

Determination of the T cell epitopes of the lipocalin allergen, Rat n 1

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Summary

Background Laboratory animal allergy (LAA) is an important cause of occupational sensitization and asthma. Rats are a frequent cause of LAA and the major rat allergen, Rat n 1, is a member of the lipocalin protein family, which includes several other animal allergens such as the cow allergen, Bos d 2. To date, Bos d 2 is the only mammalian lipocalin allergen to have been studied in detail.

Objective We undertook a cross-sectional study of a large population of individuals exposed to laboratory rats to determine the proliferative responses of peripheral blood mononuclear cells (PBMCs) to the major rat allergen, Rat n 1.

Methods Eighty-three cases (defined by a positive skin prick test (SPT) ≥ 3 mm and/or a positive RAST $\geq 2\%$ binding) and 274 referents without specific IgE to rats were tested for their proliferative responses of PBMCs to rat allergen. Cytokine release to rat urinary protein was examined in 28 sensitized and 42 non-sensitized exposed individuals.

Results Proliferation to rat urinary protein was weak in all individuals. Four regions within Rat n 1 were identified as containing potential immunodominant T cell epitopes and three of these co-localized within the conserved regions of the lipocalin molecule. All four regions within Rat n 1 overlapped considerably with the characterized epitopes of the lipocalin allergen, Bos d 2. IL-5 and ratios of IL-5/IFN- γ were significantly increased in cases.

Conclusion The response to Rat n 1 is remarkably similar to the cow lipocalin allergen Bos d 2. T cell epitopes within lipocalins appear to co-localize with the conserved regions of the molecule. LAA is characterized by an increased production of IL-5. Investigation of other lipocalin allergens will provide further information about the allergenicity of this group of proteins.

Keywords cytokines, epitopes, laboratory animal allergy, lipocalins, occupational allergy, Rat n 1, T cells

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Introduction

Acquired allergy to laboratory animal proteins – particularly those derived from rats – remains an important cause of occupational sensitization and asthma. The size of the exposed population in the UK is approximately 15000; similar numbers are likely in many countries with an active scientific community.

The annual incidence of the disease among the UK research community is estimated to be 1.56/1000 employees [1] with a prevalence of specific IgE sensitization of about 16% [2].

Rat n 1, the major allergenic protein from rats is well characterized [3] and belongs to the lipocalin family of proteins [4]. Lipocalins encompass a group of functionally and structurally related proteins and are predominantly carrier molecules involved in the transport of hydrophobic ligands. Several major allergens are members of the lipocalin

family including those from the mouse (Mus m 1) [4], dog (Can f 1 and Can f 2) [5], horse (Equ c 1) [6], cow (Bos d 2 and Bos d 5) [7, 8] and rabbit (18 and 21 kDa salivary proteins) [9]. The factors, which give rise to so many lipocalins becoming inducers of allergy, are as yet unclear.

Because allergen-specific T-helper (Th) cells of the Th2 subset are involved in IgE synthesis, the hallmark of allergic disease, information regarding allergen-specific T lymphocytes and the precise T cell epitopes they recognize is critical for understanding allergenicity. To date very little is known about the human T cell responses to lipocalins. Bos d 2, the major cow allergen, is the only mammalian lipocalin to have been studied in detail at the cellular level. This allergen was shown to induce an intense clinical allergy with a strong IgE response, but with a surprisingly weak T cell proliferation [10]. T cell epitopes were detected within three conserved regions of Bos d 2, suggesting that lipocalin animal allergens may contain only few T cell epitopes, concentrated in the conserved regions of the molecule.

In the present study we examined the peripheral blood mononuclear cell (PBMC) responses to rat urinary protein and the cytokine release patterns in sensitized and non-sensitized employees working with laboratory rats.

