

A mouse model for *in vivo* tracking of the major dust mite allergen Der p 2 after inhalation

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Keywords

allergy; Der p 2; house dust mite; protein labelling; selenocysteine

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(Received 15 February 2005, revised 2 May 2005, accepted 12 May 2005)

doi:10.1111/j.1742-4658.2005.04764.x

Inhaled environmental antigens, i.e. allergens, cause allergic symptoms in millions of patients worldwide. As little is known about the fate of an allergen upon inhalation, we addressed this issue for a major dust mite allergen, Der p 2. First, a model for Der p 2-sensitization was established in C57BL/6 J mice, in which sensitized mice mounted a Der p 2-specific IgE-response with eosinophilic lung inflammation after allergen challenge in the airways. In this model, we applied recombinant Der p 2 carrying a novel C-terminal tetrapeptide Sel-tag enabling labelling with the gamma-emitting radionuclide ⁷⁵Se at a single selenocysteine residue ([⁷⁵Se]Der p 2). *In vivo* tracking of intratracheally administered [⁷⁵Se]Der p 2 using whole-body autoradiography revealed that [⁷⁵Se]Der p 2-derived radioactivity persisted in the lungs of sensitized mice as long as 48 h. Radioactivity was also detected in kidneys, liver and in enlarged lung-associated lymph nodes. Interestingly, a larger proportion of radioactivity was found in the lungs of sensitized compared with nonsensitized mice 24 h after intratracheal instillation of [⁷⁵Se]Der p 2. A radioactive protein corresponding to intact Der p 2 could only be detected in the lungs, whereas [⁷⁵Se]Der p 2-derived radioactivity was recovered in known selenoproteins both in lung and other organs. Hence, using the recently developed Sel-tag method in a mouse model for Der p 2-sensitization, we could track the fate of an inhaled allergen *in vivo*. Based upon our findings, we conclude that the inflammatory state of the lung influences the rate of metabolism and clearance of Der p 2. Thus, an allergic response to the inhaled allergen may lead to prolonged retention of Der p 2 in the lung.

The respiratory mucosa is exposed to a wide range of antigens, pathogens as well as harmless substances. It is of major importance that the homeostasis in the airway mucosa is maintained in order to prevent respiratory infections as well as allergic manifestations. However, in an increasing proportion of the population in industrialized countries, a number of common

airborne antigens, e.g. pollen, furred animal dander and dust mites induce allergic reactions when inhaled. Why these specific antigens, defined as allergens on the basis of their capacity to induce an immunoglobulin (Ig) E-response, are particularly prone to elicit allergic symptoms is not known. Factors like solubility in the mucosa, low dose exposure, protein stability and

Abbreviations

BAL, bronchoalveolar lavage; GPx1, glutathione peroxidase 1; HDM, house dust mite; i.p., intraperitoneal; i.t., intratracheal; OVA, chicken egg albumin; TrxR1, thioredoxin reductase 1.

intrinsic biological properties of the allergens may all contribute to their allergenicity [1–4]. The intrinsic properties required for evoking an allergic immune response has only been thoroughly studied for a limited number of allergens. House dust mites (HDM), which are a common cause of allergic disease worldwide [5,6] specifically promote allergic T helper (Th) 2-driven inflammation by different mechanisms, e.g. a direct effect on lung macrophages [7] and mast cells [8]. A major HDM allergen, Der p 1, which is a cysteine protease has been shown to modulate both the adaptive and innate immune system *in vitro* and *in vivo* [9–14]. In addition, Der p 1 might contribute to HDM sensitization by degrading the airway epithelial barrier, as it was found to disrupt tight junctions and increase permeability in confluent monolayers of epithelial cells [15]. All these activities may contribute to the allergenicity of HDM by favouring a pro-inflammatory environment in the airways. In the case of most allergens though, including other dust mite allergens such as Der p 2, detailed investigations on how protein function contributes to allergenicity are still lacking. Thus, studies aiming at an understanding of how airborne allergens interact with the airway mucosa and the immune system after inhalation are of crucial importance.

Mice are used widely for *in vivo* models of allergy and asthma [16]. Common protocols for sensitizing mice involve immunization with allergen together with aluminium hydroxide followed by allergen challenge in the airways. The allergic response is usually characterized by allergen-specific IgE antibodies, eosinophilic inflammation in the lungs and a Th2-type of T-cell response to the sensitizing allergen. Although the relevance of experimental mouse models as a description for human allergic disease may be questioned, they offer excellent tools for studying the effects of allergens *in vivo* in their natural target organs [17]. In the present study, a mouse model for sensitization to a major HDM allergen, Der p 2, was established.

Technically it is generally difficult to follow the *in vivo* clearance and turnover of an allergen after inhalation. In this study we used a novel approach for specific labelling of proteins in order to investigate how an airborne allergen, Der p 2, is deposited in the airways of mice and metabolized. The labelling method involves the incorporation of a selenocysteine residue and the gamma-emitter selenium-75 (⁷⁵Se) within an engineered C-terminal tetrapeptide motif designated as a Sel-tag [18]. The metabolism of ⁷⁵Se-labelled proteins can readily be followed, as ⁷⁵Se is only incorporated into a limited number of defined mammalian selenoproteins [19,20]. Recombinant

Der p 2 with a Sel-tag was hence radioactively labelled ([⁷⁵Se]Der p 2) and instilled into the trachea of mice that had previously been exposed to HDM extract in aerosol by inhalation. The HDM extract corresponds to the naturally encountered allergen, i.e. *Dermatophagoides pteronyssinus* whole mites, and consists of all mite components, including the major allergens Der p 1 and Der p 2 [21,22]. In order to assess if Der p 2 is differentially processed *in vivo* depending on if the mice were sensitized to Der p 2 or not prior to instillation, the established mouse model for Der p 2 sensitization was applied and the tracking of [⁷⁵Se]Der p 2 was performed in sensitized, as well as nonsensitized mice. To our knowledge, this is the first report on *in vivo* tracking after intratracheal (i.t.) administration of an airborne allergen relevant for human allergic disease. The fate of Der p 2 was followed both at the whole-body level by autoradiography and at the molecular level by protein analysis of mouse tissues.

