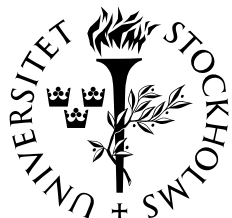


1998:15

# Purification, Characterization and Immunological Studies of Rat Urinary Proteins Causing Allergy in Humans

Second Edition

*Cecilia Bayard*



*Department of Immunology, Wenner-Gren Institute  
Stockholm University, Stockholm, Sweden  
ISBN 91-7153-848-8*

*Department of Occupational Medicine  
National Institute for Working Life, Solna, Sweden*

---

ARBETE OCH HÄLSA VETENSKAPLIG SKRIFTSERIE

ISBN 91-7045-510-4 ISSN 0346-7821 <http://www.niwl.se/ah/>



*Arbetslivsinstitutet*  
National Institute for Working Life

## National Institute for Working Life

The National Institute for Working Life is Sweden's national centre for work life research, development and training.

The labour market, occupational safety and health, and work organisation are our main fields of activity. The creation and use of knowledge through learning, information and documentation are important to the Institute, as is international co-operation. The Institute is collaborating with interested parties in various development projects.

The areas in which the Institute is active include:

- labour market and labour law,
- work organisation,
- musculoskeletal disorders,
- chemical substances and allergens, noise and electromagnetic fields,
- the psychosocial problems and strain-related disorders in modern working life.

### ARBETE OCH HÄLSA

---

Redaktör: Anders Kjellberg

Redaktionskommitté: Anders Colmsjö  
och Ewa Wigaeus Hjelm

© Arbetslivsinstitutet & författarna 1999

Arbetslivsinstitutet,  
171 84 Solna, Sverige

ISBN 91-7045-510-4

ISSN 0346-7821

<http://www.niwl.se/ah/>

Tryckt hos CM Gruppen

Om du inte bestiger berget  
får du ingen utsikt över dalen.

Okänd

# Original papers

The thesis is based on the following publications, which will be referred to in the text by their roman numerals.

- I Marshall T, Williams KM, Bayard C, Vesterberg O. Electrophoresis and silverstaining of rat urinary proteins including  $\alpha_{2u}$ -globulin (Rat n II). *Appl. Theor. Electrophor.* 1992; 2: 189-192.
- II Bayard C, Holmquist L, Vesterberg O. Purification and identification of allergenic  $\alpha_{2u}$ -globulin species of rat urine. *Biochim. Biophys. Acta* 1996; 1290:129-134 and *Biochim. Biophys. Acta* 1996; 1291: 253-255.
- III Vesterberg O, Acevedo F, Bayard C. Sensitive quantification of proteins by electrophoresis in gels by use of chemiluminescence. *Electrophoresis* 1995; 16: 1390-1393.
- IV Bayard C, Vesterberg O. Rat urinary proteins binding human IgE detected by chemiluminescence. *Electrophoresis* 1998; 19: 1642-1648.
- V Bayard C, Siddique A, Berzins K, Troye-Blomberg M, Hellman U, Vesterberg O. Mapping of IgE binding regions in the major rat urinary protein,  $\alpha_{2u}$ -globulin, using overlapping peptides. *Submitted*.

# Abbreviations

A2U	$\alpha_{2u}$ -globulin
AP	Alkaline phosphatase
APC	antigen-presenting cell
Asn	Asparagine
AU	Arbitrary units
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCR	B-cell receptor
BLAST	Basic local alignment search tool
CD	Cluster of differentiation or cluster designation
CDP	Disodium 2-chloro-5-(4-methoxyspiro(1,2-dioxetane-3,2-(5-chloro)-tricyclo(3.3.1.1)decan)-4-yl)phenyl phosphate
C-terminal/us	Carboxy-terminal end of a polypeptide chain
CTL	Cytotoxic T lymphocyte
D	Aspartic acid
Da	Dalton
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
e-RABP	Epididymal retinoic acid binding protein
Fc	Fragment crystallizable or fragment complement binding
FPLC	Fast protein liquid chromatography
G	Glycine
Gly	Glycine
HPHT	High performance hydroxyapatite
IEF	Isoelectric focusing
IFN- $\gamma$	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IU	International Units
IUIS	International Union of Immunological Societies
kDa	kilo Dalton
LAA	Laboratory animal allergy
LAK cell	Lymphokine activated killer cell
$M_r$	Molecular weight
MAb	Monoclonal antibody
Met	Methionine
MHC	Major histocompatibility complex
MUP	Mouse urinary protein, if not otherwise stated
MW	Molecular weight
N-terminal/us	Amino-terminal end of a polypeptide chain
NBT	Nitro blue tetrazolium chloride
NK cell	Natural killer cell
OD	Optical density

PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
Phe	Phenylalanine
pI	isoelectric point
p-NPP	p-Nitrophenyl phosphate
PVDF	Polyvinylidene difluoride
RAST	Radioallergosorbent test
RLU	Relative light units
SDS-PAG/E	Sodium dodecyl sulfate polyacrylamide gel / electrophoresis
Ser	Serine
SPT	Skin prick test
3-D	Three dimensional
TBS	Tris buffered saline
TCR	T-cell receptor
Thr	Threonine
TNF $\beta$	Tumour necrosis factor beta
Tris	Tris(hydroxymethyl)aminomethane
W	Tryptophan
w/v	weight/volume
WHO	World Health Organization
ZIA	Zone immunoelectrophoresis assay

# Contents

<b>1. Introduction</b>	<b>1</b>
The immune system	1
Activation of T cells	1
T-helper cells	2
Cytokines	3
B cells and Ig class switching	4
IgE and IgE receptors	4
Allergy	5
Hypersensitivity reactions	6
Exposure to laboratory animals	7
Rat allergens	7
Properties of $\alpha_{2u}$ -globulin and pre-albumin	8
Allergens from various laboratory animals	9
<b>Aims of the present study</b>	<b>10</b>
<b>2. Methodology</b>	<b>11</b>
Urine collection	11
Separation and purification	11
The Centriprep procedure	11
Precipitation of $\alpha_{2u}$ -globulin	11
SDS-PAGE and IEF as analytical tools for monitoring the purification procedure	12
Total protein determination	12
Affinity chromatography	12
Zone immunoelectrophoresis assay	12
Detection of glycoproteins in gels after SDS-PAGE	13
Detection of glycoproteins on Western Blotting membranes	13
Patients and controls	13
Skin prick testing	13
Specific IgE	14
Total IgE	14
Agarose gel electrophoresis	14
SDS-PAGE prior to immunoblotting	14
Immunoblotting	14
Colloidal gold staining	14
Dot blotting	14
Chemiluminescence procedures	15
Peptide synthesis	16
ELISA screening	16
Allergen-specific IgE analysis	16

Enzymatic cleavage	16
Molecular mass determination of the peptides	16
Computer analysis of the amino acid sequence of $\alpha_{2u}$ -globulin for prediction of epitopes, homology and peptide mass	17
<b>3. Results</b>	<b>18</b>
<b>Urine sampling and precipitation of <math>\alpha_{2u}</math>-globulin (I)</b>	<b>18</b>
<b>Protein concentration procedures (I, II)</b>	<b>18</b>
<b>Isolation and purification studies (II)</b>	<b>21</b>
<b>Identification and characterization of certain proteins (II, V)</b>	<b>21</b>
<b>Quantification by chemiluminescence (III)</b>	<b>22</b>
<b>IgE-binding to rat urine extract (IV)</b>	<b>23</b>
<b>Epitope mapping (V)</b>	<b>24</b>
<b>Homology (V)</b>	<b>25</b>
<b>4. Discussion</b>	<b>26</b>
Urine sampling	26
Concentration of proteins	27
Purification	27
Electrophoretic studies	28
Identification and molecular characterization	29
Glycosylation	29
Quantification of proteins by chemiluminescence	30
Allergenicity studies of rat urinary proteins	31
IgE binding proteins in rat saliva	33
Stability of allergenic proteins	33
Epitope mapping	34
Homology studies	35
Future aspects and significance of the studies in relation to diagnosis of allergy and immunotherapy	36
<b>Concluding remarks</b>	<b>39</b>
<b>Summary</b>	<b>40</b>
<b>Sammanfattning</b>	<b>42</b>
<b>Acknowledgements</b>	<b>44</b>
<b>References</b>	<b>46</b>



# 1. Introduction

## **The immune system**

For protection against invading pathogenic microorganisms, vertebrates have developed complex biological defense mechanisms collectively called the immune system. The primary function of the immune system is to remove infectious agents, thereby minimizing the damage they could cause. When an immune response is generated a variety of cells and tissues are involved in recognizing, attacking and eliminating foreign substances in the body.

The immune system can be divided into two compartments which interplay with each other. The first is the non-specific, innate, or natural immune response and the second the antigen-specific, acquired or adaptive. Natural immunity is comprised of physical obstacles, e.g. the skin and the mucous membranes, as well as physiological barriers which include increased body temperature, the acid pH of the stomach and secretory components such as lysozymes in tears, bactericidal fatty acids, and interferons and the complement system. A variety of chemical mediators such as histamine as well as hydrolytic enzymes also contribute to innate immunity. Cells involved in the innate immune response include granulocytes, macrophages, mast cells and natural killer (NK) cells. Phagocytosis is another important natural defense mechanism.

Acquired immunity is characterized by specificity, diversity, memory, and self/nonself recognition. Lymphocytes have a central role in all adaptive immune responses. There are two categories of lymphocytes, the antibody-producing B lymphocytes (B cells) and the T lymphocytes (T cells). Lymphocytes specifically recognize and eliminate foreign substances called antigens both inside host cells and outside, in the blood or tissue fluids. B- and T-cells combat their target antigens by humoral and/or cellular mechanisms. Both types of lymphocytes have antigen-specific surface receptors. B cells interact with intact antigen via their antigen-specific B-cell receptor (BCR), a complex of membrane-bound immunoglobulin and a disulfide-linked heterodimer. T cells express T-cell receptors (TCR) which in association with a cell membrane complex of polypeptides known as CD3 form a TCR-CD3 membrane complex. T-cell receptors recognize processed antigen associated with molecules encoded by major histocompatibility complex (MHC) and which are present on the surface of antigen-presenting cells (APC). Both B- and T-cell receptors exist in diverse forms which are generated by random rearrangements of multiple gene segments during the maturation of B- and T-cells. The communication between the cells in the immune system is regulated by cytokines, a group of low molecular weight proteins secreted by leukocytes.

## **Activation of T cells**

The recognition of antigen by certain T lymphocytes is fundamental for the generation and regulation of an effective immune response. T lymphocytes arise from the hematopoietic stem cells in the bone marrow and subsequently they

migrate to the thymus to mature. In the lymph nodes the T cells proliferate and differentiate into memory T cells and various effector T cells such as T helper-(Th) and cytotoxic (Tc) cells. These subsets are distinguished by their expression of specific surface proteins. The Th cells express the CD4 marker and Tc cells the CD8 marker. These markers strengthen the relatively weak interaction between TCR and the peptide-major histocompatibility complex (MHC). T cells are only able to recognize processed antigen which has been degraded into peptide fragments. The processed antigen in association with highly polymorphic MHC on the surface of antigen-presenting cells such as macrophages, B lymphocytes or dendritic cells is recognized by the T cell receptor on mature T cells. CD4<sup>+</sup> T cells recognize their specific antigenic peptides in association with MHC class II, whereas CD8<sup>+</sup> T cells bind antigenic peptides in association with MHC class I. To fully activate Th cells a co-stimulatory signal is needed which is provided by the antigen presenting cell. One such ligand-receptor system consists of CD28 expressed by CD4<sup>+</sup> cells and its ligands B7-1 (CD80) and B7-2 (CD86) expressed on the antigen-presenting cells (24, 83). After activation the CD4<sup>+</sup> cells express CTLA-4 (CD152) which also binds to the B7-1/B7-2 ligands but with a much higher affinity. The delivery of co-stimulatory signals prevents anergy. Interaction between the TCR-CD3 complex and the peptide-MHC molecule leads to the production of IL-2. Autocrine stimulation of IL-2 promotes the proliferation of Th cells. These cells secrete various cytokines which are crucial for the activation of macrophages, B- and Tc-cells. Cytotoxic-T lymphocytes, activated by the cytokine IL-2, mediate membrane damage to altered self-cells, leading to cell lysis.