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Materials and Methods

Subjects

Between September 1999 and May 2001 we undertook a cross-sectional survey of 776 employees working with laboratory animals at six UK pharmaceutical sites. The ethics committee of the Royal Brompton Hospital/NHLI approved the study, and written informed consent was obtained from all volunteers. Any current employee identified from health surveillance registers as having had at least 1 month's contact with rat proteins was considered eligible for the study ($n = 718$). Cases were defined as those individuals with a positive skin prick test (SPT) (≥ 3 mm) and/or a positive RAST ($\geq 2\%$ binding) [11] to rat urinary allergens. We tested the proliferative response of PBMCs to rat urine, Rat n 1 and overlapping peptides of Rat n 1 in 83 cases (72% of those eligible) and 274 referents (45% of those eligible) without evidence of rat-specific IgE sensitization. We also tested an additional 37 persons without exposure to laboratory animal proteins (all SPT and RAST negative) to rat urinary protein and overlapping peptides of Rat n 1.

Skin prick tests and symptoms

For SPTs we used physiological saline, histamine (1% solution) (Allergopharma, Reinbek, Germany), and in-house extracts of rat and mouse urines (1 mg/mL dissolved in phosphate buffered saline containing 50% v/v glycerol and filtered through a sterile 0.2 μ m filter (Acrodisc, Pall Corp., Ann Arbor, MI, USA)). Weal sizes were recorded after 10 min by measuring the mean of their longest and midpoint orthogonal internal diameters. Atopy was defined as a positive response to tests with one or more extracts of cat, grass pollen, or *Dermatophagoides pteronyssinus* (Allergopharma). Inter-observer differences were minimized by ensuring that the coefficient of variability of weal sizes, for each tester, was less than 20% for 16 repeated positive histamine controls [12]. We used a questionnaire to enquire into work-related chest and nose symptoms, defined as those occurring at work and/or improving when away from work. Of the case population tested 77% of individuals possessed nose and/or chest symptoms whereas only 14% of referents had any symptoms.

Measurement of specific immunoglobulin E

Specific IgE to an extract of rat urine was measured through use of a RAST. Briefly 3 mg of lyophilized rat urine (collected from Wistar rats) was coupled to 300 mg of activated discs by the method of Ceska et al. [13]. We incubated the sera (50 μ L) at room temperature overnight with discs in duplicate. Discs were washed and bound allergen was detected by adding 50 μ L ^{125} I anti-IgE (Pharmacia and Upjohn, Milton Keynes, UK). After 16 h incubation at room temperature, the discs were washed and radioactivity counted on a γ scintillation counter (COBRA auto- γ counter; Canberra Packard, Pangbourne, Berks, UK). Results are expressed as the percentage binding of the total ^{125} I anti-IgE added. A percentage binding of $\geq 2\%$ was considered positive [11].

Rat urine preparation

Urine was collected from male Wistar rats by the use of a metabolic cage (Techniplast, Varese, Italy) [14]. The pooled urine was filtered (grade 1 paper, Whatman International Ltd, Maidstone, Kent, UK) and concentrated with the use of an Amicon CH2A hollow-fibre system and dialyzed against distilled water and freeze dried and then stored at -20°C . We used the crude rat urine extract (w/v) for skin prick testing, measurement of specific IgE (RAST) and in primary T cell cultures.

Rat n 1

Rat urine was further purified by extensive dialysis against 2×6 L of distilled water per day over 3 days until a white precipitate was visible. Dialysis was carried out at 4°C . The precipitate was collected by high-speed centrifugation (11 000 r.p.m. for 45 min) and resuspended in distilled water and freeze dried. Purity of protein content was evaluated by means of SDS PAGE analysis, which indicated a reasonable level of purity as there were two molecular weight bands visible at 17 kDa [14]. We used the Rat n 1 extract (w/v) in primary T cell cultures.

Synthetic peptides

Thirty-eight 16-mer peptides offset by four amino acids and spanning the entire sequence of Rat n 1 were synthesized by Chiron Mimotopes (Clayton, Vic., Australia). Lyophilized peptides were dissolved in 0.1% acetic acid/water to give a concentration of 1 mg/mL. The peptides (all soluble) were then pooled into pairs, i.e. 1+2, 3+4, etc., to give a set of 19 peptide pools covering 20 amino acids in length and overlapping by eight amino acids (Table 1).