Results

Der p 2 sensitization and allergen challenge

Groups of C57BL/6 mice were injected twice intraperitoneally (i.p.) with recombinant Der p 2 followed by challenge three times with aerosolized HDM extract (Fig. 1A). Bronchoalveolar lavage (BAL) was performed 18 h after the last aerosol challenge and the leukocytes were differentially counted to determine the magnitude of allergic airway inflammation. Compared with nonsensitized mice, the sensitized animals showed an increased number of leukocytes in BAL fluid, of which 40–80% were eosinophils after receiving HDM aerosol (Fig. 1B). The nontreated healthy animals showed only a small number of leukocytes in BAL fluid, < 300 000 cells and the amount of eosinophils was less than 5% (data not shown). Challenge with HDM extract in nonsensitized mice caused no airway inflammation since the numbers of recovered cells in BAL fluid was similar to the numbers in untreated animals. No signs of inflammation were detected in lungs from mice sensitized with chicken egg albumin (OVA) and exposed to HDM aerosol, demonstrating that the airway response was dependent on a specific sensitization against Der p 2 (data not shown).

Sensitization to Der p 2 was monitored in serum by analysis of Der p 2-specific IgE antibodies. Sensitized and challenged mice displayed a Der p 2-specific IgE response, while nonimmunized control animals showed IgE-levels in the same range as the background (Fig. 1C).

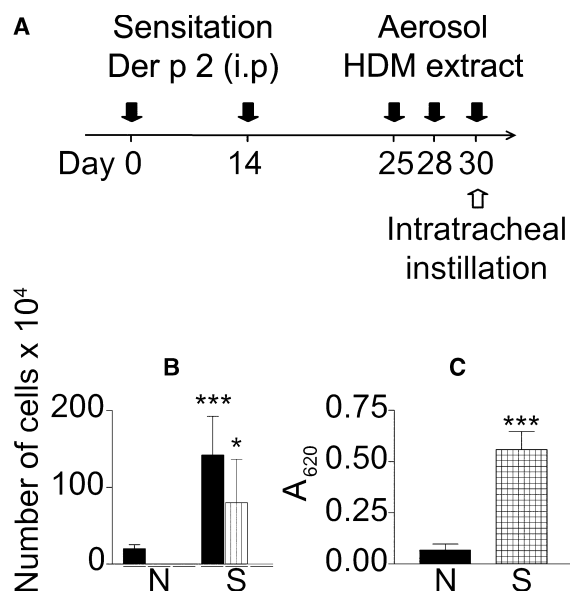


Fig. 1. The mouse model. (A) Immunization and challenge protocol for the mouse model. C57BL/6 J mice were given 1 μg of Der p 2 adsorbed to aluminium hydroxide i.p. at day 0 and 14. The mice were challenged three times with house dust mite (HDM) extract aerosol at day 25, 28 and 30. Alternatively, in Der p 2 tracking experiments the mice received an i.t. instillation of ^{75}Se Der p 2 on day 30. (B) Airway inflammation in sensitized mice. The number of total leukocytes (solid bar), eosinophils (striped bar) and neutrophils (open bar, at baseline) in bronchoalveolar lavage fluid from mice sensitized twice with 1 μg of Der p 2 and given three aerosol challenges with HDM extract was analyzed 18 h after the last challenge. Non-sensitized mice received no other treatment than the HDM aerosol challenge. (C) Der p 2 specific IgE responses. Analysis of Der p 2 specific IgE in serum (diluted 1 : 3) from C57BL/6 J mice sensitized twice with 1 μg of Der p 2 and given aerosol challenge three times with HDM extract. Non-sensitized mice received no other treatment than the HDM aerosol. N, nonsensitized mice; S, sensitized mice. Mean values $\pm$ standard deviation (SD) shown ($n = 5$). * $P < 0.05$, *** $P < 0.001$ by unpaired Student's t -test (two-tailed) for sensitized vs. nonsensitized mice.

Radioactivity persisted in lungs of sensitized mice after 48 h

The time-dependence of the tissue distribution of radioactive mite allergen in sensitized mice was assessed. Six mice were given an i.t. instillation of ^{75}Se Der p 2 instead of the last aerosol challenge at day 30. Analysis of BAL fluid from mice instilled with nonlabelled Der p 2 at day 30 displayed an airway inflammation 18 h after treatment, in the same magnitude as in animals challenged with a third HDM aerosol on day 30 (data not shown). The animals were killed after different time points (6, 24 and 48 h) and the radioactivity was tracked by whole-body autoradiography (Fig. 2). The highest levels of radioactivity