### **T-helper cells**

Mosmann originally described two types of CD4<sup>+</sup> T-helper cells in the murine system (123), denoted Th1- and Th2-cells and which differ in their biological functions based on the different cytokine profiles they secrete. T cells that do not display restricted Th1- or Th2-like cytokine profiles, but rather intermediate ones, are known as Th0 cells (58, 173). In humans the segregation between cells that produce Th1 and Th2 cytokines is not as clear as in mouse (149). Th1 cells are involved in the cell-mediated immune response. Typical human Th1 cytokines include IFN- $\gamma$ , TNF $\beta$ , and IL-2 which mainly mediate delayed-hypersensitivity reactions and activate macrophages. Cytokines produced by Th2 cells are IL-4, IL-5, IL-6, IL-10, and IL-13 (13, 124) with principal effects on B cell growth and differentiation.

Certain Th1 derived cytokines downregulate Th2 cells and vice versa (161). IFN- $\gamma$  and IL-4 are such cytokines with reciprocal effects. The Th1 cytokine IFN- $\gamma$  inhibits the IgE synthesis (139) while the Th2 cytokine IL-4 induces the IgE production and simultaneously inhibits the production of TNF- $\alpha$  and IL-1 (63). Differentiation of CD4<sup>+</sup> cells into either Th1- or Th2-cells determines the outcome of an immune response. Factors suggested to be of importance for this regulation are: cytokine environment, antigen dose and route of administration, type of antigen-presenting cells and their secretion of cytokines as well as the

activity of co-stimulatory molecules (1, 133, 161). The immune response is also highly influenced by the genetic constitution of the individual.

On the basis of the cytokine profiles there is evidence for the existence of functionally distinct subsets also among the CD8<sup>+</sup> Tc cells (155, 160).

## Cytokines

Cytokines, the regulators of the immune system, are important for the allergic reactions. The intensity and duration of the immune response are regulated by cytokines. They stimulate or inhibit the activation, proliferation and differentiation of various cells and regulate their secretion of antibodies and cytokines. In this thesis mainly cytokines involved in type I hypersensitivity reactions will be discussed, i.e. the Th2 subset of cytokines. Elevated concentrations of Th2 cytokines are generally seen in allergic diseases and in helminthic infections. The Th1 and Th2 subsets of cytokines have cross-regulating functions. The Th1 cytokine IFN- $\gamma$  inhibits the proliferation of the Th2 subset, while the Th2 cytokine IL-10 decreases the secretion of IFN- $\gamma$  by downregulating the MHC class II expression on monocytes and macrophages. IL-12 secreted by macrophages induces proliferation of Th1- and NK cells and thereby an increased IFN- $\gamma$  production. Thus, IL-12 promotes Th1-type of response while the Th2 cytokines IL-4 and IL-10 promote a Th2-type of response by inhibiting the IL-12 production. The cytokine environment present at the time when Th cells differentiate influences the type of subset developed. *In vitro* activation of Th cells by an antigen together with IL-4 results in a Th2 subset whereas activation by the same antigen but in the presence of IFN- $\gamma$  gives rise to a Th1 subset.

Here follows an overview of principal features of certain cytokines.

**IL-4**, secreted by Th2 cells is involved in immunoglobulin class switching from IgM/IgG to IgE as well as enhancing proliferation and differentiation of activated B cells. In resting B cells and macrophages the expression of class II MHC is up-regulated by IL-4 (135). The macrophages' phagocytic activity is also increased by the pleiotropic IL-4. It stimulates growth of mast cells and co-stimulates activation of antigen-primed B cells. Further, the cytokine induces proliferation of thymocytes and T cells. Overproduction of IL-4 is seen in allergic individuals, possibly due to polymorphism within the promotor of the IL-4 gene (165).

**IL-5**, secreted by Th2 cells (177) stimulates proliferation and differentiation of activated B cells, induces Ig class switch to IgA and promotes growth and differentiation of eosinophils.

**IL-10**, secreted by Th1-, Th2- and activated B cells and macrophages, suppresses IFN- $\gamma$  production in macrophages and thus indirectly reduces cytokine production by Th1 cells (122). In antigen-presenting cells the expression of MHC class II molecules is down-regulated by IL-10.

**IL-12**, secreted by macrophages, B cells (182) and dendritic cells, stimulates proliferation in NK cells and lymphokine activated killer (LAK) cells as well as activated Th1 cells (112, 159). In activated Tc cells IL-12 acts synergistically with IL-2 to induce differentiation of cytotoxic T lymphocytes (CTL).

**IL-13**, secreted by Th cells, is an important regulator of inflammatory response as it inhibits activation and release of inflammatory cytokines in macrophages (42). Ig class switching to IgG4 or IgE in B cells is regulated by IL-13 (100, 144).

**IFN- $\gamma$** , secreted by Th1-, Tc- and NK cells, enhances the activity of macrophages (25). IFN- $\gamma$  increases the expression of MHC class I and II molecules in many cell types. The cytokine inhibits the proliferation of Th2 cells and blocks the IL-4-induced class switch to IgE.

### **B cells and Ig class switching**

The B lymphocytes arise from hematopoietic stem cells in the bone marrow. Naive B lymphocytes leave the bone marrow with unique antigen-binding receptors on their membrane, i.e. antibody molecules often of both IgM and IgD isotypes but with the same unique antigen specificity. This specificity is determined prior to contact with the antigen by random rearrangements of gene segments encoding the variable regions of the antibody molecule. Upon activation B cells will differentiate into antibody forming cells. During the primary immune response IgM molecules of rather low affinity are secreted and the IgD expression is downregulated. The B cells will then migrate from the primary follicles in the spleen or lymph nodes into the germinal centers where the secondary immune response takes place. In the germinal center B cells will undergo somatic mutation i.e. small changes like deletions or additions of nucleotides in the immunoglobulin gene sequence. To complete the maturation of affinity B cells will be selected by their ability to bind antigen. In the germinal center B cells with high-affinity receptors will interact with antigen on follicular dendritic cells and as a result the bcl-2 gene is expressed. The bcl-2 protein prevents B cells from undergoing apoptosis. Antigenic stimulation of the B cell results in Ig class switching i.e. changed biological function of the antibody. However, the antibody specificity is maintained after class switching. The isotype switching from IgM/IgG to IgE is dependent on signals from CD4<sup>+</sup> Th cells, the CD40 ligand (82), and the cytokines IL-4 or IL-13 (193), secreted by antigen-activated Th2 cells. Likewise IL-10 promotes IgG1 and IgG3 switching (122). After the switch the B cells will leave the germinal center to become memory- or plasma cells. The signalling through the CD40 marker is crucial for the differentiation of B cells into memory cells. In the absence of this signal, B cells undergo terminal differentiation into plasma cells (14).

### **IgE and IgE receptors**

When allergens - that is, antigens that elicit allergic reactions - enter the body a local reaction occurs and IgE-secreting plasma cells will move to the site. Allergens trapped in the lymph nodes may give rise to large quantities of locally produced IgE molecules, secreted by medullary plasma cells, which will leave the lymph nodes through the efferent lymphatic vessel. The antibodies enter the circulation and are thus distributed throughout the body. Free serum IgE has a half-life of a few days but remains for many months when bound to the high affinity binding IgE receptor Fc $\epsilon$ RI which is present on mast cells or basophils (121). In normal individuals the serum level of total IgE is within the

concentration range 0.1-0.4 µg/ml compared to 10 mg/ml for IgG. In type I hypersensitive individuals the total concentration of IgE is often increased but seldom higher than 1 µg/ml.

The IgE heavy chain contains, besides one variable domain, four constant domains, one more than IgG, and no hinge region. Secreted IgE antibodies bind with high affinity to IgE specific receptors on mast cells and basophils. The IgE receptor interacts with the Fc region of the  $\epsilon$  heavy chain, i.e. the C $\epsilon$ 2 and/or C $\epsilon$ 3 domains. After sensitization and upon repeated exposures to the allergen, the membrane bound IgE molecules become cross-linked. This cross-linkage of the Fc $\epsilon$ RI receptor-bound IgE triggers the degranulation of the mast cell. The degranulation causes the release of various pharmacologically active mediators which in turn mediate type I allergies, such as asthma, hay fever, and eczema. In addition to the high affinity receptor there are two forms of the low affinity receptor: Fc $\epsilon$ RIIa and Fc $\epsilon$ RIIb. These two forms of the low affinity receptor are also known as CD23. Fc $\epsilon$ RIIa is expressed on B cells before they differentiate into plasma cells. Fc $\epsilon$ RIIb is expressed on B cells, T cells, monocytes, and eosinophils but only after induction by the cytokine IL-4 (43). The Fc $\epsilon$ RII receptor has a regulatory role for the intensity of the IgE response. Atopic individuals have higher levels of Fc $\epsilon$ RII on their lymphocytes and macrophages than nonatopics. Crosslinking of Fc $\epsilon$ RII with allergens or IgE-immune complexes leads to the release of leukotrienes, TNF and IL-1 (47). Fc $\epsilon$ RII also plays a role in IgE-mediated reactions by enhancing antigen presentation to T cells (188).

## Allergy

The word allergy originates from the Greek language and means "other action" or "changed reactivity" and was originally coined by von Pirquet in 1906 (22, 145). By definition, an allergy or hypersensitivity reaction is an exaggerated physical response to certain antigens, typically common environmental substances that normally produce little or no response in the general population. In sensitive individuals, however, antigens elicit reactions that may cause tissue damage and lead to various symptoms in the airways, gut, and skin. The most common allergic diseases are rhinitis, asthma, atopic dermatitis, urticaria, and food allergies.

IgE antibodies are thought primarily to have evolved to play a role in the defense against parasitic worm infestations. In the absence of Th1 inducing infections the Th1/Th2 balance could be disturbed and when that occurs Th2 mechanisms could be triggered and promote allergic disorders. Consequently, in industrialized countries where parasitic worms are rare, the synthesis of IgE antibodies is regarded as an unwanted immune response. Hence, allergy is often said to be an unfortunate side effect. It is suggested that the decline of the frequency of viral and bacterial infections, due to improved living standards and immunization programs, could cause an increase in the incidence of allergy (39, 162). Environmental factors may also play important roles in the development of allergic diseases. Thus, pollutants in the air may increase mucosal permeability and thereby enhance allergen entry (178). The level of allergen exposure, the nutrition status of the individual, intestinal flora, stress, and passive smoking are other factors claimed to contribute to the allergic disorder (8, 23, 80).

Consequently, allergy affects the quality of life for many persons. Reduced ventilation and changed dietary habits are also suggested to be adjuvants enhancing IgE synthesis (178, 203). Thus the "Western lifestyle" can be an important risk factor for the development of allergic diseases.

There is a genetic predisposition for atopy and the development of specific IgE antibodies against common environmental antigens. Parental history of allergy is thus a risk factor for atopy. When both parents have an atopic disease there is a 50% risk that the children will develop allergy and with one allergic parent there is a 30% risk. The atopy concept has recently been discussed since there is still some controversy about the precise clinical definition (102).

### **Hypersensitivity reactions**

According to Coombs and Gell there are four types of hypersensitivity reactions (65). This thesis deals exclusively with type I hypersensitivity reactions. The type I hypersensitivity reaction is IgE-mediated and induced by specific antigens referred to as allergens. The allergens activate Th2 cells which will start to secrete IL-4. This will induce Ig class switching in B cells followed by production of IgE molecules, which will bind to specific IgE-Fc receptors on mast cells and basophils. Later, when exposed to the same allergen, the cell-bound IgE antibodies will become crosslinked, resulting in degranulation of mast cells and the release of pharmacologically very potent mediators such as histamine, serotonin, heparin and various enzymes. Also other mediators appear simultaneously, for instance the platelet-activating factor, chemotactic factors, and inflammatory mediators such as prostaglandins and leukotrienes. The effects caused by the mediators are inflammation, vasodilation, smooth muscle spasm and chemotaxis. In some individuals the immediate phase of IgE-dependent hypersensitivity reactions or the first stage of inflammation is followed by a late reaction or the second stage of inflammation. This is due to invasion of inflammatory cells associated with the mucosal edema and secretion of inflammatory activators.

The type II reaction develops when IgG binds to either self- or foreign antigen on cells, leading to phagocytosis, complement-mediated lysis or antibody-dependent cell-mediated cytotoxicity.

Type III reactions occur when large quantities of immune complexes are formed and not cleared away thoroughly. This type III hypersensitivity reaction is often observed when large amounts of antigen enter the bloodstream and when the level of circulating antibodies specific for that particular antigen is high.

Types I-III are immediate reactions, appearing within a few minutes up to several hours after exposure to an antigen.

Type IV is a delayed hypersensitivity reaction (DTH) where symptoms occur within a few days and is mainly mediated by T-cells or macrophages. Many contact-dermatitis reactions are of type IV or  $T_{DTH}$ -mediated.

## Exposure to laboratory animals

Handling of laboratory animals involves increased risk of developing allergy (11, 33, 69). This is a world-wide occupational problem. Mice and rats are the most frequently used laboratory animals. In Sweden 131 496 mice and 118 404 rats were used during 1996 in research laboratories, pharmaceutical companies and industries, and at universities and hospitals (54). Thus, employees at such workplaces, students, veterinarians and breeders are exposed to laboratory animals. In addition, mice and rats are becoming more and more popular as pets, which may contribute to development of allergy.