Table 1. Amino acid sequence and numbering of peptide pools

Peptide pool	Amino acid sequence	Amino acid sequence numbering
1	EEASSTRGNLDVAKLNGDWF	1–20
2	NLDVAKLNGDWFVIVASNK	9–28
3	GDWFSIVVASNKREKIEENG	17–36
4	ASNKREKIEENGSMRVFMQH	25–44
5	EENGSMRVFMQHDVLENSL	33–52
6	FMQHDVLENSLGFKFRIKE	41–60
7	ENSLGFKFRIKENGECRELY	49–68
8	RIKENGECRELYLVAYKTPE	57–76
9	RELYLVAYKTPEDGEYFVEY	65–84
10	KTPEDGEYFVEYDGGNTFTI	73–92
11	FVEYDGGNTFTILKTDYDRY	81–100
12	TFTILKTDYDRYVMFHILNF	89–108
13	YDRYVMFHILNFKNGETFQL	97–116
14	LINFKNGETFQLMVLVGRTK	105–124
15	TFQLMVLVGRTKDLSSDIKE	113–132
16	GRTKDLSSDIKEKFAKLCEA	121–140
17	DIKEKFAKLCEAHGITRDN	129–148
18	LCEAHGITRDNIDLTKTDR	137–156
19	RDNIIDLTKTDRCLQARG	145–162

Proliferation assays

Proliferation to rat urinary protein and Rat n 1 were optimized in terms of time of incubation and antigen concentration. PBMCs were separated on Ficoll paque (Amersham Pharmacia Biotech, Uppsala, Sweden) by density gradient centrifugation. Isolated cells were washed and resuspended in RPMI 1640 media (Life Technologies, Paisley, UK) supplemented with 5% human AB serum (Sigma, Poole, Dorset, UK), 2 mM L-Glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin (Life Technologies) and seeded out at 2×10^{-5} cells/well in triplicate in a final volume of 200 µL in round-bottomed 96-well microtitre plates (Nunc, VWR International Ltd, UK). For a positive control we used phytohaemagglutinin (PHA) (Life Technologies, Gibco, Paisley, UK) at three different dilutions (1:10, 1:100, and 1:1000). Rat urine and Rat n 1 were added at a concentration of 100 µg/mL and the pooled peptides at a concentration of 5 µg/mL each. The cultures were incubated for 7 days in a humidified 5% CO₂ incubator at 37 °C, then pulsed for 16 h with 5 mCi of [³H]thymidine per well (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The cells were harvested onto filter paper using an automated cell harvester (Perkin-Elmer, Wellesley, USA) and radionuclide uptake was measured by scintillation counting on a β counter (Perkin-Elmer) and the results expressed as a stimulation index (SI: ratio between the mean counts per minute (c.p.m.) in cultures stimulated with antigen, and the mean c.p.m. in cultures without antigen). We used a stimulation index of over 3 as a positive result.

Measurement of cytokine production

We collected 100 µL of supernatants from PBMC proliferation assays on day 7 and stored them at -70 °C until cytokine analysis was carried out. Cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ) were measured from the PBMC cultures of 28 cases, 42 referents and 18 non-exposed controls using a cytometric bead array (CBA) kit according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Acquisition of sample data was carried out using a FACS calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and results were analysed using BD CBA analysis software. Cytokine measurements to Rat n 1 were similar to rat urinary protein in the seven subjects where both rat urinary protein and Rat n 1 were analysed, we therefore chose to restrict our cytokine analysis to pooled supernatants from rat urinary protein. Cytokine measurements were also carried out for unstimulated cultures but in most cases were barely detectable.

Statistical analysis

The χ^2 test was used to compare the percentage of cases and referents responding to each of the 19 peptide pools. Kruskal-Wallis tests were used to compare cytokine concentrations and stimulation indices with rat urine, Rat n 1 and peptides of Rat n 1 between cases, referents and non-exposed controls; pairwise comparisons between cases and referents for cytokine concentrations and stimulation indices and cases and non-exposed controls for cytokine concentrations were undertaken using the Mann-Whitney *U*-test.