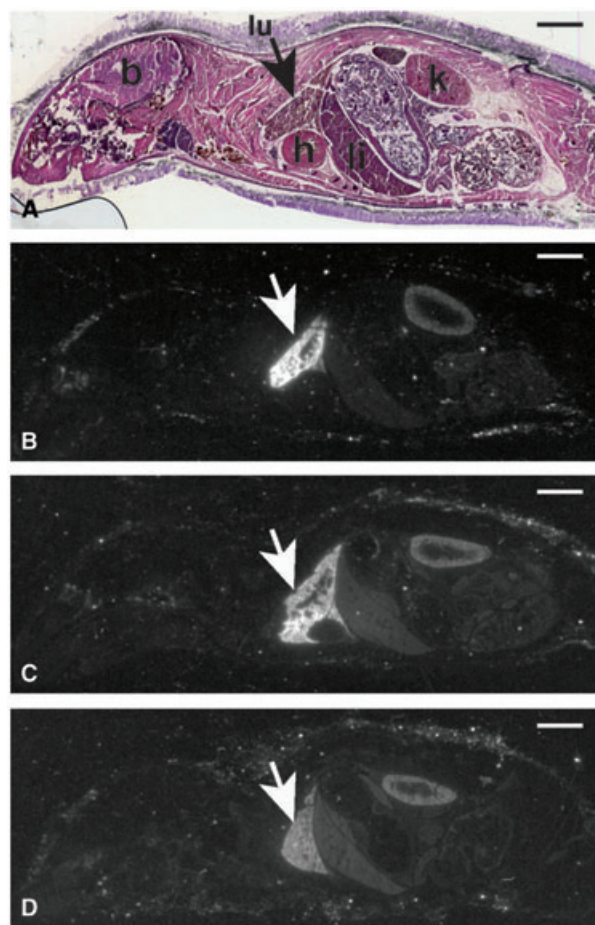


Fig. 2. Tracking of ^{75}Se Der p 2 at the whole body-level. Sagittal tape-section whole-body autoradiography of Der p 2-sensitized mice at different time points after i.t. instillation of ^{75}Se Der p 2 (21 μg ; 0.13 μCi -mouse $^{-1}$). Top panel (A) shows a hematoxylin/eosin stained tape-section that corresponds to the autoradiogram in (B). Autoradiograms are from mice killed 6 h (B), 24 h (C) and 48 h (D) after i.t. instillation of ^{75}Se Der p 2. White areas correspond to high levels of radioactivity. Tissues indicated: lu, lung; k, kidney; li, liver; h, heart; b, brain. Bars correspond to 5 mm.

were found in lung, kidney cortex and liver. At the earlier time points the lung showed the strongest radioactivity labelling. The radioactivity decreased with time and at 48 h the radioactivity detected in lung was approximately of the same intensity as that of the kidney cortex. Separate radioactivity labelled enlarged lung-associated lymph nodes were identified in mice killed after 24 and 48 h (data not shown). There was no radioactivity found in blood or heart tissue at any of the time points studied and no major differences were found between the duplicate animals at each time point. Based on this experiment and earlier published studies showing that fluorescence derived from fluorescein isothiocyanate (FITC)-labelled OVA

accumulates in airway-derived lymph node dendritic cells with a peak fluorescence labelling 8–24 h after i.t. instillation of FITC-OVA [23,24], we chose the 24 h time point for a closer evaluation of the tissue distribution of [^{75}Se]Der p 2 upon i.t. administration.

Tissue distribution of radioactivity in sensitized and nonsensitized mice

Sensitized and nonsensitized mice received an i.t. instillation of [^{75}Se]Der p 2, instead of the last aerosol challenge at day 30 and all mice were killed 24 h later. On whole-body autoradiogram the radioactivity pattern was similar to the result from the initial time-dependence experiment at the time point of 24 h. Thus, the radioactivity was detected mainly in lungs, kidney cortex and liver, and at low levels in spleen. Only in the sensitized mice could an enlarged, radioactively labelled, lung-associated lymph node structure be found (Fig. 3).

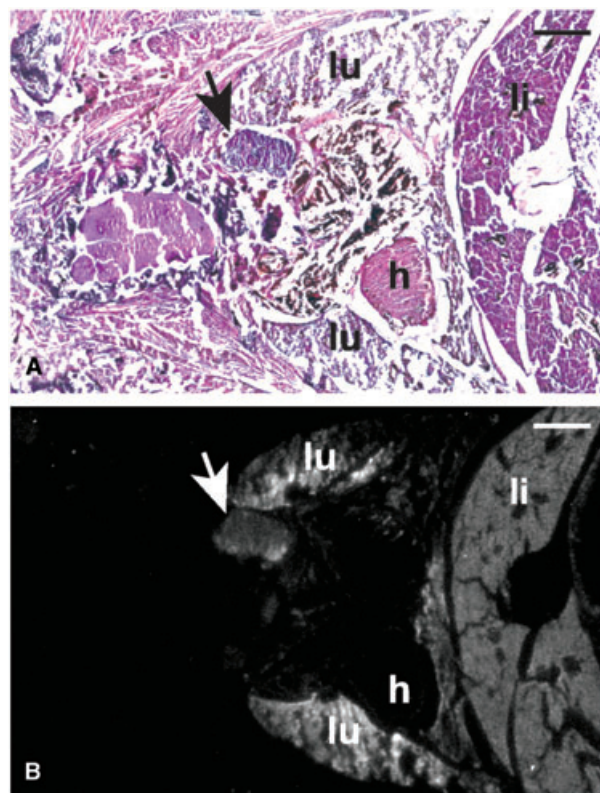


Fig. 3. Labelling of an airway-associated lymph node in Der p 2-sensitized mice. Horizontal tape-section of a Der p 2-sensitized mouse 24 h after an i.t. instillation of [^{75}Se]Der p 2 (7 μg ; 1 μCi). (A) shows a hematoxylin/eosin stained tape-section that corresponds to the autoradiogram in (B). White areas correspond to high levels of radioactivity. Tissues indicated: lu, lung; li, liver; h, heart. The arrow points at an enlarged thoracic lymph node containing radioactivity. Bars correspond to 2 mm.

Light microscopic autoradiography of lung sections confirmed the observation from whole-body sectioning that the radioactivity was evenly distributed in the lung tissue of both sensitized and nonsensitized mice. Silver grains were observed in both alveolar and bronchiolar tissue, as well as in the airway lumen (Fig. 4). In this context, it should be noted that no radioactivity could be seen in the trachea or larger bronchi, as shown on whole-body autoradiograms (Figs 2 and 3). In addition, an increased number of eosinophils were observed in lung interstitium of sensitized mice, confirming the eosinophilic response following Der p 2 challenge (Fig. 4).

The tissue levels of radioactivity differ between sensitized and nonsensitized mice

Isolated mouse tissues (lungs, kidneys, liver, spleen and thoracic lymph nodes) were homogenized and analyzed for total protein content and radioactivity. In agreement with the results from whole-body autoradiography, thoracic lymph nodes were not enlarged in nonsensitized animals and were thus only possible to isolate from sensitized mice. These lung-associated lymph nodes were found to contain radioactivity. It was clear from the quantitative analysis of radioactivity in the tissues that a significantly larger proportion of radioactivity was present in lungs of sensitized mice compared with nonsensitized animals. When comparing the distribution of radioactivity between lung and kidney in sensitized mice 4.5 times higher (mean ratio, $n = 5$) radioactivity was found in lung than in kidney, while in nonsensitized mice the ratio between radioactivity in lung and kidney was close to 1 (mean ratio, $n = 5$) (Table 1). In contrast the distribution of radioactivity between kidney and liver did not differ significantly between sensitized and nonsensitized animals (Table 1). To assess the nature of the radioactivity in the tissues, size fractioning by gel filtration was performed, showing that essentially all radioactivity was eluted in the protein fractions whereas no radioactivity was detected in the low molecular weight fractions (data not shown).