Within the first three years of exposure to laboratory animals allergic symptoms like rhinitis, conjunctivitis, asthma or urticaria occur in sensitized individuals with a prevalence of 20-30% (11, 76, 164).

Another factor, besides exposure and work task, that may affect the allergy response is physical work load, because the inhaled air volume increases the more strenuous the work. The inhaled volume may be more than three times higher than at rest. It should also be mentioned that the fume cupboards used in many workplaces are often designed to protect the experiment rather than the person doing the experiment, i.e. the air blows straight into the face of the personnel, carrying allergens with it.

Many epidemiological and prospective studies of exposure and risk factors for developing allergy to laboratory animals have been carried out (76, 147, 164). This contributes to the understanding of the problem and our ability to counteract laboratory animal allergy.

## Rat allergens

Allergens involved in rat hypersensitivity reactions have been found in fur, saliva, urine, feces, skin, and serum of rats (130, 198, 199). However, the major source is considered to be the urine since stronger skin test reactions in rat sensitive patients are obtained with urine extract compared to preparations from other rat materials (198). Protein excretion into the urine due to spontaneous nephropathy is increased with the age of the rats (27, 202). When the urine dries, the allergens may become airborne as small particles (49, 50, 131). Thus, individuals are exposed to the allergens either via direct skin contact or when they inhale airborne particles. The allergens in the fur and the skin are most probably contaminations transferred from urine or saliva. One of the most well studied and most dominant protein in fertile male rat urine is  $\alpha_{2u}$ -globulin (67, 105, 108, 130). The name  $\alpha_{2u}$ -globulin originates from the observation that the protein's electrophoretic mobility is similar to that of serum  $\alpha_2$ -globulin (93, 152), and u stands for urine. In accordance, another protein in rat urine has been called pre-albumin, since this protein migrated similarly to serum prealbumin in horizontal starch-gel electrophoresis (152). Longbottom and co-workers introduced another term for  $\alpha_{2u}$ -globulin, namely  $\alpha_2$ -euglobulin, which originates from its tendency to precipitate when dialyzed for 4-5 days against deionized water (105). The names have been used in parallel. However, this has later been found confusing and misleading. To make matters worse, the rat urinary  $\alpha_{2u}$ -globulin is registered as alpha-2u-globulin or major urinary protein (MUP) in the SWISS-PROT protein

sequence database with the accession number P02761. This may cause even more confusion since the mouse major urinary protein is also abbreviated MUP. These two MUPs are not the same protein even though they share high sequence homology (34). Pre-albumin was not renamed.

Both pre-albumin and  $\alpha_{2u}$ -globulin were shown to be potent allergens (105, 130) and in addition isoforms (21). It was recently proposed that these two rat isoallergens should be called Rat n 1.01 and Rat n 1.02, respectively (171). According to the WHO/IUIS Allergen Nomenclature Subcommittee (86) the first three letters in the nomenclature name correspond to the first letters of the genus name. The fourth is the first letter of the species name. The Arabic numerals represent the order in which the allergens have been identified and described. In recent years it has often been found that a certain allergenic protein can occur in molecular forms that differ only slightly, e.g. by a few residues in the amino acid sequence. Allergens with very similar physical, chemical and immunochemical structures but slightly different isoelectric points, and sharing at least 67% of the amino acid sequence identity are termed isoallergens (98). Isoallergens are indicated by an additional two numerals. Thus, Rat n 1.01 stands for Rat (*Rattus*), n (*norvegicus*) and 1 (the first allergen identified). The figures 01 indicate that this is the first isoallergen identified in *Rattus norvegicus*. Despite the dominance of Rat n 1.01 and Rat n 1.02, also other proteins present in rat urine may be allergenic (67, 77, 78).

### **Properties of $\alpha_{2u}$ -globulin and pre-albumin**

Important characteristics for description of proteins are isoelectric point (pI) and molecular weight ( $M_r$ ), which is unitless, or molecular mass given in units of Dalton. The latter two terms are used interchangeably to indicate the size of proteins. The pI is determined by the composition of the protein i.e. the relative proportions of different amino acids, their charged groups and some other molecular moieties that may occur, e.g. bound carbohydrates. A variety of mainly chromatographic procedures have been used to purify rat urinary proteins, particularly,  $\alpha_{2u}$ -globulin (51, 89, 94, 95, 105, 152, 192).

$\alpha_{2u}$ -Globulin is synthesized in the liver, secreted into the blood and excreted in the urine (153, 154). It represents 30% of the total urinary protein (89) and is produced in adult male rats under androgen control. However, a very similar protein in urine from female rats has been reported to occur but at a much lower concentration; approximately 120 times less (192). Further, this protein is also present in other secretory tissues like the submaxillary, lachrymal and mammary glands as well as in the anterior pituitary both in female and male rats, although it is not as abundant as in male rat urine (10, 27, 70, 110, 205). Mature  $\alpha_{2u}$ -globulin has a molecular weight of 18 700 Da and contains 162 amino acids (183). Earlier published pI values are 5.1, 5.3 and 5.5 (105). The pI values for pre-albumin were reported to be 4.2 and 4.3 (105) and its molecular weight 21 000 Da as determined by SDS-PAGE (67, 107).

Diet has been found to influence the excretion of  $\alpha_{2u}$ -globulin. In rats, a protein-free diet i.e. 0% casein, was shown to reduce significantly the concentration of  $\alpha_{2u}$ -globulin in urine (129). Excretion of the protein is age-dependent in rats (67,



128). After the age of 160 days the albumin becomes the major component of male rat urine. The excretion of albumin accelerates with age due to spontaneous development of renal lesions and nephropathy. Protein droplet nephropathy and renal tumors also occur in the male rats and are caused by several chemical compounds, for instance d-limonene and dichlorobenzene which are present in unleaded gasoline. This results in an accumulation of  $\alpha_{2u}$ -globulin in the proximal tubular cells (27).

$\alpha_{2u}$ -Globulin is a transport protein for hydrophobic molecules and it belongs to the lipocalin family. The lipocalin name refers to the capability of binding lipophilic molecules as well as the binding mode in which the ligand is enclosed by the protein (140).  $\alpha_{2u}$ -Globulin in rat urine and the closely related major urinary protein (MUP) in mouse urine, serve as carriers for odorants, which could act as sexual attractants or for territorial marking (18, 59). The proteins may also bind degraded sex hormones i.e. pheromones. Pheromones are messenger substances used for communication between members of the same species. These pheromones are volatile components of low molecular mass which are easily released from drying urine. The three-dimensional structure of  $\alpha_{2u}$ -globulin has been determined and to some extent also its ligand binding properties (34, 81, 97). The lipocalins are a rapidly growing protein family which includes the previously named  $\alpha_{2u}$ -globulin superfamily (31, 140). Proteins in a superfamily have similar folding patterns and a sequence identity < 50%. For lipocalins the sequence homology is about 20% (60). Typical members of the  $\alpha_{2u}$  superfamily are low molecular weight transport proteins with a similar three-dimensional structure where the polypeptide chain forms a  $\beta$ -barrel (27). Included in this superfamily are human, rat and mouse urinary proteins,  $\beta$ -lactoglobulins, and serum retinol-binding proteins (31). Most of the lipocalins are found in vertebrates but some have been identified in arthropods and even in bacteria (59) and the database PROSITE, release 15. Because of the continual expansion of this family, the lipocalins are no longer primarily classified as transport proteins. Currently known family members possess a variety of different functions as for example in enzymatic synthesis, cell regulation and pheromone signalling (59).

### **Allergens from various laboratory animals**

Not only people who handle rats and mice but also those who handle other animals are at risk for development of allergy. Thus, saliva, fur, and dander from rabbits and guinea pigs are other sources of allergens (105, 109, 142, 143, 200). These allergens are also proteins, mainly in the molecular mass range 10-40 kDa (101). Yet other species used as laboratory animals which could cause allergy are cats and dogs. The major cat allergen, Fel d 1, with a molecular weight of 35 kDa, has been detected both in saliva and in skin epithelium (7, 20, 32, 40). Can f 1, the major dog allergen, has been characterized as well (61, 68, 158, 169).

# **Aims of the present study**

Although our knowledge about allergenic proteins has increased during recent years, still further biochemical and immunological studies are needed at the molecular level to better understand laboratory animal allergies.

The aim of this thesis was to improve the knowledge about allergens present in rat urine with special emphasis on:

- 1) isolation, identification and characterization of the major urinary proteins;
- 2) studying the allergenicity of rat urinary proteins using improved methods;
- 3) examination of, in some detail, the IgE binding regions of the major allergens.

## 2. Methodology

The methods used in papers I-V are described briefly below. For itemized explanations see the *Materials and Methods* sections in the respective papers. Methods not mentioned in papers I-V are described in greater detail in this section.

### *Urine collection*

Urine was collected in separate portions, day time from 8 a.m. to 4 p.m. and during nights from 4 p.m. to 8 a.m., from fertile male 14 - 15 week old Sprague-Dawley rats housed individually in metabolic cages. These cages are constructed to separate urine and faeces in special containers. The cages are also specially designed for collection of food- and drinking-water waste to avoid contamination of urine and faeces samples. Bladder urine samples were obtained by bladder puncture as described in paper I.

### *Separation and purification*

The collected urine samples were processed as described in paper II followed by concentration by ultrafiltration centrifugation (paper I) or a chromatographic method utilizing hydroxyapatite (78), (paper II). Subsequently, rat urinary proteins were separated and purified by liquid chromatography using the Pharmacia FPLC system which involved molecular size separation by gel filtration on a Hiload Superdex column followed by charge separation on an ion exchange column, Mono Q (II). The two purified dominating proteins were identified as different forms of the same parent protein,  $\alpha_{2u}$ -globulin, by using amino-acid compositional analysis and sequence analysis as well as mass spectrometry.

Rat albumin (Nordic Immunological Laboratories, Tilburg, The Netherlands) was purified further by gel filtration as described in paper IV, (21).

### *The Centriprep procedure*

Centriprep™ concentrators (Amicon, W. R. Grace & Co., Danvers, MA, USA) are disposable ultrafiltration devices for use in centrifuges. They are suitable for concentrating and desalting biological samples in the 5-15 ml volume range. Concentration and desalting to a certain extent occur simultaneously. Dialyzed rat urine was spun in a Centriprep™ C30 unit with a nominal membrane cutoff corresponding to 30 000 MW. The C30-filtrate was re-spun in a Centriprep™ C10 unit with a nominal membrane cutoff of 10 000 MW (I).

### *Precipitation of $\alpha_{2u}$ -globulin*

The day-time and night-time urine samples were centrifuged at 3000g for 20 min. Aliquots of the resulting supernatant were then thoroughly dialyzed against deionized water (105) or against 50 mM ammonium hydrogen carbonate (107) or against 4 mM sodium phosphate buffer pH 6.8 (78). The dialyzed urines were

centrifuged (3000g, 20 min), the supernatants decanted and the precipitates resuspended in 50 mM ammonium hydrogen carbonate.

#### *SDS-PAGE and IEF as analytical tools for monitoring the purification procedure*

Protein extracts and liquid chromatography fractions were separated under reducing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (78) using gel slabs (10-20% linear gradient). Ready-made Phast SDS-PAGE gradient gels (10-15%) and the Phast System<sup>®</sup> (Pharmacia Biotech) were also used. Proteins in the gels were stained with Coomassie Brilliant Blue R250 (2, 78) or by silver as described earlier (2) and the Phast System<sup>®</sup> manual, respectively. Horizontal SDS electrophoresis with ExelGel<sup>™</sup> SDS, gradient 8-18% (Pharmacia) were run as well. Silver staining was done according to the manufacturer's instructions.

Urinary proteins concentrated by Centriprep<sup>™</sup> i.e. ultrafiltration centrifugation, were analyzed under slightly different conditions (I) namely by placing samples in precasted 1% (w/v) agarose wells on linear 4-25% (w/v) polyacrylamide gradient gels (75 x 75 x 3 mm) (116, 117). The gels were silver stained (115).

Native rat urinary proteins (untreated sample) were analyzed by isoelectric focusing in the Phast System<sup>®</sup> using ready-made gels with the pH ranges 3-9 and 4 - 6.5. The Phast gels were silver stained in accordance with the Phast System<sup>®</sup> manual.

#### *Total protein determination*

For quantification of total protein concentrations to ensure adequate sample loading in electrophoresis and liquid chromatography both the BCA (Bicinchoninic acid) protein assay (Pierce, Rockford, IL, USA) and the Coomassie<sup>®</sup> Protein Assay (Pierce), a variant of the Bradford method, were used according to the manufacturer's recommendations.

