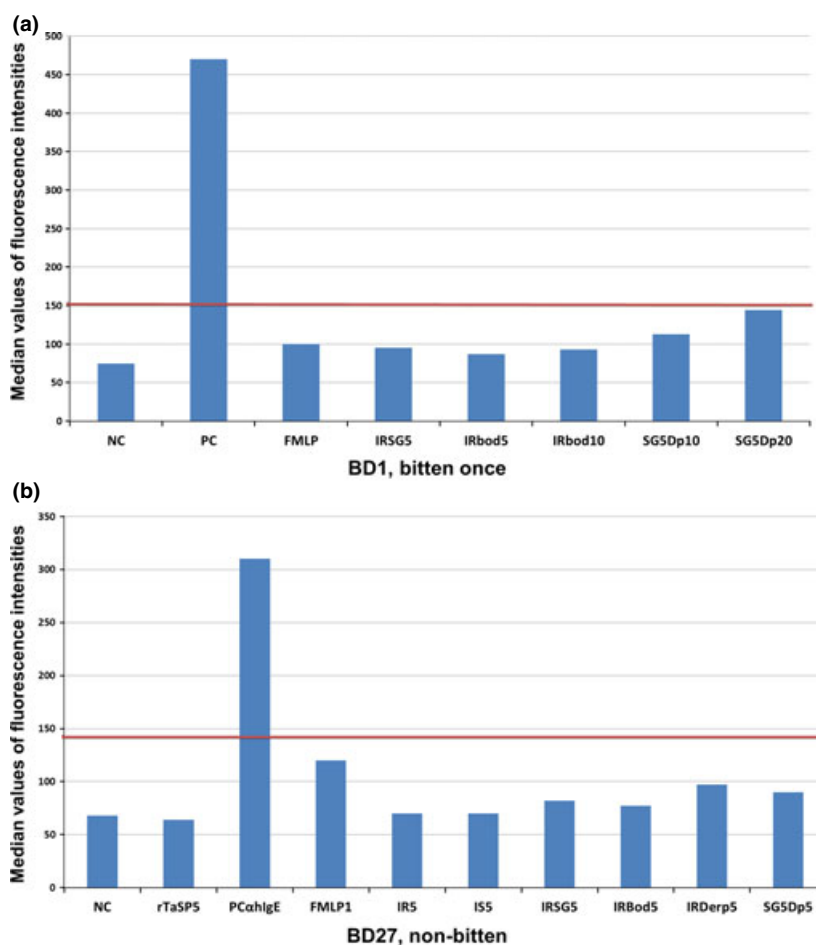


Fig. 4. Subjects who were only once tick-bitten alike the ones who recalled no tick contact show no significant basophil responsiveness, as displayed by the mean values of fluorescence intensities (MFI), after whole-blood incubation with tick antigenic lysates and IRDer-p2. No noticeable basophil stimulation could be measured following whole-blood incubation with the indicated stimuli (see Fig. 3) in the case of single tick exposures, a, or no tick contact, b; IRbod10 – *I. ricinus* body lysate, 10 µg/ml; SG5Dp10, 20 – *I. ricinus* salivary gland extract, 5 µg/ml + IRDer-p2, 10 and 20 µg/ml, respectively; horizontal line marks the cut-off value; BD – blood donor

is based on personal history, skin testing and the determination of allergen-specific IgE serum antibodies. In the last years, a considerable progress in the development of novel functional *in vitro* tests took place, and the flow cytometric basophil activation test (BAT) has been validated and is now accepted as an additional and reliable diagnostic assay. Similar to CD63, basophil CD203c expression rapidly increases after allergen challenge in sensitized individuals (Sturm et al., 2010). Thereby, this test was used for analysing basophil activation in blood from healthy human donors after stimulation with tick antigens present in cell lysates derived from 2 hard tick cell lines, along with *I. ricinus* salivary glands and whole-body lysates.

Along with the above-mentioned stimuli, IRDer-p2 from the castor bean tick was recombinantly expressed as a 15-kDa histidine-tag fusion protein, purified and included as a stimulus for our screening trials. This molecule,

belonging to a protein family possessing a lipid-recognition motif, was found to be partially related to major allergens of house dust mites. In ticks, genes encoding proteins of this family were gradually identified. Yet, their protein counterparts have not been concordantly characterized. Analysis of the expression pattern showed that the gene encoding the tick Der-p2 allergen-like protein is strongly induced by the blood meal in gut and hemolymph throughout all tick developmental stages. According to our findings, only low stimulation was observed using recombinant *I. ricinus* Der-p2 allergen-like protein as a stimulus, possibly because this protein is not present in the salivary glands and therefore not inoculated into the host during tick feeding. The presence of N-terminal signal peptide and its gene expression in the tick's gut during blood intake could be an indicator of protein expression in secretory cells and its possible secretion into the gut lumen (Inohara