As the radioactivity shown in Table 1 corresponds to high molecular weight fractions in the gel filtration analysis, we performed SDS/PAGE followed by autoradiography as a qualitative analysis of ^{75}Se -labelled proteins in the different mouse tissues. A comparison between lung and kidney samples revealed dissimilar patterns of radioactively labelled proteins in these two organs (Fig. 5). In the lung, protein bands corresponding to estimated molecular weights of 56, 25 and 16 kDa were detected, while in kidney only a 25 kDa

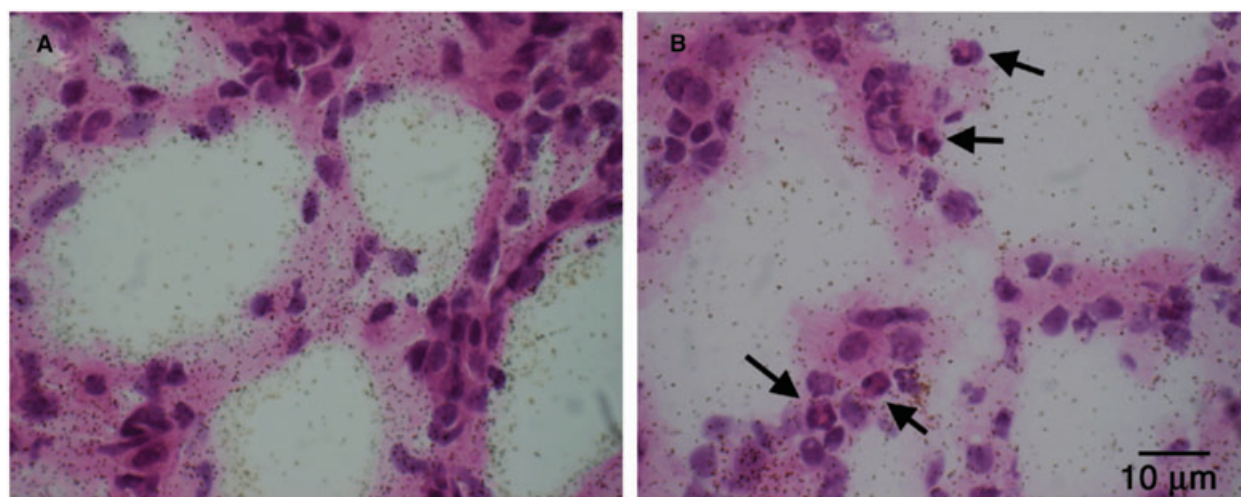


Fig. 4. Airway inflammation and distribution of [^{75}Se]Der p 2 in lung tissue as shown by light microscopic autoradiograms. Sections of lung tissue from a nonsensitized (A) and a sensitized (B) C57BL/6 J mouse 24 h after an i.t. instillation of [^{75}Se]Der p 2. The tissues were processed for light microscopic autoradiography and radioactivity visualized by the dark silver grains. The tissue sections were stained with hematoxylin/eosin. Eosinophils are indicated by arrows in (B).

Table 1. Distribution of radioactivity in tissues. Tissues were isolated from sensitized ($n = 5$) and nonsensitized ($n = 4$) mice, given [^{75}Se]Der p 2 i.t. 24 h before killed. The radioactivity per mg of total protein in each tissue was measured and the ratio between lung/kidney and liver/kidney was determined in sensitized and nonsensitized mice. Mean values $\pm$ SD are shown. * $P < 0.05$ by unpaired Student's *t*-test (two-tailed) for sensitized vs. nonsensitized mice.

	Ratio	
	Lung/kidney ($n = 5$; mean $\pm$ SD)	Liver/kidney ($n = 4$; mean $\pm$ SD)
Sensitized	$4.6 \pm 3.5^*$	0.8 ± 0.6
Non-sensitized	0.9 ± 0.2	1.2 ± 0.2

band was clearly visible (Fig. 5B). The 16 kDa protein migrated in the gel identically to [^{75}Se]Der p 2 and this band could only be detected in the lung. Autoradiograms of separated liver and thoracic lymph node proteins revealed a radioactive band of ≈ 25 kDa in liver and bands of ≈ 30 and 56 kDa in thoracic lymph nodes (Fig. 5C). No radioactive protein bands could be detected in spleen samples. An attempt was made to identify the 16 kDa protein found in lung with anti-Der p 2 Igs by western blot analysis. However, due to the lower sensitivity of this method compared with autoradiography, it was not possible to detect the 16 kDa protein by western blot. We could in fact show that autoradiography of SDS/PAGE is at least 10