#### *Affinity chromatography*

For the affinity purification of hydroxyapatite concentrated rat urinary proteins (HPHT-extract) the monoclonal antibody, mAb(6) specific against rat urinary allergen, Rat n 1 i.e. both isoallergens, (148) was used. The monoclonal antibody was immobilized to an AminoLink column (Pierce). The AminoLink column was processed according to the manual of ImmunoPure<sup>®</sup> Antigen/Antibody Immobilization Kit 1 (Pierce).

#### *Zone immunoelectrophoresis assay*

For determination of specific proteins, zone immunoelectrophoresis assay, ZIA, was used in principle as previously described (194). Briefly, a 1.3% (w/v) solution of agarose in 0.04 M Tris-Tricine buffer pH 8.6 was mixed with antibodies at 55°C. Antibodies specific to rat albumin (Nordic) or transferrin (Organon Teknika-Cappel, Turnhout, Belgium) were used. The twenty glass tubes in the zone immunoelectrophoresis apparatus were filled up to a few centimeters from their tops with the antibody-containing agarose solution. Dilutions of standards and rat urinary protein samples were applied on the upper gel surface in each tube and the electrode vessels were subsequently filled with buffer. Electrophoresis

was performed overnight. The antibodies formed complexes with the antigens giving immobile antigen-antibody precipitation zones in the gel rods. The rods were then pushed out of the glass tubes and placed on a piece of GelBond foil (FMC, Glostrup, Denmark). Immunoprecipitates could be seen directly in oblique light or visualized with Coomassie blue (79) or silver staining (196). The amount of protein in a sample was linearly related to the distance from the upper gel surface to the front of the immunoprecipitation zone. Samples were analyzed in duplicate. Standard curves were obtained with different concentrations of rat serum (DAKO, Glostrup, Denmark) when analyzing transferrin and different standard concentrations of rat albumin (Nordic) for albumin determination.

#### *Detection of glycoproteins in gels after SDS-PAGE*

Sodium dodecyl sulfate-polyacrylamide gels were stained for glycoproteins according to Dubray *et al.* (46) using periodic acid-Schiff reagent (PAS) (Sigma, St. Louis, MO, USA) omitting the enhancing step i.e. the simplified silver staining, due to non-specific staining of the rat urinary proteins with this enhanced detection method. Ribonuclease B (Sigma) was used as a positive control and  $\beta$ -lactoglobulin (Sigma) as a negative control.

#### *Detection of glycoproteins on Western Blotting membranes*

Detection of carbohydrate moieties in glycoproteins immobilized to a membrane was executed according to the principle of the GlycoTrack™ kit (Oxford GlycoSystems Ltd, Abingdon, U. K.). Briefly, the sample immobilized to a PVDF membrane after Western blotting is oxidized with periodate. Biotinylation using biotin-hydrazides followed by probing with a streptavidin-alkaline phosphatase conjugate was used for detection. Subsequently, visualization was performed with the substrate NBT/BCIP (Bio-Rad, Hercules, CA, USA) giving a colored precipitate.

#### *Patients and controls*

Serum samples used were from patients with a clinical history and clear symptoms of allergy to rat, verified by skin prick test (SPT) with the rat extract Soluprick® from (ALK, Hørsholm, Denmark) and also confirmed by specific serum IgE determination using RAST as described (4). The patients had been working as laboratory technicians or as animal keepers. Sera from persons without any allergic symptoms were used as controls. The samples were taken with informed consent and the studies were approved by the Ethics Committee of Lund Hospital, University of Lund, Sweden.

#### *Skin prick testing*

Skin prick tests with gel filtration fractions corresponding to Rat n 1.01 (pre-albumin) or Rat n 1.02 ( $\alpha_{2u}$ -globulin) (170) as well as the HPHT-extract were performed on the volar aspect of the forearm as described (4, 78). The tests were carried out in collaboration with the Department of Occupational Dermatology, University Hospital, Lund.

### *Specific IgE*

Radio allergosorbent test (RAST) was used to quantify serum IgE antibodies specific to rat urinary proteins. The test was performed at the Department of Clinical Immunology, Karolinska Hospital, Stockholm.

### *Total IgE*

The total concentration of serum IgE was determined by paper radioimmunosorbent test (PRIST<sup>®</sup>) (Pharmacia) and the results were expressed in kU/l. These analyses were performed at the Department of Occupational Dermatology, University Hospital, Lund.

### *Agarose gel electrophoresis*

Electrophoresis was run vertically in 1.5% w/v agarose gels in a Miniprotean II apparatus (Bio-Rad) (2). The upper buffer contained 0.1% w/v SDS, 100 mM glycine and 25 mM Tris. The lower buffer contained 100 mM sodium acetate. From each SDS-treated sample (non-reduced) 10 µl was applied (III).

### *SDS-PAGE prior to immunoblotting*

Rat urinary proteins were separated by SDS-PAGE under non-reducing conditions in a Mighty Small<sup>™</sup> apparatus (Hoefer, San Francisco, CA, USA) using gel slabs (10 - 20% linear gradient) (IV). Samples were mixed with an equal volume of a cocktail of 2% w/v SDS, 0.1% w/v bromophenol blue, 20% w/v sucrose and then heated at 100°C for 5 min. Electrophoresis was performed vertically with cooling at 15°C in principle as described (90).

### *Immunoblotting*

After electrophoresis the proteins were transferred to a PVDF membrane (0.2 µm) (BioRad) by electroblotting in a Mighty Small Transfer Unit (Hoefer) (IV). Briefly, a sandwich was built up with foam sponges, blotting paper, membrane and gel and placed in a cassette. The cassette was inserted into a vertical slot in the buffer chamber, and the Western blotting procedure started. If more than one serum was studied at the same time the membrane was after completion of the transfer cut into strips. The strips could then be analyzed individually. Subsequently the membrane was blocked and thereafter incubated, in diluted patient sera, at 37°C for at least 3 1/2 h, but often overnight for practical reasons. After washings the strips were further incubated for at least 1 1/2 h at 37°C with a 1:2000 dilution of alkaline phosphatase conjugated goat-anti-human IgE (Tago, Biosource, Camarillo, CA, USA).

### *Colloidal gold staining*

AuroDye<sup>™</sup> forte (Amersham, Little Chalfont, UK) ready-to-use solution was used for total protein staining of rat urinary proteins transferred to PVDF membranes (IV).

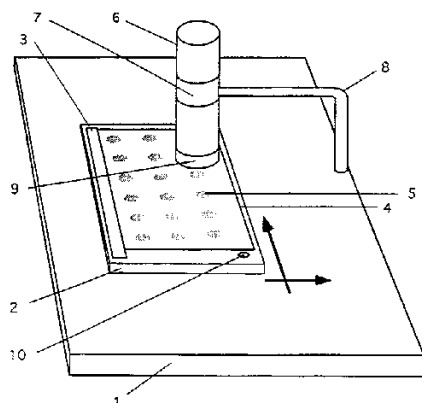
### *Dot blotting*

On a moist PVDF membrane (0.2 µm) protein samples were applied as dots. The sample volume was between 10 µl and 200 µl. Volumes over 40 µl required sequential application. Blocking, antibody incubation and washing procedures

were in accordance with the ones for Western blotting as described above. Biotinylated goat anti-human IgE (Vector Laboratories, Burlingame, CA, USA) diluted 1:1000 was used as secondary antibody. Visualization of the dots was performed by color development using the Vectastain ABC-AP kit (Vector) and the substrate BCIP/NBT (BioRad) (IV). Alternatively, visualization was done by chemiluminescence using the substrate CDP-Star™ (Tropix, Bedford, MA, USA) and an alkaline phosphatase conjugated goat anti-human IgE antibody diluted 1:2000 (IV). Additionally, after washing off the luminescence reagents the membrane was stained preferably with BCIP/NBT (BioRad).

#### *Chemiluminescence procedures (III, IV)*

In the chemiluminescence reaction light is released after enzymatic dephosphorylation of the substrate. The light emission persists for hours, allowing multiple film exposures and measurements. Before incubation in the substrate solution the membrane was equilibrated in TBS pH 7.5 followed by submersion in the reaction buffer. Thereafter the membrane was placed on a plastic foil, GelBond, and the substrate solution was added. Another piece of GelBond was placed on top and excess reagents were squeezed out by passing a roller over the plastic foil-sandwich. A light-sensitive film, Hyperfilm™ (Amersham), was used to capture the light signals where IgE-antibodies had bound rat urinary proteins in the membrane. Only a few minutes exposure time in the dark room was needed to visualize the protein bands satisfactorily. The light was also measured in a luminometer apparatus using a photomultiplier tube and a slit 4 x 1 mm. For details see Fig. 1. Scanning was made perpendicular to the protein bands along the lanes and each position was measured for 1.0 second. Light signals were stored in a computer and expressed in relative light units (RLU). Calibration graphs were constructed by using an Excel® program (Microsoft, CA, USA).



**Figure 1.** Luminometer for measuring luminescence in spots on blotting membranes. The support (1) carries an x-y positioning table (2). A clamp (3) fastens a membrane (4) with the luminescent spots (5) which are measured by the photo multiplier (6) supported by the holder (7) and the steel arm (8). The diaphragm (9) is easy to replace with one having a suitable light aperture. A light emitting diode (10) is used to check luminometer performance.

### *Peptide synthesis*

Octapeptides, overlapping four amino acids, corresponding to the  $\alpha_{2u}$ -globulin sequence were synthesized on derivatised polyethylene rods arranged in a microtiter plate format using the Multipin™ Peptide Synthesis Kit (Chiron Technologies, Clayton, Australia) (V). Briefly, Fmoc-amino acid active esters were coupled to the pins by repetitive cycles; one amino acid per cycle from the C-terminus to the N-terminus of the peptide chain.

### *ELISA screening*

The set of synthesized peptides was scanned for sequential epitopes in an ELISA system as follows (V). The pins with the peptides were blocked in a pre-coating buffer and thereafter incubated overnight at 4°C in a 50-fold dilution of patient sera. After washing, the pins were incubated at room temperature in a dilution 1:500 of alkaline phosphatase conjugated antibody, goat anti-human IgE-AP (Biosource). Subsequently the pins were washed and immersed in freshly prepared substrate solution of p-Nitrophenyl Phosphate, pNPP, (Sigma). The optical density was read at 405 nm in a V<sub>max</sub> ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). The peptides, covalently linked to the pins, could be repeatedly tested by careful removal of bound antibodies after each assay.

To confirm the specificity of the binding, inhibition studies with serum samples preincubated with a HPHT-extract, i.e. concentrated rat urinary proteins, were performed. Briefly, the blocked pins were incubated overnight with preincubated serum followed by washing and incubation with the secondary antibody as described above.

### *Allergen-specific IgE analysis*

Purified proteins corresponding to the allergens Rat n 1.02 ( $\alpha_{2u}$ -globulin) or Rat n 1.01 (pre-albumin) (21) were tested in ELISA (V). Microtiter plates were coated with the respective proteins and then separately incubated with different dilutions of individual patient sera. Alkaline phosphatase conjugated goat anti-human IgE (Tago), diluted 1:500, was added. The substrate pNPP (Sigma) was used and the optical density was read at 405 nm in a plate reader (SPECTRAMax™ 250, Molecular Devices).

### *Enzymatic cleavage*

The purified proteins corresponding to the allergens Rat n 1.01 or Rat n 1.02 were after separation by SDS-PAGE digested using the enzyme endoproteinase Lys-C (74, 75). Resulting peptides were separated by reversed-phase liquid chromatography on a Smart System (Pharmacia Biotech) and then sequence analysed with a 494A PE-Applied Biosystem instrument (Applied Biosystems, Foster City, CA, USA) (V).

### *Molecular mass determination of the peptides*

Molecular masses of isolated peptides were determined by mass spectrometry on a BIFLEX MALDI-TOF (Bruker Franzen Analytik GmbH, Bremen, Germany) according to the manufacturer's recommendation (V).



*Computer analysis of the amino acid sequence of  $\alpha_{2u}$ -globulin for prediction of epitopes, homology and peptide mass*

For B-cell epitope prediction the model developed by Chou and Fasman (37) was utilized (V).

The BLAST program (Basic Local Alignment Search Tool) (6) was used to search comprehensive protein sequence databases for other proteins having amino acid sequence homology to  $\alpha_{2u}$ -globulin (V).

Theoretical enzymatic cleavage of  $\alpha_{2u}$ -globulin with Lys-C was performed with the program PeptideMass (12), (V).