Correlation between proliferation and cytokine release was analysed using the Spearman rank test. SAS (SAS Institute Inc., Cary, NC, USA) and GraphPad InStat version 3 (San Diego, CA, USA) statistical software packages were used for these analyses.

Results

Magnitude of the peripheral blood mononuclear cell proliferative responses in cases and referents to rat urine and Rat n 1

The proliferative responses of PBMCs to rat urinary protein and Rat n 1 were weak. Cases showed the highest proliferation with stimulation indices ranging from 0.1 to 37.8 (median = 1.9) for rat urinary protein and 0.01 to 22.2 (median = 1.3) for Rat n 1 (Figs 1 and 2). There was little difference in the magnitude of the proliferative response to rat

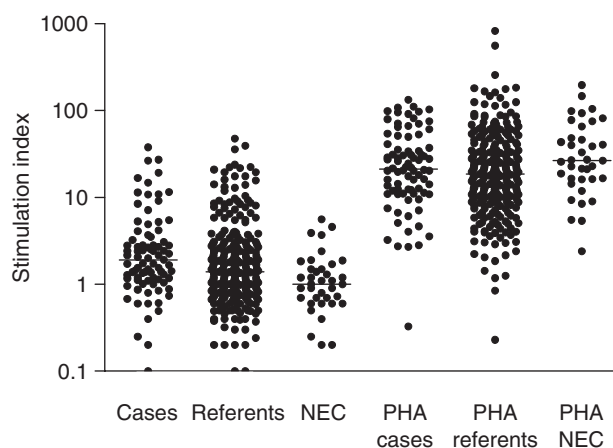


Fig. 1. Proliferative responses of peripheral blood mononuclear cells to rat urine in cases ($n = 83$), referents ($n = 274$) and non-exposed controls (NEC) ($n = 37$). Responses to phytohaemagglutinin (PHA) are also shown for all three groups. Horizontal lines represent median values. (Range of proliferation for unstimulated cultures = 87–8604 c.p.m.)

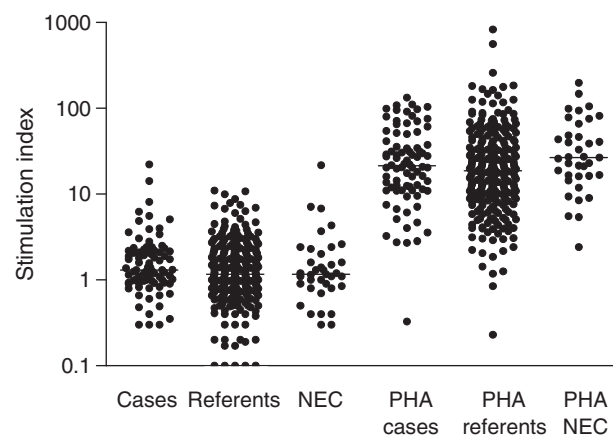


Fig. 2. Proliferative responses of peripheral blood mononuclear cells (PBMCs) to Rat n 1 in cases ($n = 83$), referents ($n = 274$), and non-exposed controls (NEC) ($n = 37$). Responses to phytohaemagglutinin (PHA) are also shown for all three groups. Horizontal lines represent median values. (Range of proliferation for unstimulated cultures = 87–8604 c.p.m.)

urinary protein in cases (median stimulation index 1.9) compared with referents (median = 1.4); however, this difference did achieve statistical significance ($P = 0.02$). No difference was seen in the response to Rat n 1 between the two groups. Vigorous proliferative responses were seen with PHA for cases and referents (median = 21.2 and 18.7, respectively).