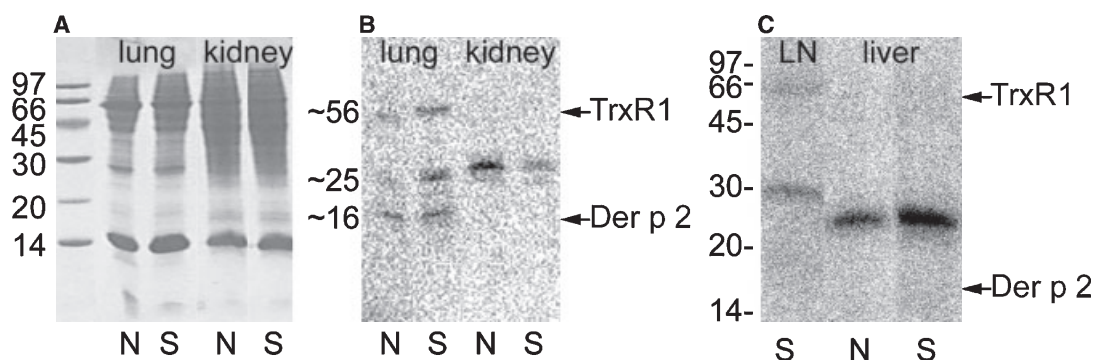


Fig. 5. Radioactive proteins in mouse tissues. Homogenized tissues from mice that had received [^{75}Se]Der p 2 i.t. 24 h before being killed were run on SDS/PAGE. Proteins were stained with Coomassie and radioactive protein bands were visualized by autoradiography. Lung and kidney proteins from sensitized and nonsensitized mice are shown on a Coomassie-stained gel (A) and autoradiogram (B) of the same SDS/PAGE. Proteins from lymph node (LN) of a sensitized mouse (one representative experiment out of two) and liver of sensitized and nonsensitized mice shown by SDS/PAGE autoradiogram (C). The positions for recombinant ^{75}Se -labelled rat TrxR1 and [^{75}Se]Der p 2 on SDS/PAGE are indicated. N, nonsensitized mice; S, sensitized mice.

times more sensitive than western analysis for detecting [^{75}Se]Der p 2.

Discussion

In this study, we tracked a major HDM allergen, Der p 2, after deposition in the airways of Der p 2-sensitized and nonsensitized mice. The fate of the allergen could be followed *in vivo* both at the whole-body level and at the molecular level, through the application of a newly developed technique for specific labelling of recombinant proteins by means of incorporating a radioactive selenocysteine residue in a C-terminal Sel-tag [18].

Der p 2 carrying the Sel-tag had an intact core sequence and maintained allergen-specific IgE-binding epitopes and the use of a Sel-tag enabled labelling with the gamma-emitting radionuclide ^{75}Se at a single pre-defined selenocysteine residue ([^{75}Se]Der p 2) [18]. This is the first example of an *in vivo* application of a protein produced by this novel labelling procedure. The advantage of this labelling method over, e.g. chemical ligation of radioactive or fluorescent probes to proteins is that the metabolism of ^{75}Se -labelled proteins can readily be followed through identification of newly synthesized selenoproteins, as the major endogenous murine selenoproteins are few and relatively well characterized [19,20]. We demonstrate here that the Sel-tag can be used for qualitative assessments both by whole-body autoradiography, light-microscopic autoradiography of tissue sections and SDS/PAGE analysis of tissue proteins containing the labelled selenocysteine.

In order to track the Der p 2 allergen in sensitized mice a mouse model for sensitization with Der p 2 was established. In contrast to OVA, which is commonly used in mouse allergy models, Der p 2 represents a major inhalant allergen causing allergic symptoms, in particular allergic asthma, in many patients world-wide [5,6]. The mice were immunized twice with Der p 2 followed by challenge with HDM extract in the airways. Thus, in this model the mice were sensitized to a specific HDM allergen, Der p 2, and then exposed to whole HDM extract, mimicking inhalation of the natural allergen. The fact that i.t. instillation of [^{75}Se]Der p 2 led to deposition of radioactivity in alveoli and bronchioli with no detectable allergen remaining in the trachea demonstrates that the model is suitable for studies of inhaled allergens. The i.t. instillation route was used to minimize the loss of [^{75}Se]Der p 2 during the exposure. Although this administration technique does not entirely represent physiological inhalation of airborne allergens, the even distribution of [^{75}Se]Der p 2 in the lower airways as demonstrated in

our tracking experiments indicates that the deposition is similar to more physiological inhalation routes.

The main finding in this study was that i.t. administered Der p 2 becomes differently distributed in the tissues depending on if the mouse was presensitized or not. A larger proportion of radioactivity was detected in the lungs of sensitized mice than in nonsensitized animals. The distribution of radioactivity in the other investigated organs did not differ due to the sensitization. Thus, the allergen-induced airway inflammation in sensitized mice apparently leads to an increased local retention and an altered metabolism of the inhaled allergen. This effect may result from interactions between allergen and inflammatory cells present in the inflamed airways and possibly add to the allergenic properties of HDM. In this context it is interesting to note that exposure to HDM allergens has been shown to be associated both with HDM sensitization and disease severity [25–27].

Administration of protein antigens generally results in uptake by dendritic cells, followed by antigen presentation to the immune system. It has been shown that the turnover of airway dendritic cells is influenced by the inflammatory state of the lungs [24] and that these cells play an essential role both in the induction and maintenance of allergen-driven eosinophilic airway inflammation [28–30]. Trafficking of dendritic cells to the airways and the lung epithelium was also demonstrated to be dramatically increased in mice with an allergic airway inflammation, partly due to induced activity of matrix metalloproteinase-9 [31]. In addition, we have previously demonstrated increased levels of B-cells and allergen-specific IgG and IgA antibodies in BAL fluid of mice with established allergic inflammation [32]. Thus, it is evident that the inflammatory condition is associated with enhanced capability to bind the antigen through extracellular immunoglobulins and a more efficient cellular uptake through antigen-presenting pathways. This provides an immunity-based hypothesis for an increased retention of the allergen in the lungs. However, the observed retention may also be due to a disturbed physiological clearance of inhaled proteins in sensitized animals.

Antigens are transported by dendritic cells from the airway mucosa to thoracic lymph nodes with a peak appearance of antigen-derived label 24 h after administration of labelled antigen [23]. In accordance with these findings we found that lung-associated lymph nodes were radioactively labelled 24 h after i.t. instillation of [^{75}Se]Der p 2. However, only a small fraction of radioactivity was recovered in lung-associated lymph node structures compared with lung, liver and kidney. As only the C-terminal tetrapeptide of Der p 2

contained ^{75}Se , it is possible that partly degraded Der p 2 was taken up, processed and presented as peptides by dendritic cells in lymph nodes. Different mouse strains react to different Der p 2-derived peptides but in C57BL/6 mice peptides spanning the entire sequence, in particular the N-terminal part of Der p 2, have been shown to stimulate T-cell responses [33,34]. The C-terminal tetrapeptide of Sel-tagged Der p 2 containing selenocysteine might not be presented by the major histocompatibility complex (MHC) but rather metabolized into selenocysteine-containing proteins. This is consistent with our finding of high-molecular weight radiolabelled proteins in the lymph nodes.