### 3. Results

#### **Urine sampling and precipitation of $\alpha_{2u}$ -globulin (I)**

The influence of the collection method and the type of urine sampled in the starting material was investigated. Rat urine samples were collected partly in metabolic cages (day-time and night-time) and partly by bladder puncture. Electrophoretic analysis under reducing conditions showed that rat urine contained a complex mixture of proteins as revealed by silver staining. Both the collection method and the time of sampling influenced the pattern and the protein contents in the bands. For instance, proteins with a molecular weight of 14 400 or less were missing in the day-time cage urine. The bladder urine contained the highest protein concentration and the day-time cage urine the lowest.

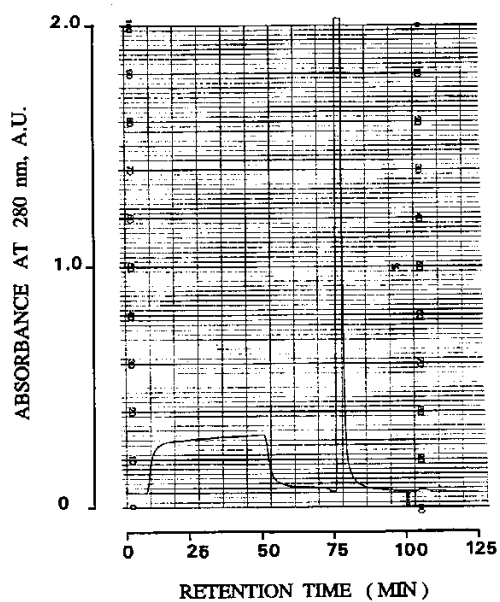
The effect of different storage conditions of the urinary samples was also examined. Hence, protein modification due to prolonged incubation time at room temperature was studied. It was found that incubation times as long as 72 h had little effect on the urine proteins in bladder and day-time samples. Night-time urine was more affected by this procedure, however, and displayed reduced amounts particularly of low molecular weight proteins and above all the proteins with  $M_r$  10 000, 16 000, and 32 000 which were abundant in bladder urine. In addition, long time storage in the freezer at  $-70^\circ\text{C}$  resulted in a change of the distribution pattern towards lower molecular weight components, especially proteins below  $M_r$  32 800 (unpublished results). This change was seen both with night- and day-time urine. Bladder urine, however, was not investigated with regard to this aspect.

Three different buffers were tested to evaluate the effectiveness of precipitating  $\alpha_{2u}$ -globulin by dialysis. 50 mM ammonium hydrogen carbonate buffer, 4 mM phosphate buffer pH 6.8 and deionized water were used. Precipitation of  $\alpha_{2u}$ -globulin occurred only after dialysis against deionized water. The precipitate was removed by centrifugation and the supernatant was analyzed for the presence of  $\alpha_{2u}$ -globulin.  $\alpha_{2u}$ -Globulin was not totally precipitated, since the electrophoretic analysis of the supernatant showed presence of this protein. Nor did the precipitate contain solely  $\alpha_{2u}$ -globulin as indicated by electrophoresis following ultrafiltration concentration of the resuspended precipitate. Thus several low molecular weight polypeptides co-precipitated with the  $\alpha_{2u}$ -globulin protein.

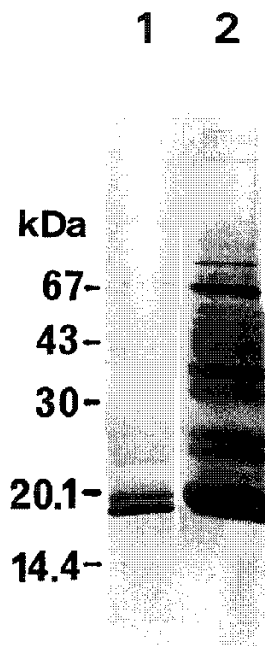
#### **Protein concentration procedures (I, II)**

One centrifugation method utilizing ultrafiltration and one chromatographic method were tested for the concentration of urinary proteins. Two molecular weight cut-off sizes were used in the Centriprep ultrafiltration concentration method (I), 10 000 and 30 000, respectively. The fraction retained after Centriprep 10 000 contained preferably proteins of molecular weights  $< 30\ 000$ . The Centriprep method was quick but gave incomplete recovery of low molecular weight proteins, possibly due to absorption of proteins to the membrane surface. By the Centriprep method non-protein components were also concentrated. Electrophoretic analyses revealed that  $\alpha_{2u}$ -globulin obtained by precipitation was more pure than that prepared by the Centriprep method.

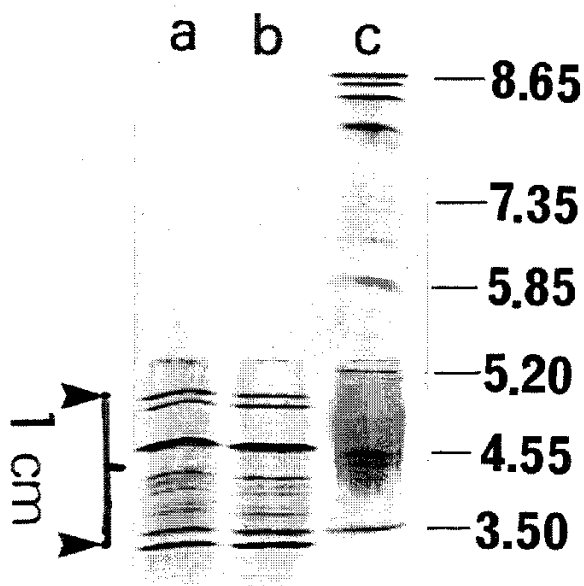
When using the chromatographic concentration method (78), utilizing hydroxyapatite (Fig. 2), a protein preparation was obtained containing all components in the rat urine except the non-protein substances. This mild and relatively rapid method could preferably be automated. Both SDS-PAGE (Fig. 3) and IEF (Fig. 4) revealed, after staining, complex protein patterns confirming our own earlier findings (78) and partially those of others (67, 92).



**Figure 2.** Light absorption curve obtained when rat urinary proteins were concentrated on a hydroxyapatite column (7.8x100 mm). Light absorbance at 280 nm is shown in arbitrary units, A.U.



**Figure 3.** Rat urinary proteins separated by SDS-PAGE and visualized by Coomassie Brilliant Blue before (lane 1) and after (lane 2) concentration on a hydroxyapatite column.



**Figure 4.** Proteins separated by isoelectric focusing in the Phast System using ready-made gels pI 3-9 and visualized by silver staining. Samples, each 4  $\mu$ l: a) dialyzed rat urine; b) rat urine concentrated on hydroxyapatite, eluted and diluted 60 times; c) IEF-standards (Pharmacia).

## **Isolation and purification studies (II)**

Efforts were made to elaborate a sufficiently powerful isolation and purification method which was applicable for proteins in urine. High resolving chromatographic methods are important for successful isolation of proteins with similar properties. Hydroxyapatite concentrated rat urinary proteins were purified by gel filtration and ion exchange chromatography. Electrophoretic studies by SDS-PAGE of samples from the two major peaks in the gel filtration chromatogram resulted in similar molecular weights for the two protein fractions of 20 000 and 18 000, respectively. The gel filtration column used is particularly suitable for separation of low molecular weight proteins. Determination by mass spectrometry demonstrated that the proteins had molecular masses between 18 500 and 18 700 Da. In the gel filtration chromatogram the A<sub>280</sub> peak II was three times higher than peak I. Wavelength scanning in a spectrophotometer of samples corresponding to the two peaks also indicated that approximately three times more protein was present in the fractions of peak II than of peak I. Separation by ion exchange chromatography showed that the protein in the two fractions constituted charge variants. To improve the ion exchange separation conditions further different buffer systems were evaluated as well as another ultrafilter. The BA 83 filter previously used before sample application on the anion-exchange column was exchanged for the OE 66 filter (Schleicher & Schuell, Dassel, Germany) giving much higher recovery (unpublished data).

Two different extracts, the HPHT-extract and rat urine concentrated through YM-2 ultrafiltration, were studied by affinity chromatography. The coupling efficiency of the monoclonal antibody, mAb(6) with specificity for rat urinary allergens, to the AminoLink column was determined according to the instructions of the manufacturer and calculated to be 65%. The two fractions containing the immunosorbent purified proteins were studied by Phast SDS-PAGE followed by silverstaining. Two dominating proteins in the region between 14 400 and 20 100 could be seen and obviously the two isoallergens coeluted, i.e. mAb(6) thus recognized and bound both proteins (148) and unpublished results). Similar results were obtained by Western blotting utilizing biotinylated mAb(6) (148). On the other hand the antibody mAb(1) with specificity for rat urinary allergen bound only one protein. There was also a minor contamination of a protein with a size similar to that of albumin, 67 000, as revealed by Phast SDS-PAGE. When the fractions were separated in a gradient gel, 10-20%, no band at 67 000 could be seen, indicating that this was most likely a contamination in that particular Phast electrophoresis run.

## **Identification and characterization of certain proteins (II, V)**

Amino acid composition and sequence analysis conclusively showed that the two proteins corresponding to the two major peaks in the gel filtration chromatogram were closely related and actually different forms of the same parent protein,  $\alpha_{2u}$ -globulin. Peptide mapping after enzymatic digestion of these two allergens revealed two different peptide maps (V), which clearly verified our findings of the different isoforms of  $\alpha_{2u}$ -globulin. Subsequent amino acid sequencing of the proteolytic fragments confirmed the close relationship. It was further

demonstrated that the protein previously named pre-albumin is not transthyretin, which is a completely different protein with certain well described properties (44).

Isoelectric focusing of the two isoproteins on Phast gels showed that the isoelectric points were 4.55 for the allergen Rat n 1.01 (earlier called pre-albumin) and 5.2 for the allergen Rat n 1.02 ( $\alpha_{2u}$ -globulin), respectively (unpublished data).

Rat urinary albumin and transferrin in the high molecular weight fraction seen after gel filtration were identified by zone immunoelectrophoresis assay (ZIA). Sets of 20 glass capillaries filled with agarose gel solution containing specific antibodies against rat transferrin and albumin were used. Narrow immunoprecipitates with sharp zone fronts were produced with migration distances directly proportional to the concentration of the respective proteins. This phenomenon can be used for their quantification. Changes in the transferrin concentration in unprocessed rat urine related to the age of the male rats were also studied by ZIA. The highest concentration of transferrin was obtained in the urine of the oldest rats, and the night-time urine contained a higher concentration of transferrin than day-time urine (unpublished data).

The allergenic potency of the two isoproteins was confirmed by strong skin prick test reactions in patients allergic to laboratory animals. Rat n 1.01 seemed to be slightly more reactive than Rat n 1.02. In contrast to the reported dot blot results (IV) the response with the further purified rat albumin was weak.

Search for carbohydrate moieties in the glycoproteins immobilized on Western blotting membranes indicated that the two main proteins in rat urine, in the molecular weight range 18-20 kDa, were not glycosylated (unpublished data). Direct glycostaining of SDS-polyacrylamide gels confirmed the results obtained with Western blotting. No glycosylation of  $\alpha_{2u}$ -globulin and its modified form, previously termed pre-albumin, could be detected. However, with both these methods two other rat urinary proteins were detected which were shown to be most likely glycosylated. The 23 400 protein was revealed by Western blotting and another protein with an approximate molecular weight of 40 000 by the direct glycostaining procedure (unpublished data).

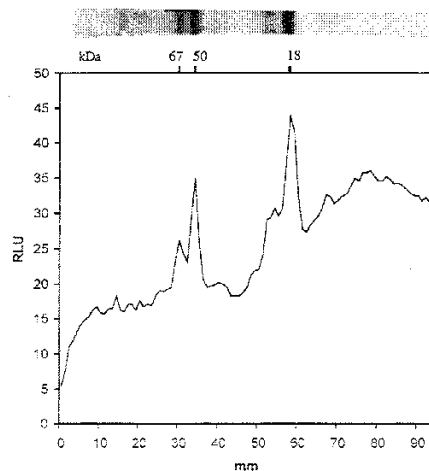
### **Quantification by chemiluminescence (III)**

Modification and evaluation of the chemiluminescence detection method as well as the luminometer previously described in (195) were performed. Here agarose gel electrophoresis under non-reducing conditions followed by Western blotting was used. The gray and black bands on the light sensitive film represent the protein bands on the blotting membrane producing light signals due to the chemiluminescence reaction. A good quantitative and linear relationship was achieved for the IgE 150 kDa protein band over the concentration range of 0.01 to 0.6 IU IgE and the relative light units (RLU) measured. Each position on the blotting membrane was measured for only 1.0 second in the luminometer apparatus. Equally comparable detection limits and linear relationships between the concentration of the antibody enzyme complex and RLU measured were obtained for dot blots as well. Both a single and a double antibody system, goat anti-human-IgE AP or rabbit-anti-human-IgE plus goat anti-rabbit-IgG AP, respectively, were found to have similar sensitivity. However, the double antibody system tended to give higher background probably due to extra incubation steps.