Comparison of peripheral blood mononuclear cell responses to Rat n 1 overlapping peptide pools in cases, referents and non-exposed controls

Stimulation indices to overlapping synthetic peptides of Rat n 1 were low and there was a diverse peptide recognition pattern between individuals. None of the peptide pools elicited a proliferative T cell response in all individuals indicating that the peptides were not mitogenic. On the whole the proliferation to overlapping peptides of Rat n 1 was similar between cases and referents in terms of the magnitude of the proliferative response and the prevalence of individuals responding to the 19 peptide pools. However, the frequency of cases responding to peptide pool 7 was borderline

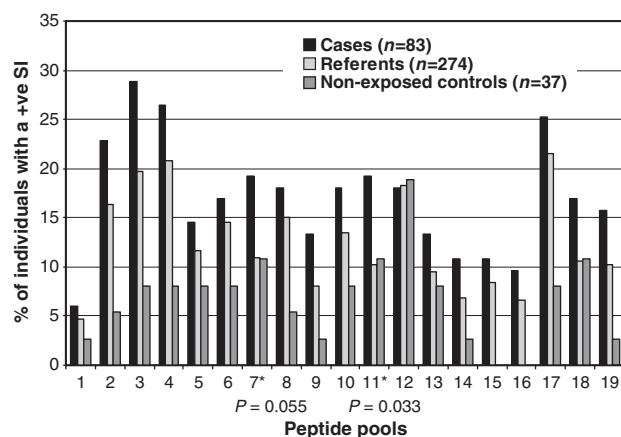


Fig. 3. Frequency of cases ($n = 83$), referents ($n = 274$) and non-exposed controls ($n = 37$) with a positive stimulation index (SI) (>3) to overlapping peptide pools of Rat n 1. P -values are for differences between cases and referents. Asterisks indicate differences between the frequency of cases and referents with a positive SI to the peptide pools.

significantly increased ($P = 0.055$) compared with referents and significantly increased for peptide pool 11 ($P = 0.03$) (Fig. 3).

The unexposed controls responded to overlapping Rat n 1 peptide pools in a similar manner to the exposed cases and referents with the frequency of positive proliferative responses being greatest for the same peptide pools that induced the highest frequency of responses in cases and referents (Fig. 3).

There was little difference in the magnitude of the proliferative responses to overlapping peptide pools of Rat n 1 between cases and referents. Cases had a significantly increased magnitude of response to peptides 1 (median = 1.42) and 5 (median = 1.5) compared with referents (peptide 1, 1.2, peptide 5, 1.27) $P = 0.02$ and 0.01 , respectively. Four areas within Rat n 1 (peptide pools 2–4, 6–8, 10–12, 17–19, Fig. 3) induced a high frequency of positive responses in individuals. The peptide pools within the four areas that stimulated the highest number of positive T cell responses, namely peptide pools 3, 7, 11 and 17, were similar to the previously defined epitopes A, C, E and G of Bos d 2 [10] (Fig. 4). Peptide pools 3, 11 and 17 of Rat n 1 were localized within the structurally conserved regions as defined by Flower [4].

Cytokine concentrations of peripheral blood mononuclear cells cultures from cases, referents and non-exposed controls

Levels of IL-5 were significantly increased in the PBMC culture supernatants from cases (median = 13.2 pg/mL) compared with those from both referents (median = 1.7 pg/mL) and non-exposed controls (median = 0 pg/mL), $P = 0.003$ and $P < 0.001$, respectively (Fig. 5).

Correlation between proliferation to rat urine and IL-5 production was analysed and a significant correlation was found in cases ($r = 0.65$, $P < 0.001$) and to a lesser extent in referents ($r = 0.47$, $P = 0.003$).