All radioactivity extracted from tissues was found in protein fractions. When analyzed on SDS/PAGE followed by autoradiography distinct radioactive protein bands were noticed. The pattern of labelled protein bands differed between the tissues. Only in lung a 16 kDa protein band was detected 24 h after i.t. administration of [^{75}Se]Der p 2. The 16 kDa band, which could be observed in lung tissue from both sensitized and nonsensitized mice most likely corresponded to nondegraded [^{75}Se]Der p 2 as it had the same mobility on SDS/PAGE as Sel-tagged Der p 2. This assumption is also supported by other studies reporting that no selenoproteins with the same molecular mass as Der p 2 have been identified in mice [35,36]. The detection of intact Der p 2 in the lungs implies that a fraction of Der p 2 had not been processed by antigen-presenting cells or metabolized 24 h after inhalation. Thus Der p 2 remained nondegraded for a remarkably long time period in the lung and this prolonged allergen exposure of the lung tissue might contribute to the ability of Der p 2 to promote allergic inflammation. The other protein bands detected in lung, kidney, liver and lymph nodes displayed higher molecular masses than Der p 2. Except for the 30 kDa protein in lymph nodes, they all correspond to molecular masses of easily identified known selenoproteins. There are 25 mammalian selenoproteins identified [20]. The two prominent selenoproteins in most major mouse tissues are thioredoxin reductase 1 (TrxR1) and glutathione peroxidase 1 (GPx1), with molecular weights of 57 and 25 kDa, respectively [20], corresponding to the radioactive protein bands detected in our study. Furthermore, ^{75}Se -labelling of normal mouse tissues and separation by SDS/PAGE has previously revealed the 25 kDa GPx1 to be by far the most abundant selenoprotein in liver and kidney [35,36], in agreement with our labelling of these tissues. Thus, metabolic degradation of [^{75}Se]Der p 2 and incorporation of the liberated ^{75}Se into newly synthesized selenoproteins appears to have occurred within

24 h in all tissues examined. The higher relative retention of radioactivity in the lungs of sensitized mice compared with nonsensitized (Table 1) was thereby derived from both remaining intact ^{75}Se -labelled Der p 2 as well as newly synthesized TrxR1 and GPx1 (Fig. 5B), perhaps at increased levels as a result of the inflammatory process. The levels of the latter enzymes, that can only have been synthesized utilizing selenium derived from the administered [^{75}Se]Der p2, indicate together with its own remaining intact levels (the 16 kDa band) that the clearance and further metabolism of the allergen was altered as a result of the inflammation in the lungs of sensitized animals.

Up to now there are few data available on the fate of an allergen after inhalation. In this study, we tracked inhaled Der p 2 *in vivo* using the recently developed Sel-tag method in a mouse model for Der p 2-sensitization. Based upon our findings we conclude that the inflammatory state of the lung influence on the rate of metabolism and clearance of Der p 2. Thus, an allergic response may lead to prolonged retention of the allergen in the airways. This raises the possibility that a vicious circle is triggered, yielding enhanced lung exposure to inhaled Der p 2 in sensitized subjects, which thereby may contribute to the observed clinical severity and persistence of allergy to HDM allergens [5,6].

Experimental procedures

Mice

All experiments were performed using female C57BL/6 J mice 8–10 weeks old when experiments were initiated. The mice, originally obtained from Jackson Laboratories (Bar Harbor, ME, USA), were bred in the animal facility at the Swedish Defence Research Agency (FOI NBC Defence), Umeå, Sweden, and fed with standard chow and water *ad libitum*. The study was approved by the Regional Animal Research Ethics Committee according to national laws.

Preparation of allergen

House dust mite extract was prepared from *D. pteronyssinus* mites (obtained from Allergon AB, Ängelholm, Sweden) as described previously [37]. The HDM extract contained 2 ng Der p 2 per mg total protein, as determined by ELISA (Mite2 ELISA kit, Indoor Biotechnologies, UK; performed according to the instructions provided by the manufacturers), and 14.3 ng endotoxins per mg total protein, measured by a Limulus Amebocyte Lysate Endochrome assay (Charles River Endosafe, Charleston, SC, USA).

His₆-tagged recombinant Der p 2 was expressed in *Escherichia coli* as described previously [18] and purified from solubilized inclusion bodies by affinity chromatography using TALON metal affinity resin (Clontech Laboratories Inc, Palo Alto, CA, USA) followed by dialysis against NaCl/P_i, pH 7.4. For purification from endotoxins a Detoxi-Gel™ Endotoxin Removing Gel (Pierce, Rockford, IL, USA) was used according to the manufacturer's protocol with 0.2 M NaCl in NaCl/P_i pH 7.4 as buffer. Subsequently the endotoxin content was determined using Limulus Amebocyte Lysate Endochrome assay (Charles River Endosafe) and was found to be 12.3 ng endotoxins per mg Der p 2. Samples were filtrated through a 0.2 µm sterile filter (MILLIPORE, Molsheim, France) before given to the mice.

Recombinant Sel-tagged Der p 2 was produced essentially as described previously [18], with the exception of the induction step, which here was performed at late exponential phase at an D_{600} of 2.4 to increase the efficiency of selenocysteine incorporation [38]. In the case of ^{75}Se -labelling, 0.75–1.5 mCi isotope (^{75}Se), approximately 1500 mCi·mg⁻¹ Se, obtained from the Research Reactor Center, University of Missouri-Columbia) was added to 50–100 mL culture medium. Radio-labelled or nonlabelled Sel-tagged Der p 2 protein was purified from solubilized desalted inclusion bodies either by gel filtration using a Sephadex G50 column (Amersham Pharmacia Biotech, Uppsala, Sweden) and NaCl/P_i pH 7.4 buffer or an affinity chromatography method developed for Sel-tagged proteins, applying phenyl arsine oxide sepharose, which bind specifically to the selenenylsulfide motif of the Sel-tag [18]. The fractions were assayed for protein content with Coomassie-stained 8–16% SDS/PAGE and samples containing a Der p 2 protein band were collected. The radioactivity was determined using a gamma counter (Cobra II Auto-Gamma, Packard Instrument Company, Meriden, CT, USA). The labelled allergen (^{75}Se Der p 2) was purified from endotoxins and prepared for *in vivo* application in the same way as His₆-tagged Der p 2.