Lower concentrations of gelatin and Tween 20 were also found to contribute to higher background. Optimization of the experimental conditions of the assay was done by dot blot assay.

#### IgE-binding to rat urine extract (IV)

In order to study the allergenicity of rat urinary proteins they were separated by SDS-PAGE using a linear gradient under non-reducing conditions followed by Western blotting. As has been shown (I, IV) rat urine constitutes a complex mixture of proteins and several of these proteins demonstrated reactivity with IgE antibodies as revealed by immunoblotting. Chemiluminescence was preferred over color development using the Vectastain ABC-AP kit and the substrate BCIP/NBT for detection of IgE antibodies bound to blotting membranes due to the low abundance of antibodies. Precise side-by-side comparisons of IgE reactivity profiles to rat urinary proteins were obtained by cutting the membranes into strips before probing them individually with sera from rat-allergic subjects. Each allergic individual showed a specific pattern of IgE binding to the separated rat urinary proteins. All patients had IgE binding to Rat n 1.02,  $\alpha_{2u}$ -globulin. Albumin and other proteins with approximate molecular weights of 50 000, 60 000 and 205 000 were also recognized by IgE antibodies from the allergic patients. Scanning with the luminometer of Western blotting membranes or strips was performed along the lanes and not across them as was previously done (III) (Fig. 5). This was an improvement, giving more reliable measurements.



**Figure 5.** Luminometric scan of a PVDF membrane strip after immunoblotting. A film visualization of the strip is shown at the top of the figure where the dark bands represent rat urinary proteins binding IgE antibodies. The scanning was performed perpendicular to the protein bands along the lane and 1 h after CDP-Star substrate application.

Quantitative relationships for measured signal intensity, RLU, and the amount of rat urinary proteins were obtained. However, due to the varying avidities of the IgE molecules of individual sera the measured RLU values for a certain urinary protein are not directly comparable. Also here a good quantitative and linear relationship over the concentration range of 0.02 to 0.6 IU IgE was achieved for the IgE band, 150 000, and the RLU measured.

Studies of non-denatured allergens by the dot blot technique revealed feeble IgE binding to the proteins except for the albumin fraction which did bind IgE antibodies relatively strongly. Even with proteins concentrated by membrane centrifugation the binding was weak. On the other hand the HPHT-extract displayed strong IgE antibody binding even when diluted 100-fold. Non-concentrated rat serum albumin (Nordic), Soluprick (ALK) diluted 1:100, nondialyzed and dialyzed unconcentrated fertile male rat urine responded positively in the dot blot assay. Weak IgE antibody binding to the concentrated protein fractions was seen in Western blotting. Our results indicate that dot blot maintains more of the native protein conformation, which favors IgE binding. Furthermore, it was found that repeated thawing and freezing of the samples reduced their antibody binding capacity.

Three chemiluminescence substrates were tried. With CDP-Star 10 times stronger light signals were obtained, allowing shorter film exposure times.

In the dot blot assay no specific IgG binding to the rat urinary proteins separated by liquid chromatography could be seen despite the high abundance of total IgG as compared to IgE.

Furthermore, in dot blotting, sera from rat-allergic patients showed IgE antibody binding to proteins in saliva from atropinized and electrically stimulated rat salivary glands. In contrast the proteins in saliva from non-atropinized and non-stimulated rats displayed almost no antibody binding (unpublished results).

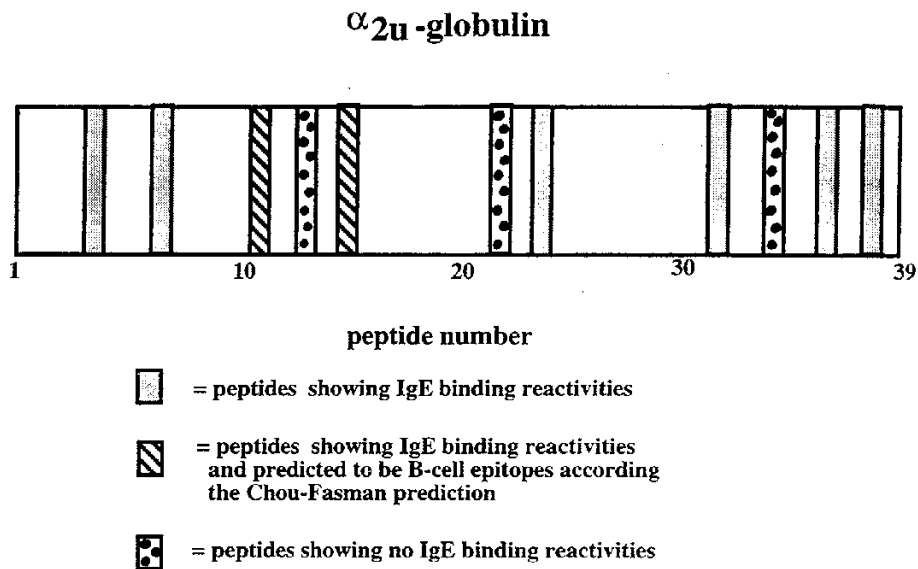
### **Epitope mapping (V)**

To investigate if certain regions of  $\alpha_{2u}$ -globulin preferentially might be involved in IgE binding, sera from rat-allergic donors were assayed using overlapping synthetic peptides representing the  $\alpha_{2u}$ -globulin amino acid sequence. Generally low absorbance values, representing specific IgE antibody binding, were obtained with the individual peptides. Only a few peptides were more frequently recognized by the IgE antibodies than others. After correction for the net absorbance of the synthetic peptides, an arbitrary cut-off of OD 0.05 was used to sift out the antibody binding peptides. By this criterion peptides no. 4, 7, 11, 15, 24, 32, 36, and 38 showed reactivity with 5 to 7 of the 15 sera studied. Likewise peptides 13, 22, and 34 were not recognized by any of the sera (Fig. 6). The specificity of the IgE reactivity was confirmed by inhibition studies using a concentrated rat urinary protein extract.

Also the IgG binding to the overlapping peptides was analyzed for a few sera from both allergic and non-allergic individuals but no specific pattern could be seen due to considerable variation in the individual sera.

B-cell epitope prediction analysis indicated that the most probable epitope region in the protein sequence is between the amino acids 50 and 102, which corresponds to the synthetic peptides no. 8-20.





**Figure 6.** Illustration of synthetic octapeptides showing IgE binding to different parts of the  $\alpha_{2u}$ -globulin molecule. Octapeptides spanning the protein sequence from the N-terminal end with four amino acids overlap were tested for IgE binding with sera from rat allergic individuals.

### Homology

Amino acid sequence homology to  $\alpha_{2u}$ -globulin among various proteins was studied. Fifty-nine alignments to  $\alpha_{2u}$ -globulin (with the access number P02761 in the Swiss protein database) were found using the BLAST program. This search engine explores all available sequence databases regardless of whether the query is protein or DNA. The BLAST program uses well-defined statistical methods (5, 85) to distinguish real matches from random background hits. Among the found homologs were several allergens, for instance bovine dander major allergen (Bos d 2), horse allergen (Equ c 1) and minor dog allergen (Can f 2) as well as mouse major urinary protein (Mus m 1), sharing respectively 27, 49, 30, and 65% identity with  $\alpha_{2u}$ -globulin. All these allergens including  $\alpha_{2u}$ -globulin are members of the lipocalin family - an interesting finding worth considering.

## 4. Discussion

Allergic reactions are complex, multifactorial processes. Knowledge about the biochemical and biological properties of allergenic proteins are of importance for the understanding of allergic diseases. Certain features might predispose proteins to become allergenic. Such information could be helpful when trying to understand the underlying causes of the progressive increase of allergic diseases. Early identification of persons at high risk of developing allergy is essential for prevention. For therapeutic purposes immunotherapy forms, with low risk-to-benefit ratio which not cause anaphylactic reactions, are desirable. In this context well characterized allergenic proteins are an advantage. Accordingly, the isolation, identification and characterization of the two closely related potent allergenic  $\alpha_{2u}$ -globulin proteins corresponding to the allergens Rat n 1.01 and Rat n 1.02, which this thesis is focused on, is hoped to contribute to the understanding of laboratory animal allergy, LAA.

### Urine sampling

Collection and handling of the raw material prior to purification could influence the properties of the urinary proteins and their subsequent identification and characterization. Thus, two different collection methods were studied; in metabolic cages and by bladder puncture. In addition two different sampling modes, day- and night-time urine, were studied. It was decided not to use preservatives and enzyme inhibitors, as this would more closely mimic normal conditions in experimental animal housing. Differences in the protein composition of the various types of urine samples were analyzed by electrophoresis and subsequent silverstaining. Each sample was shown to contain numerous separated protein bands as has been shown previously (78). There was variability among the samples with respect to the number of bands and the amount of protein in the bands. Both the contents and the relative distribution of protein in the bands were partly influenced by the collection method and partly by the time of sampling. Prolonged storage of urine in room temperature for 72 h seemed only to have effect on the night-time collected urine. Some bladder specific proteins of low molecular weight were not found in the night-time urine. These proteins are obviously particularly sensitive to different kinds of degradation upon exposure to air, which is what normally occurs in the cages. The reasons for these discrepancies between day- and night-time urine could be that night-time urine was collected for 18 h whereas day-time urine was collected during 6 h. Thus, the night-time urine could have been exposed to hydrolytic enzymes for a much longer period. The urine collected by bladder puncture had protein patterns somewhat different from urine samples collected in the cages.

The electrophoretic protein patterns of urine stored for a long time at  $-70^{\circ}\text{C}$  was somewhat altered as compared to that of fresh rat urine sampled in the same way. Thus, for aged urine as well as for long-term stored urinary protein fractions an occasional partial displacement of proteins towards lower molecular weights was

seen (unpublished data). Possibly this was due to enzymatic degradation. However, it was not within the scope of this study to examine in greater detail whether the differences seen were caused by oxidation, proteolysis by enzymes originally present in the urine, or by microbial growth.

### **Concentration of proteins**

Two different concentration methods were used: One chromatographic procedure utilizing hydroxyapatite (paper II) and an ultrafiltration method using Centriprep (paper I). The Centriprep method was rapid but gave incomplete recovery, and non-protein components were as well concentrated by this method. It should also be mentioned that precipitated  $\alpha_{2u}$ -globulin is more pure than that obtained by Centriprep concentration.

Chromatographic concentration on hydroxyapatite is both fast and gentle (78) and can be used for labile proteins (38). Hydroxyapatite has a high adsorbing capacity for proteins and accumulation of non-protein substances naturally occurring in urine is avoided. Thus, the hydroxyapatite method was milder, and contamination and denaturation of the urinary proteins was avoided to a greater extent; therefore this concentration method was preferred.

### **Purification**

There are several reports describing purification of  $\alpha_{2u}$ -globulin using various chromatographic methods (89, 94, 95, 105, 192). Nevertheless, these separation techniques were found not enough high resolving for the isolation of  $\alpha_{2u}$ -globulin. Therefore, another strategy was selected where hydroxyapatite-concentrated rat urinary proteins were purified by gel filtration and ion exchange chromatography using the FPLC system (paper II). In a previous study this separation procedure had been shown useful (78). The chromatogram of the gel filtration separation showed two major peaks, which were separable despite quite similar molecular protein masses. This might be due to different conformations of the proteins. Ion-exchange chromatography as well as isoelectric focusing revealed that there were charge variants or mixtures of closely related proteins. These minute differences between proteins could be caused by amino-acid microheterogeneity or C-terminal differences, or by point mutations that resulted in exchange of a few amino acids. The anion-exchange chromatography technique was further improved by modifying the buffer system and by the use of another type of ultrafilter prior to sample application on the Mono Q column.

Later an affinity chromatography method was tried, which used an AminoLink column and a mouse monoclonal antibody specific against rat urinary allergen, Rat n 1 (148). However, this immunosorbent technique did not separate the two isoallergens, Rat n 1.01 and Rat n 1.02 (148 and unpublished data), since the monoclonal mAb(6) specific against rat urinary allergen Rat n 1, used was shown to recognize both proteins. Rat n 1.01 and Rat n 1.02 correspond to the two isoproteins described above which were fractionated by liquid chromatography.

The preferred method for purification of rat urinary allergens utilize the unique concept of the FPLC system which comprise high performance chromatography at low pressure for the separation of biologically active molecules. In conclusion,

high resolving chromatographic methods are needed for isolation of isoallergens and proteins with similar properties. This purification procedure should also be suitable for allergens from other sources.