The concentration of IFN- γ in PBMC culture supernatants was considerably higher than for any of the other cytokines measured. IFN- γ levels tended to be higher in cases

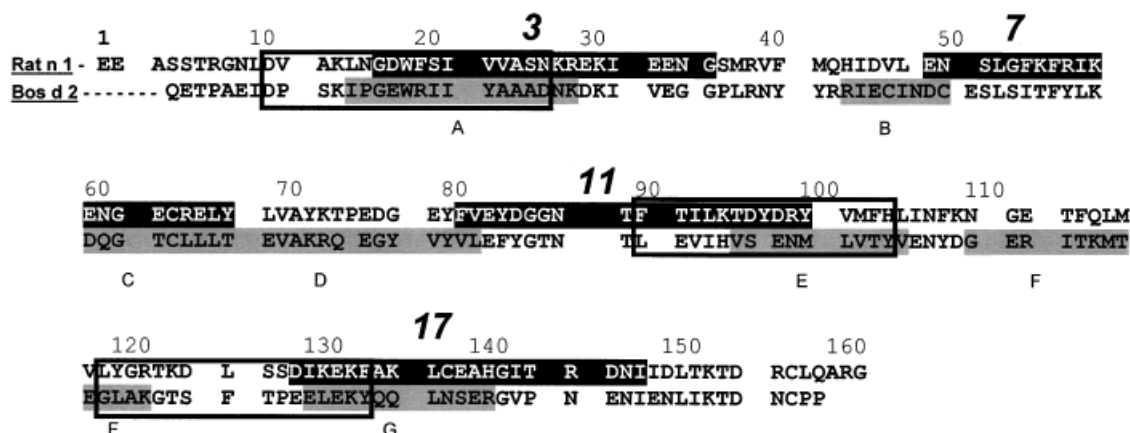


Fig. 4. Sequence alignment of Rat n 1 with Bos d 2. The boxed regions denote the three structurally conserved regions for the lipocalins. The experimentally verified T cell reactive regions of Rat n 1 determined using peripheral blood mononuclear cells are shown as numbered shaded areas. Bold italic numbers represent the Rat n 1 peptide pools. Amino acid numbering of Rat n 1 is shown directly above the sequence. Verified epitopes of Bos d 2 are shown as shaded areas labelled A–G.

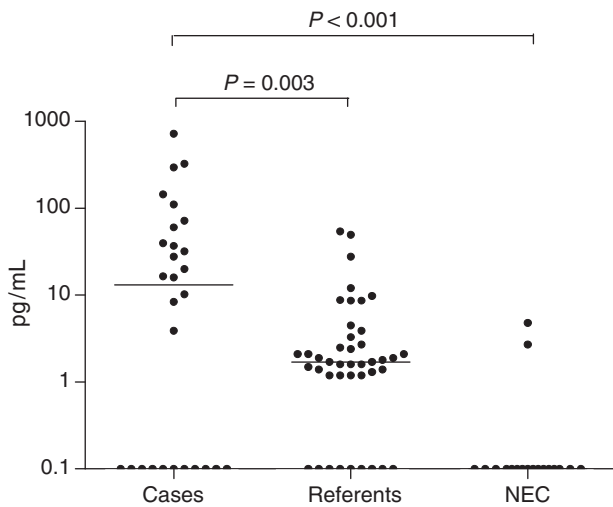


Fig. 5. Concentrations of IL-5 in the supernatants of cultures stimulated with rat urinary protein from cases ($n = 28$), referents ($n = 42$) and non-exposed controls (NEC) ($n = 18$). Horizontal lines represent median values.

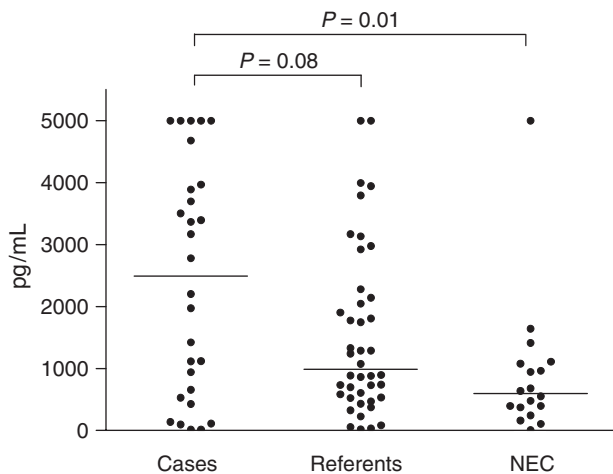


Fig. 6. Concentrations of IFN- γ in the supernatants of cultures stimulated with rat urinary protein from cases ($n = 28$), referents ($n = 42$) and non-exposed controls (NEC) ($n = 18$). Horizontal lines represent median values.