and Nunez, 2002; Mullen et al., 2003; cited by Horácková et al., 2010). Another reason for the absence of stimulatory capacity of this protein could be that the recombinant protein is not correctly folded, as correct folding, meaning an appropriate tertiary structure, is required to ensure protein functionality. It might also play another role as stated by Horácková et al., namely recognition and transport of lipids or interaction with lipidic molecules of microbes entering the tick body, as it possesses a putative lipid-binding site, according to the comparisons with the related proteins. Furthermore, this protein could be involved in the neutralization of IgE antibodies taken up with blood from repeatedly tick-bitten individuals. This might represent another mechanism of clearing the host antibodies by the tick instead of involving IgG-binding proteins, according to the mechanism known from *Rhipicephalus appendiculatus* (Wang et al., 1998; Wang and Nuttall, 1999; cited by Horácková et al., 2010). Considering the expression of the gene after a blood meal, it is likely that this protein is not expressed, for example, in the investigated tick cell lines. The original primers used for isolating Der-p2 gene were designed according to the known partial nucleotide sequence of the gene encoding *I. ricinus* allergen-like protein (AJ547805). This approach might include some deviations from the normal genomic sequence, which could explain the inability to amplify the corresponding product using the genetic material of IRE11 cell line. Another argument could be that mutations may have occurred at one of the primer annealing sites, thus hampering primer annealing and synthesis of the sequence. Genetic instability has been described to be associated with some of the embryonic-derived tick cell lines (Bell-Sakyi et al., 2007).

Of the total of thirty-six donors, twenty-eight recalled to have been bitten by a tick. Only eight of them displayed a positive reaction as judged by an MFI value exceeding the set-up threshold. This might be an indication of a sensitization reaction mounted towards putative tick allergens. Eight blood donors declared they did not experience or did not remember to have suffered from a tick bite; however, two of them showed a positive reaction as pictured by their basophil activation profile. One of these donors, namely BD6, showed a slight reactivity to cell line lysates, which might be due to cross-reactivity of acarid antigens, taking into consideration his allergenic symptoms in response to house dust mites exposure. The other one, BD3, might have been tick-exposed, although he indicated no direct tick encounter or he may represent an atopic patient, therefore positively reacting to various allergenic molecules.

High basophil responsiveness to all tested stimuli could be recorded in the case of 8 tick-bitten donors of whom 7 have experienced more than one tick bite, whereas 6 of the non-bitten individuals displayed no basophil activation. The basophils of those donors, who were bitten only once,

did not show a significant response. An exception was noted for BD21, who displayed a high degree of reactivity particularly against whole-tick-body lysates. However, both cell lysates had no stimulating effect on BD21 (positively reacting donor, but only once bitten).

Another interesting observation was that 4 of the reactive donors displayed a markedly increased sensitivity towards IDE8 lysate, which may seem inconsistent with the fact that *I. scapularis* is a North American species, and the possibility of a tick bite in Europe is less likely. However, cross-reactivity has been observed by some researchers between different hard tick species and even across several tick genera and among different families of the same order (Acari) with respect to allergenic proteins (Rolla et al., 2004). On the other hand, it could well be possible that the eliciting allergens of a hypersensitivity reaction present in their natural source – tick saliva – are not necessarily expressed by the analysed tick cell lines or that they may be present in a very low amount and thus unable to induce a quantifiable stimulatory reaction that may be detected within BAT sensitivity range.

Hence, further investigations have to be conducted in order to establish a clear conclusion regarding the allergic status of the blood donors with respect to tick antigens expressed in the cell line-derived lysates, which served as one of the testing material within the frame of BAT.

In addition, several serum samples originating from the analysed donors have been already tested and screened for the presence of IgE antibodies directed against tick antigens, including IRDer-p2, through immunoblotting as well as ELISA (data not shown). The preliminary conducted experiments suggest that a meaningful IgE-driven immune response was mounted upon tick sensitization, pointed towards salivary gland components, but not against the recombinant protein.

In conclusion, basophil reactivity could be demonstrated for several human subjects who were repeatedly bitten by ticks, most probably *I. ricinus*, which is the dominant species coming across in the area of North Germany. Tick cell line, salivary gland and whole-body lysates were able to induce upregulation of basophil surface marker CD203c via cross-linking of the FcεRI-bound IgE, as it was quantified through flow cytometric analyses. This may be regarded as an indication of human host sensitization occurring as a result of tick bites. Surprisingly, high upregulation levels of the analysed marker could be detected even in the case of persons who experienced tick bites traced back as far as 4–10 years. In this case, the persistence of such an immune response could be explained by the existence of memory T and B lymphocytes, which make sure that basophils will be continuously kept active and be capable of exerting their role as effector cells upon encounter of a previous stimulus. On the other hand, the recombinantly expressed

construct seem to possess no ability of inducing basophil activation, and therefore, it should not be considered a true allergen, as it was also suggested by the authors initially isolating its sequence.

Acknowledgements

Authors wish to thank Dr. Maria Kazimirova, Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia, for providing us with most of the hard tick samples and Dr. Lesley Bell-Sakyi from the University of Edinburgh, currently at Pirbright Institute, UK, for supplying us with the tick cell lines, representing biological material of the Tick Cell Biobank. We acknowledge here also Dr. Ulrike Munderloh, University of Minnesota, USA, who firstly initiated these tick cell lines. We are very grateful for all the support provided by the technical staff from Research Center Borstel, of both the Division of Veterinary Infection Biology and Immunology and Division of Immune Cell-Analytics, respectively. We are also thankful to Carola Schneider, Division of Biochemical Immunology, for organizing the blood donations.

First author is a PhD student supported by the POSTICK ITN (Post-graduate training network for capacity building to control ticks and tick-borne diseases) within the FP7-PEOPLE – ITN programme (EU Grant No. 238511). The current project has received community research funding.

Conflicts of interest

The authors have no conflicts of interest to declare.

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