### **Electrophoretic studies**

Electrophoresis is a valuable tool for identification of proteins with regard to size and charge irrespective of sample origin. Analysis by electrophoresis is particularly suitable for complex samples such as urine. The purification procedure was monitored by electrophoretic methods and the proteins in the chromatographic fractions were analyzed with respect to isoelectric point (pI) by isoelectric focusing (IEF) and relative molecular mass ( $M_r$ ) by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and non-reduced conditions. In IEF, horizontal gels with the respective pH-gradients 3-9 and 4-6.5 (Phast-system) were used. The pI value obtained for the allergen Rat n 1.01 (4.55) is in agreement with previous studies (105, 106). The pI-value for Rat n 1.02 was slightly more acidic (5.2) than earlier reported by Longbottom (5.4, 5.6 and 5.8, as presented in different publications). The calculated pI for Rat n 1.02 is 5.48 (Swissprot, release 35). Here it should be mentioned that the pI value of a specific protein is influenced by certain experimental parameters. Thus when measurements are carried out at 4°C a value about 0.5 pH units higher than at 25°C may be obtained (197). Therefore fairly large discrepancies are to be expected, unless the conditions in the investigations compared are quite similar. Furthermore, to get good agreement between experimentally determined and calculated pI values the computer program used must be able to handle all parameters correctly.

In the SDS-PAGE, commercially available horizontal gels or vertical gel slabs made in our laboratory were used with a linear gel gradient of increasing acrylamide concentration and hence decreasing pore size. A gradient gel is most suitable for complex mixtures, as it gives higher resolution and sharper individual bands than homogeneous gels (113). In this study non-reducing conditions means the absence of 2-mercaptoethanol, which reduces the disulphide bonds in proteins, resulting in incomplete denaturation and binding of fewer SDS molecules to the polypeptide (9). Compared to the anionic detergent SDS, non-ionic detergents like Triton X-100 preserve the immunological activity and the native structure of the protein better because the protein is not disrupted into subunits (9).

Different molecular weight values are usually obtained in SDS-PAGE in the presence and absence of reducing agents. In the presence of reducing agents large amounts of the anionic detergent SDS are bound to the protein molecules resulting in dissociation into subunits. With large amounts of charged detergent molecules the net charge of each protein molecule per unit mass becomes approximately constant. The electrophoretic migration in the acrylamide gels is then proportional to the molecular weight of the protein. For proteins containing intra-disulphide bonds the absence of reducing agents results in incomplete unfolding of the polypeptide chain and thereby less binding of SDS molecules (9). This leads to changed migration of the protein in acrylamide gels. The positions of protein bands in such gels were different when reduced and non-reduced sample

preparations of the same protein were compared. The non-reduced samples showed protein bands with increased electrophoretic mobility having moved further down the gel. This could be misinterpreted as decreased  $M_r$ . Moreover, the extra bands that appeared in the reduced samples are probably due to oxidation products of the reducing agent (174) and/or dissociation of the proteins into subunits (9).

### **Identification and molecular characterization**

Throughout the purification steps, fractions and samples were analyzed with various electrophoretic methods. ZIA (194) was used to identify albumin and transferrin in the high molecular mass protein fraction eluted from the Hilo column. One advantage of this immunoprecipitation method is that large and varying sample volumes can be used. The method is also quite insensitive to variations in the molecular conformation and aggregation states of proteins. The age dependent increase in the excretion of urine proteins seen for the transferrin concentration is in accordance with the excretion of both total urinary proteins and  $\alpha_{2u}$ -globulin as reported by Neuhaus (128). The results obtained concerning the protein concentration in the day- and night-time urine as well as the transferrin contents measured by ZIA corroborate with those reported in another study by Neuhaus of the circadian rhythm of protein excretion (127).

Amino-acid compositional and sequence analysis followed by mass spectrometric determination were used for identification and characterization of the purified proteins described in Paper II. The results showed that the allergenic rat urinary protein denoted pre-albumin is not the same as the well characterized protein transthyretin (44) which has been thoroughly studied in blood samples. We have shown that this "non-transthyretin" protein and  $\alpha_2$ -euglobulin are different forms of the same protein,  $\alpha_{2u}$ -globulin. Our findings have bearing on the modern allergen nomenclature for these two proteins called Rat n 1.01 and Rat n 1.02, respectively (170, 171). Correct identification, characterization and nomenclature of individual allergens are important especially to avoid mistakes and misunderstanding. Multiple isoforms of  $\alpha_{2u}$ -globulin have been described to occur in various tissues (27, 70, 110, 151, 205). Hormonal, developmental (70), or age-dependent regulations (126, 151) have been suggested to explain these variations of  $\alpha_{2u}$ -globulin expression.

### **Glycosylation**

Glycosylation of a protein may be of significance for its antigenic properties and contribute to immunogenicity (172). Many allergens share characteristics displayed by viral and bacterial proteins to which the body has developed non-adaptive defence mechanisms. Macrophages possess a variety of different receptors, for example carbohydrate receptors (119). Thus, binding of the carbohydrate moiety of the glycoprotein to the receptor results in endocytosis followed by the release of mediators (172), which might lead to the development of hypersensitivity reactions. The carbohydrate binding receptors are important in host defence, particularly in the removal of bacteria (172). In this regard, the possible existence of glycosylated forms of  $\alpha_{2u}$ -globulin was studied in some

detail. According to the Swissprot database, release 35, there is a possible glycosylation site at the position 54, asparagine (Asn) in the full length protein of  $\alpha_{2u}$ -globulin. Essential for the N-glycosylation is the tripeptide Asn-X-Thr/Ser, where X is any amino acid except proline (64, 114). Glycosylation is inhibited by proline residues in position X and in the position next to Thr/Ser (64). A site lacking proline residues in these two positions has been estimated to be glycosylated in 93% of the cases studied (64). The putative proline free glycosylation site in  $\alpha_{2u}$ -globulin is the four amino acid long peptide sequence Asn-Gly-Ser-Met. Folding/unfolding of the protein is also suggested to be important in the regulation of the N-glycosylation (141). Exposure to the carbohydrate-transferring enzyme, oligosaccharyl transferase, which is found in the lumen of the endoplasmic reticulum, may also be of significance (64, 84, 99).

No evidence for glycosylation of the two main proteins in rat urine was obtained from Western blotting or direct glycostaining after SDS-PAGE (unpublished data).

It has been suggested that the carbohydrate content in  $\alpha_{2u}$ -globulin could be caused by unsuccessful cleavage of sugar residues prior to secretion of the protein from the liver (36, 93). Two glycosylated derivatives with the molecular weights 21.2 and 20.6 kDa have been described (36) as well as two non-glycosylated forms of the processed protein (18.8 kDa and 18.1 kDa) initially synthesized as two unprocessed forms containing a 19 amino acid signal sequence which is cleaved off prior to secretion (27). Other investigators have suggested that there are three forms of  $\alpha_{2u}$ -globulin in rat hepatocytes of which two are glycosylated, one with the molecular weight 75 kDa and another of 50 kDa (72). Female rats do not synthesize the non-glycosylated form of the 20 kDa  $\alpha_{2u}$ -globulin protein (152, 192). No real evidence for the existence of two glycosylated high molecular weight forms of  $\alpha_{2u}$ -globulin was obtained by us although two forms were reported by Haars and Pitot (see ref. 72). In contrast, we have indications supporting the presence of the two glycosylated unprocessed forms, 20.6 and 21.2 kDa, in both concentrated and untreated rat urine which is in agreement with results of Chatterjee *et al* (see ref 36).

### **Quantification of proteins by chemiluminescence**

Sensitive measurements of proteins blotted on membranes after Western blotting as well as after dot blotting were performed by chemiluminescence. A specially built luminometer was used. This instrument can be utilized for measurements directly on blotting membranes, in microtiter plates and test tubes (195). The chemiluminescence detection method and the newly developed luminometer were modified and evaluated (paper III). For evaluation of proteins separated by SDS agarose gel electrophoresis, non-reducing conditions were used to maintain the biological activity of the proteins. Agarose gel has a more open pore structure and is thus preferable to polyacrylamide gel for studies of large proteins such as IgE of 150 kDa. More protein can be applied per lane in agarose gels as compared to polyacrylamide gels, due to a lower risk of clogging the gel pores.

Major advantages of the chemiluminescence detection methods are that they do not require use of radioactive isotopes and still have very low detection limits and

wide measuring ranges. Further, the fact that the light signals are stable and are emitted for hours allows multiple measurements on a membrane. Permanent records of the blotting results can be generated by capturing the emitted light directly on film with the possibility of repeated film exposures. In addition, the luminescence reagent can be washed off and the membrane can be probed again with the substrate. This second probing gives almost equally good light signals. The membrane can also be processed to expose precipitated stained enzymatic products after the luminescence reaction (3).

The detection level for the IgE band in the immunoblotting membrane after agarose gel electrophoresis (paper III) was lower, 0.005 IU, than in the PVDF membrane after SDS-PAGE (paper IV), 0.02 IU. This could be explained by the more open pore structure in the agarose gel. Transfer of larger proteins to a membrane is more complete from agarose gels than from polyacrylamide gels (19). Thus, since some of the IgE molecules remain in the polyacrylamide gel this might result in a lower detection limit when this gel type is used.

The risk of contamination with phosphatase activity of microbial origin must be eliminated by the use of fresh and sterile filtered solutions as well as ethanol washed incubation trays and properly cleaned GelBond. It is important that the enzyme is saturated with substrate in order to obtain light signals proportional to the amount of enzyme. The white areas occasionally seen in films in the center of the dots or the bands could be due to too high concentration of the protein. When there is too much protein, large amounts of antibody-conjugated alkaline phosphatase are bound resulting in rapid dephosphorylation of the chemiluminescent substrate so that less light emission is seen after a while. The intensity of the signal then begins to decay shortly after substrate application, resulting in an underexposed area on the photographic film. To avoid this the amounts of protein at each position studied should be limited. Therefore we have examined various protein loads per lane in the electrophoresis gels as well as at dot blotting.

### **Allergenicity studies of rat urinary proteins**

Western blotting is often used to study separation of protein antigens present in complex mixtures such as rat urine, and also for their identification and comparison without prior isolation and purification. In addition to physicochemical characteristics such as  $M_r$  and pI of IgE-binding proteins, blotting provides important information on the occurrence and intensity of IgE antibody binding to allergens. Human IgE binding components in rat urine were thus studied by immunoblotting (paper IV). Non-reducing conditions were chosen to preserve the allergenic activity and because non-reduced proteins were shown to be transferred more easily to the blotting membrane than under reducing conditions. Individual IgE-binding patterns, reactivity profiles, were obtained and investigated in sera from rat-sensitive persons. The variability in the profiles seen in paper IV is probably due to different avidity of the antibodies which in turn may depend on the individual's different exposure or boosting to the allergens. Another factor which might influence the immune response to specific allergens is the genetic predisposition of the subjects (26).

Minor components which would not be visualized when using a serum pool could be detected when using individual sera. It should be noted that IgE antibodies from some patients were almost entirely directed against such urinary proteins with molecular weights below 35 kDa, of which there are only small amounts in rat urine. The binding profiles, exhibiting reactivity with both the low molecular weight constituent in the 20 kDa range as well as the albumin area, are in agreement with observations made by others (67, 77). Hollander et al (77) obtained the strongest reaction with the 15, 24, and 66 kDa proteins, while Gordon et al (67) observed the most prominent IgE-binding to the 17, 21, 23, 63, and 68 kDa proteins. The differences between the reactivity profiles seen in the three studies may reflect not only the above mentioned conditions but also variations in the methodologies, in the origin or preparation of the proteins. For instance, different rat strains were used which varied slightly in age. Sprague Dawley rats 15 weeks old were used in our study and Wistar rats 12 weeks old in the study by Gordon et al (67). In the study by Hollander et al (77) Wistar rats were used. This could be of some significance, since it is known that there may be species variations in the expression of urinary proteins (73). In addition, the sample preparations and analysis conditions were not exactly the same in the three studies. In our study and in the study by Gordon et al (67) non-reducing conditions were used, whereas in the study by Hollander et al (77) reducing conditions were utilized, which would most likely influence the reactivity patterns due to altered electrophoretic mobility of the proteins as discussed above. Further, the visualization systems used in these studies were different as well as the antibodies used for detection.