(median = 2492.4 pg/mL) compared with referents (median = 985.7 pg/mL, $P > 0.05$) and non-exposed controls (median = 596.5 pg/mL, $P = 0.01$) (Fig. 6). A significant correlation between proliferation and IFN- γ production was found for cases ($r = 0.4$, $P = 0.04$) and referents ($r = 0.5$, $P = 0.001$).

The ratios of IL-5 to IFN- γ were low; but significant differences between cases and referents were seen. The median ratio of IL-5 to IFN- γ was 0.008 (range 0–1.0) in cases and 0.002 (range 0–0.15) in referents (Fig. 7). This difference was highly significant ($P = 0.001$) indicating the cases were producing higher concentrations of IL-5 in relation to IFN- γ than referents.

IL-4 production was barely detectable in the majority of samples (median = 0 pg/mL) and IL-10 levels were low in both cases (median = 9.8 pg/mL) and referents (median = 7.2 pg/mL), with no significant difference between the groups. TNF- α levels were detectable in both cases and referents, although there was no difference between the two groups (data not shown).

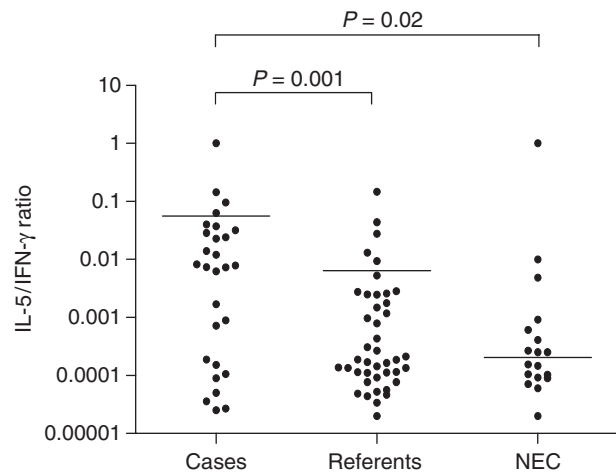


Fig. 7. Ratios of IL-5/IFN- γ in cases ($n = 28$), referents ($n = 42$) and non-exposed controls (NEC) ($n = 18$). Horizontal lines represent median values.

Cytokine levels were compared in atopic ($n = 38$) and non-atopic ($n = 31$) individuals (data not shown). No significant differences were observed between the levels of any of the cytokines analysed between the two groups.

Discussion

This study is the first to investigate, in detail, the cellular responses to rat urine, Rat n 1 and overlapping peptides of Rat n 1 in individuals exposed to laboratory animals in the workplace. One of the most striking features of the study was the similar cellular response to the major rat and cow allergens, namely Rat n 1 and Bos d 2, of individuals exposed to these allergens. Both allergens elicit only a weak PBMC response in cases and referents, in contrast to the high levels of specific IgE antibody in cases. There was also a remarkable parallel between both allergens in the regions likely to contain immunodominant T cell epitopes. The four regions identified in Rat n 1, which induced the highest frequency of responses, overlapped with epitopes A, C, E and G of Bos d 2 [10]. Three of these regions were found within the structurally conserved regions of both Rat n 1 and Bos d 2 lipocalin allergens.

The structurally conserved regions of lipocalin molecules appear to be important in eliciting a T cell response. Our study was not designed to fine map T cell epitopes because of the fact that the peptides were pooled; however, the conserved regions of the Rat n 1 molecule (peptide pools 3, 7 and 11) are those inducing a more frequent response in individuals exposed to rats. The frequency of cases responding to peptide pools 7 and 11 of Rat n 1, localized within the conserved region, was increased compared with referents.