Sensitization and aerosol challenge

Mice were sensitized to Der p 2 employing a sensitization procedure that was modified after a method for OVA-sensitization previously described by Svensson *et al.* [32]. In brief, mice were sensitized with 200 µL His₆-tagged Der p 2 adsorbed to aluminium hydroxide gel (1 : 3) i.p. at day 0 and 14. Two doses of Der p 2, 0.2 or 1 µg per mouse, were initially evaluated for sensitization but the 1 µg dose was chosen for the subsequent experiments since this dose gave more stable responses for Der p 2-specific serum-IgE and cell infiltrates in BAL fluid. On days 25, 28 and 30 mice were challenged in the lungs by inhalation of aerosolized HDM extract using a nose-only Batelle exposure chamber. Aerosols were generated by a compressed-air nebulizer

(Collision 6-jet) at an airflow of 7 L·min⁻¹ using a nebulizer concentration of 2.5 mg protein·mL⁻¹ dissolved in NaCl/P_i, pH 7.4. The sensitization and challenge protocol is outlined in Fig. 1A. Control mice were given no other treatment than aerosolized HDM extract at day 25, 28 and 30. As a control for the antigen specificity of the airway inflammation, mice were immunized with OVA [32] prior to challenge in the lungs with HDM extract.

Analysis of leukocytes in bronchoalveolar lavage fluid

Mice were killed by cervical dislocation 18 h after the last aerosol challenge. The trachea was cannulated with polyethylene tubing and BAL was performed using 1 mL aliquots of Hank's balanced salt solution to a total recovered volume of 4 mL. The BAL fluid was centrifuged (400 g, 10 min, 4 °C), the cells were resuspended in 0.4 mL NaCl/P_i pH 7.4 and total leukocytes were counted using trypan blue exclusion in a Bürker chamber. Duplicate Cytospin (Cytospin 3, Shandon, Runcorn, UK) preparations of BAL fluid cells were made for differential counts, using standard morphological criteria after May Grünwald Giemsa staining.

Analysis of Der p 2 specific IgE antibodies

Serum samples were obtained by orbital puncture 18 h after the last aerosol challenge and the amount of Der p 2 specific IgE was analyzed with a capture ELISA using biotin-labelled Der p 2. Ten milligrams Der p 2 in 1 mL NaCl/P_i pH 7.4 was mixed with 2.5 mg biotinamidocaproic acid 3-sulpho-*N*-hydroxy-succinimide ester (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.25 mL distilled water by stirring for 2 h at room temperature. To remove un-reacted biotin the mixture was dialysed against NaCl/P_i, pH 7.4, at 4 °C in 0.1% sodium azide.

For the capture ELISA, Nunc-Immuno Plates with Max Sorb surface (Tamro MedLab AB, Mölndal, Sweden) were coated with 100 µL anti-IgE monoclonal antibody (mAb) (8 µg·mL⁻¹, clone R35-72, BD Biosciences Pharmingen, San Diego, CA, USA) and incubated with 100 µL mouse immune sera (diluted 1 : 3) for 2 h at room temperature. Bound anti-Der p 2 Igs were quantified after incubation with 100 µL biotinylated Der p 2 (2 µg·mL⁻¹), by using a ready-to-use peroxidase substrate system (Sigma) where 100 µL streptavidin-peroxidase conjugate (0.05 U) and finally 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) substrate were added. The soluble product was analyzed after 40 min at A_{620} in a Thermo Labsystems iEMS ELISA reader (Vantaa, Finland). Washing solutions used were saline/0.1% (v/v) Tween. The background level of the ELISA as determined in uncoated wells to which all substrates and serum were added, was subtracted from all data.

Tracking experiments

For tracking experiments mice were sensitized with 1 μg Der p 2 at day 0 and 14 and challenged on day 25 and 28 with aerosolized HDM extract (2.5 $\text{mg}\cdot\text{mL}^{-1}$). Instead of the last aerosol challenge at day 30, the mice were anesthetized with enfluran (Efrane®, Abbott, Solna, Sweden) and i.t. instilled with [^{75}Se]Der p 2 in 50 μL NaCl/P_i pH 7.4.

An initial experiment was set up to examine the distribution of the radioactivity at different time points. Six sensitized mice were given an i.t. instillation of 21 μg [^{75}Se]Der p 2, ≈ 0.13 μCi . Mice were killed after 6, 24 and 48 h, two mice at each time point, with an overdose of pentobarbital (150 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) and processed for tape-section autoradiography.

Two tracking experiments were then set up, where we compared sensitized and nonsensitized mice at the 24 h time-point:

(a) Intratracheal instillation of 7.5 μg ^{75}Se -labelled Sel-tagged Der p 2, ≈ 1.1 μCi , into two sensitized and two non-sensitized mice. Twenty-four hours after the instillation the mice were killed. The mice which were subjected subsequently to whole-body autoradiography were killed with an overdose of pentobarbital (150 $\text{mg}\cdot\text{kg}^{-1}$, i.p.). Tape-section autoradiography on one sensitized and one nonsensitized mouse was performed. The right lung from one sensitized and one nonsensitized mouse was processed for light-microscopic autoradiography. The left lung and both kidneys were immediately frozen in liquid nitrogen and kept in -80 °C until analysis of radioactivity distribution in the tissues.