Within Bos d 2, epitope G was regarded as immunodominant as all patients with cow allergy recognized this epitope [10]. Another lipocalin molecule, Bos d 5, the major allergen of cow's milk, has also been shown to have a T cell epitope within the equivalent region of epitope G of Bos d 2 [15, 16]. The similarities in the cellular studies to lipocalin allergens to date, suggest that other lipocalin allergens may provoke a similar allergic response and contain epitopes in the structurally conserved regions of the molecule. Indeed T cell

clones raised to mouse urinary allergen have been found to respond to rat urinary allergen [17], suggesting cross reactivity at the T cell level between these two lipocalins.

The dichotomous response to both Rat n 1 and Bos d 2 is intriguing, as the IgE production is intense but the cellular response is particularly weak. Zeiler et al. have suggested that endogenous lipocalins could be involved in the modulation of the immune response [10, 18, 19]. Self-reactive T cells to endogenous lipocalins, which have escaped thymic deletion, but are under peripheral regulatory mechanisms, may recognize epitopes on exogenous lipocalins but in an inefficient way. This may result in sub-optimal T cell receptor-mediated activation of the T cells resulting in deviated production of cytokines. There was some evidence of sub-optimal recognition of Bos d 2, as a peptide analogue of an immunodominant peptide was able to induce a 100-fold greater proliferation response in a T cell clone [20]. We, however, were unable to detect any T cell response to the endogenous lipocalin retinol binding protein in rat-sensitized individuals (data not shown).

Despite the weak proliferative responses to rat urinary allergen in PBMCs, we have demonstrated a significantly higher IL-5 production in relation to IFN- γ , in cases compared with referents. IL-5 production is a key mediator in the pathogenesis of asthma and allergic disease and enhanced IL-5 has been shown to correlate directly with disease severity and airway hyper-responsiveness, its production is regarded as a characteristic feature of asthma [21, 22]. We ruled out the possibility that IL-5 was associated with atopy, a known risk factor for laboratory animal allergy (LAA) [23], as there was no difference in the IL-5 level between atopics and non-atopics. Thus despite weak proliferative responses, IL-5 production was increased in LAA cases in comparison with their referents.

Previous studies with other lipocalin molecules, Bos d 2 and Bos d 5 have detected cytokine responses with T cell clones and lines. The majority of T cell clones (55%) to Bos d 2 were classified as Th2-like clones, secreting IL-4, IL-5 and low IFN- γ , whereas 37% secreted IL-4, IL-5 and IFN- γ , and were classified as Th0-like clones [10]. Although in a study of cow's milk hypersensitivity where Bos d 5 is a major allergen, enhancement of IFN- γ and IL-4-secreting cells in cow's milk-sensitive patients, compared with control subjects, was seen in the lamina propria; however, the number of cells secreting IFN- γ were 10-fold larger than cells secreting IL-4, showing a dominance of Th1-type responses in these individuals [24].

In the current study, despite the increased ratio of IL-5:IFN- γ in LAA cases compared with referents, there was a high level of IFN- γ production. Production of this archetypal Th1 cytokine has been shown to be common for a variety of allergens [25–27]. The presence of endotoxins in our rat urinary extract were considered as a possible factor in the production of high IFN- γ levels; nevertheless, there was evidence that allergen-driven cytokine release also occurred that was different between cases and referents.

In conclusion, we have demonstrated that the response to Rat n 1 is similar to that seen with Bos d 2 with strong IgE production and a weak proliferative response. Despite weak proliferative responses, we were able to demonstrate an increased IL-5:IFN- γ ratio in cases compared with referents. The similarities between Rat n 1 and Bos d 2 are intriguing.

Further work is required to determine whether other lipocalin animal allergens, including mouse, rabbit, dog, horse and cow may provoke similar allergic responses and, like Rat n 1 and Bos d 2, contain epitopes within their conserved regions. These studies may further our understanding of the allergenicity of animal lipocalin molecules and have implications for a common therapeutic strategy.

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