(b) Intratracheal instillation of 25 μg [^{75}Se]Der p 2, approximately 1.7 μCi , into four sensitized and four non-sensitized mice. The mice were killed by cervical dislocation 24 h after the instillation. Lung, liver, spleen, thoracic lymph nodes and both kidneys were dissected from the eight mice and immediately frozen in liquid nitrogen and kept in -80 °C until analysis of radioactivity distribution in the tissues.

In order to confirm airway inflammation in the model one group of mice received an i.t. instillation (50 μL) of 13 μg nonlabelled Der p 2 instead of [^{75}Se]Der p 2 in parallel to tracking experiment 1 ($n = 4$) and 2 ($n = 5$). After 18 h the mice were killed, BAL was performed and leukocytes differentiated.

Tape section autoradiography

The mice were embedded in aqueous carboxymethyl cellulose and frozen in a CO_2 /hexane bath. The frozen tissues were processed for tape-section autoradiography as described [39,40]. Series of 20 or 60- μm sections were collected on tape through the body followed by freeze-drying. The sections were then pressed against X-ray film (Structurix, Agfa, Mortsel, Belgium), exposed at -20 °C and developed

using D19 (Kodak, Rochester, NY, UK). Selected sections were stained in hematoxylin (Sigma) and eosin (BDH Ltd, UK).

Light-microscopic autoradiography

Lungs were excised from animals and injected with 0.3 mL Tissue Tek® OCT (Sakura Finetek, Zoeterwoude, the Netherlands)/NaCl/P_i pH 7.4, 1 : 3 before they were frozen in Tissue Tek® OCT in liquid petroleum gas. The tissues were freeze sectioned, rinsed in 4% phosphate buffered formaldehyde, pH 7.4 (2×5 min) followed by rinse in phosphate buffer pH 7.4 (2×5 min) and dip in deionized water. The slides were dried and dipped in liquid film emulsion/water, 2 : 1 (NTB-2; Kodak, Rochester, NY, USA). After exposure to D19 (Kodak), the sections were stained in hematoxylin (Sigma) and eosin (BDH) and evaluated in a light microscope (Nikon Eclipse E400) equipped with a digital camera (Nikon DXM1200) and imaging software (Nikon ACT-1).

Measurements of radioactivity in tissue and analysis of ^{75}Se -containing proteins

The frozen tissues were thawed, weighed and subsequently homogenized in 1 mL 50 mM Tris pH 7.5, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethanesulphonyl fluoride, 20% glycerol, 0.5% Nonidet on ice for 30 s with a PCU-2 Homogenizer (Kinematica, Luzern, Switzerland). The homogenates were cleared by centrifugation, 10 000 g , 20 min at 4 °C, and the supernatants were analyzed in a gamma counter (Cobra II Auto-Gamma, Packard Instrument Company). The protein concentrations in the supernatants were thereafter determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA) using BSA as standard. The distribution of radioactivity in the tissues was expressed as the ratio of c.p.m. $\cdot\text{mg}$ protein $^{-1}$ between different tissues in each mouse, in order to compensate for individual differences of recovered radioactivity. Equal amounts of protein from all tissues were loaded on 8–16% SDS/PAGE (Bio-Rad). ^{75}Se -labelled proteins were visualized by autoradiography using a PhosphorImager with the IMAGE QUANT software (both from Molecular Dynamics, Sunnyvale, CA, USA). As standards for SDS/PAGE autoradiography, ^{75}Se -labelled recombinant rat TrxR1 [41] and [^{75}Se]Der p 2 in crude bacterial extracts were run on the same gels as the mouse tissues.

High and low molecular mass components in the supernatants were separated by gel filtrations on NAP-5 columns (Amersham Pharmacia Biotech) in TE-buffer (50 mM Tris pH 7.5, 2 mM EDTA). Fractions (0.5 mL) were assayed for radioactivity with a gamma counter and protein contents were determined by the Bradford assay.

Western blot experiments were performed as described previously [42]. In order to detect Der p 2 in mouse tissues a mouse mAb against Der p 2 (MA-1D8 from Mite2

ELISA kit, Indoor Biotechnologies) was used, followed by detection with a rabbit-anti-(mouse IgG) conjugated with alkaline phosphatase (DAKO A/S, Glostrup, Denmark) and AP Conjugate Substrate Kit (Bio-Rad). Alternatively, a serum from a HDM sensitized patient (48 kU·L⁻¹ IgE against *D. pteronyssinus* as determined with Pharmacia CAP SystemTM, Pharmacia Diagnostics, Uppsala, Sweden) was used for detection as earlier described [42]. In control experiments with Sel-tagged Der p 2 blotted onto the membrane, the Sel-tagged Der p 2 was recognized by both the anti-Der p 2 mAb and patients' serum, showing that the Sel-tagged Der p 2 had a preserved protein structure with maintained IgG and IgE-binding epitopes.

For the comparison of sensitivity of [⁷⁵Se]Der p 2 detection by autoradiography and western, a dilution series (10-fold dilutions) of ⁷⁵Se-labelled Der p 2 in crude bacterial extract was applied to two SDS/PAGE gels. One gel was subsequently Coomassie stained and subjected to autoradiography while the other gel was used for western blot analysis as described above.

Statistical analyses

Statistical comparisons were performed by analysis of means using unpaired Student's *t*-test (two-tailed). All values are shown as mean ± standard deviation (SD). *P* < 0.05 was regarded as significant.

Acknowledgements

We thank Bo Lilliehöök (FOI NBC Defence) and Margareta Mattsson (Department of Environmental Toxicology, Uppsala University) for excellent technical assistance. This work was supported by grants from the Swedish Foundation for Health Care Sciences and Allergy Research, the Swedish Research Council for Medicine (projects 14527 and 14528), Hesselmanns foundation, Magnus Bergvalls foundation, Konsul Th C Berghs foundation, Lars Hiertas Foundation, Åke Wibergs foundation, the King Gustaf V 80th Birthday Foundation, and the Karolinska Institutet.

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Supplementary material

The following supplementary material is available online for this article.

Figure S1. Autoradiography is at least ten times more sensitive than western blot analysis for detection of ⁷⁵Se-labelled Der p 2.