All patients in our study had IgE binding to Rat n 1.02, some more pronounced than others. However, the detection signal was relatively weak for purified Rat n 1.01 and Rat n 1.02, even after concentration by membrane centrifugation, in both Western blotting and the non-denaturing procedures using dot blot or line blot. The low detection signals could be due to losses by adsorption of the proteins to the walls of the vessels used for storage (glass/plastic) (111). This problem can be more pronounced at low protein concentration. Another possible cause for the weak signal is that some IgE-binding epitopes may be non-accessible for the IgE antibodies when the protein is bound to the membrane. In addition, transfer from the gel to the blotting membrane is not always quantitative (180) and thus some proteins will not be completely transferred. The agent used to block antigen binding sites in the membranes could also influence the detection of IgE-binding components, particularly those of low  $M_r$ . Blocking with Tween-20 would allow the detection of proteins with low  $M_r$  (180). In our studies gelatin was used for blocking, giving relatively low background and reduced the artefactual non-specific antibody binding. Loss of antigenicity due to conformational changes during the purification (17) could be another reason for low detection signals. This was evidenced by the weaker IgE antibody binding to the purified antigenic proteins compared to the corresponding native proteins present in the rat urine extract when analyzed by Western blotting. Furthermore, during the sample preparation before electrophoresis the proteins are exposed to heating and denaturation which will destroy their tertiary conformations; the proteins open out into linear forms that might only partly refold again in the PVDF membrane.



However, allergens have been reported to be multivalent and the IgE antibody response to them is probably polyclonal which means there is a good chance that sufficient determinants on the antigen molecules can survive the denaturing conditions to allow detection of each allergen under dissociating conditions (181). Another problem could be the presence of mainly low affinity IgE antibodies. Such antibodies may be lost during washing of the membrane resulting in weak detection signal. The varying avidities of the IgE molecules in individual sera as well as the quality of the secondary antibody are also important parameters. Anti-IgE antibodies used for detection but of lower specificity might result in unspecific binding and the appearance of bands in the PVDF membrane even with negative control sera due to unspecific binding.

When the Rat n 1.01 and Rat n 1.02 were used in skin prick tests strong reactions were obtained confirming earlier studies (78). *In vivo* tests are generally considered to be more sensitive than *in vitro* tests (189) but they rely on completely different mechanisms. Therefore they do not necessarily give concordant results. Thus, even though the reliability of these *in vivo* and *in vitro* methods is generally good, it is sometimes difficult to make an accurate allergic diagnosis by using only measurement of allergen-specific IgE concentrations. For example, low values of specific IgE do not necessarily imply that the patient is non-allergic. In sera from some rat allergic individuals a significant specific IgE-binding in Western blotting was observed despite low RAST values (unpublished data). In conclusion, Western blotting is an important tool for allergenicity studies of proteins. The method gives a good general view of the strongly varying individual IgE-binding patterns in sera from rat allergic patients regarding both distribution and intensity of the reactivity for different proteins.

### **IgE binding proteins in rat saliva**

In dot blot studies sera from rat allergic patients showed positive reaction with rat saliva, indicating presence of allergenic components. It may be mentioned that  $\alpha_{2u}$ -globulin has been reported by others to be present in rat saliva (62).

### **Stability of allergenic proteins**

Compared to other proteins most allergenic proteins are relatively resistant to enzymatic attack and chemical denaturation. The suggested reason for this is that these mostly environmental components are naturally subjected to degradation processes and thereby may resist host defence mechanisms more effectively (45, 176). Moreover, many of the food allergens, which are often storage proteins in plants, having molecular weights ranging between 10 and 70 kDa, are often stable molecules resistant to processing, cooking and digestion (98). Within the lipocalin family the conserved tertiary structure, the eight-stranded  $\beta$ -barrel conformation, forms a compact protease-resistant framework (137). Further,  $\alpha_{2u}$ -globulin is classified as a stable protein according to a computed instability index (71). The stability may allow these allergenic proteins to persist in various environments for comparatively longer times. On the other hand, allergens in aqueous extracts sometimes show limited stability upon storage. In this case the stability can be improved by lyophilization or addition of 50% glycerol (45). In addition, storage

of proteins at neutral pH may result in isoaspartic acid formation causing modification or loss of certain properties of the protein (204). Decreased stability of an allergen extract might involve loss of allergenic activity, which perhaps could explain the relatively low IgE-antibody binding obtained with the purified allergens in Western blotting and in dot blot assay.

Repeated thawing and freezing of the sample may also affect the binding of antigens with murine monoclonal antibodies (148) as well as human IgE antibodies (paper V and unpublished data). Storage of allergen extracts can sometimes influence the detection of the allergens with monoclonal antibodies (148). This is probably due to break down of proteins examined during storage as discussed above.

Human exposure to allergenic rat urinary proteins involves both the native proteins in the urine directly after excretion and proteins that have been more or less modified due to oxidation or microbial activity when urine dries and ages. However, as shown in paper I, electrophoretic studies of urine before and after special treatment (i.e. prolonged incubation at room temperature) showed that these treatments seemed to have minor effects on the protein composition. In addition, others (147) using mouse monoclonal antibodies specific against rat urinary allergen, Rat n 1, have recently shown that there is only a marginal effect on the detection of allergenic rat urinary proteins subjected to accelerated degradation (87). The purpose of accelerated degradation was to mimic what might happen with proteins in excreted urine in animal houses keeping rats.

### **Epitope mapping**

In this study we investigated in some detail IgE binding determinants of  $\alpha_{2u}$ -globulin, Rat n 1.02. Overlapping peptides of Rat n 1.02 were synthesized on solid support and screened by a modified ELISA procedure (paper V). There are several advantages with synthesized peptides. For instance, they can be produced reproducibly in large amounts and thus offer more reliable and controllable results than peptide fragments obtained by enzymatic digestion of native and purified protein.

Sera from individuals who were allergic to rats were tested. Two possible IgE binding regions were identified: one located in the N-terminal and one in the C-terminal part of the protein, respectively. The N-terminal region involved peptides 4 and 7 and the C-terminus peptides 32, 36, and 38. However, relatively weak IgE binding with the synthetic octapeptides was seen as compared to the results obtained with the native whole protein molecules of  $\alpha_{2u}$ -globulin in direct ELISA. This observation indicates that allergen specific IgE molecules mainly recognize conformational epitopes and to a lesser extent primary sequences. If the synthetic fragment only constitutes a portion of the correct epitope this will cause weak IgE binding (181). In the full length protein this might be overcome since it contains several antigenic determinants to which the antibody could bind. The results also indicate that the IgE antibodies are directed to native Rat n 1.02 and, therefore, may bind less strongly to the unfolded protein. Another suggestion would be a quenched activity signal due to relatively high background reactions resulting mainly from cross-reacting antibodies as well as different exposure histories in the allergic individuals (35).

Other studies have shown low IgE binding to synthetic peptides of allergens like cat major allergen, Fel d 1, (190). Nevertheless, synthetic peptides have been used by many scientists to determine IgE epitopes in several allergens (15, 16, 52, 53, 118, 201).

Even though the peptides probably represent only parts of a more complex discontinuous epitope (as is the case for the vast majority found in proteins) these linear fragments may have characteristic conformational features resembling those of the closely related structures in the parent protein and, therefore, it is relevant to identify them. Furthermore, a suitable IgE binding conformation may be formed in the peptide when it binds to the antibody paratope (138). However, T cell epitopes should also be studied to confirm useful and relevant allergen specific epitopes for immunotherapy as discussed below.

Another theoretical explanation for the low seroreactivity of the synthetic peptides could be that the IgE epitopes might be blocked by IgG antibodies due to their higher abundance in serum as compared to the IgE antibodies.

## Homology studies

Comparisons of amino acid sequences in a homology search with the BLAST program revealed 59 homologies, most of them from mammalian species (paper V). Several of them were recognized as allergens, for instance, bovine major dander allergen (Bos d 2), mouse major urinary protein (Mus m 1), horse allergen (Equ c 1) and dog allergen (Can f 2). Allergenicity might be a general property shared by a group of lipocalins with one or several epitopes in common. This is an interesting notion which ought to be investigated in more detail.

Three-dimensional protein profile analysis has been proven to be useful for homology modelling (29). This profile-search technique has been applied to lipocalins and related proteins and the results suggest that there are two distinct lipocalin sub-families (156), one that corresponds to e-RABP (epididymal retinoic acid binding protein) and the other comprising, among other proteins, A2U ( $\alpha_{2u}$ -globulin) as well as MUP (mouse urinary protein). By profile searching a total of 70 lipocalins and 15 related non-lipocalin proteins were shown to have the characteristic lipocalin fold (156). Since the sequence homology within the lipocalin family on the average is only about 20% (60) the BLAST program search resulted in fewer lipocalin members than other investigators have found by 3-D searching (156).

In contrast, the structural homology is greater. The lipocalins have a phylogenetically conserved structure with an eight-stranded  $\beta$ -barrel conformation which is lined with hydrophobic side chains containing aromatic residues (27, 34, 66, 206). The composition of the side chains differs between the proteins in the  $\alpha_{2u}$ -globulin superfamily and thereby determines their ligand specificity (27). In  $\alpha_{2u}$ -globulin the pocket is formed by the amino acids 59, 73, 75, 109, 122, 137, and 139 (using the numbering in the SWISS-PROT Protein Sequence Database) (34). The residues in the  $\alpha_{2u}$ -globulin protein which form the base of the binding site for the natural ligand are Phe 73, 109 and 122 (34, 81) which noncovalently can bind lipophilic compounds, for example metabolites of compounds present in unleaded gasoline (175). Although the binding site of  $\alpha_{2u}$ -globulin has been

studied (81) the natural ligands have not yet been identified. The conserved folding pattern contains three characteristic sequence forms, so-called motifs, corresponding to the three largest structurally conserved regions (60). Motifs 1 and 2 lie close to each other in the three-dimensional structure, and they are also near the surface of the protein (132) and thereby they most likely constitute a binding site. The residues Phe 109 and 122 seem to be located in motif 2 but Phe 73 is not located in any of the three motifs. Further, the IgE binding synthetic peptides 4 and 7, corresponding to the N-terminus amino acids no. 33 - 40 and 45 - 52, respectively, in the  $\alpha_{2u}$ -globulin protein, would probably be located within motif 1. In the C-terminus part of the protein sequence peptide 32, corresponding to amino acids no. 145 - 152, might be located within motif 3. Peptide 22 (amino acids no. 105 - 112), not recognized by any of the sera tested according to the criteria used here to define an IgE binding peptide, could partly be located in motif 2. Also peptide 15, amino acids no. 77 - 84, appeared to be located near this cavity. However, the IgE binding to the peptides corresponding to these three motifs was relatively low in sera of most of the individuals studied. Further investigations are required to define if the IgE binding regions found in this study could be located close to this pocket in the tertiary structure. In this connection structure comparison of a certain protein antigen with known allergens and their allergenic epitopes could be worth further studies.

The alignment GXW, where X is usually an acidic amino acid, is a key sequence conserved in most lipocalins (163). In  $\alpha_{2u}$ -globulin this sequence is assigned to GDW and most likely located within motif 1 (60), i.e. the amino acid positions 36-38.

Binding of different substances such as metabolites of fuel and solvent derived hydrocarbons, various chemicals or pheromones could induce structural alterations of the protein (27, 34, 96, 134). Due to this conformational changes owing to the ligand binding new epitopes may be exposed or some epitopes may even be concealed. This could affect the recognition by human immunocompetent cells or antibodies. However, ligand studies were not within the scope of this thesis.

### **Future aspects and significance of the studies in relation to diagnosis of allergy and immunotherapy**

Enhanced production of the cytokines IL-4 and IL-13 by allergen-specific T cells is responsible for increased IgE synthesis and the development of allergic diseases (41) which affect a large proportion of the individuals in a population. There are several mechanisms by which allergic symptoms are believed to be reduced. It is believed that this could be achieved by diminishing the production of Th1 and Th2 cytokines or forcing the Th2 response towards a Th1 response (88). Desensitization involving repeated low-dose subcutaneous injections of standardized allergen extracts has been shown to induce a temporary tolerance to normal allergen doses in some allergic individuals (28). This might be due to a shift towards IgG production (28). The IgG antibodies bind to the allergen, forming a complex which could be removed by phagocytosis, and the allergen is thus no longer available for the IgE antibodies. A major drawback of this procedure is that in the allergen extracts commercially available the content and

relative abundance of individual proteins, i.e. the antigen composition, can vary markedly both between manufacturers and between batches from the same manufacturer (180, 185). Careful physicochemical and immunological characterization of the proteins in such extracts would therefore be helpful for standardization, as has been pointed out by others (45, 191). In this context, the use of recombinant proteins offers an alternative for therapy and diagnosis of allergic diseases (88, 185). Large quantities of well-defined recombinant allergens can be produced using DNA-technology (157). This implies more reliable diagnosis and safer treatment. However, one disadvantage with the use of recombinant allergens is that the IgE-binding could be less than for their natural counterparts (30, 56, 57, 184, 187). Allergy diagnosis has been performed with various recombinant proteins, such as grass and tree-pollen allergens (91, 120, 136, 184, 186). Antibodies specific to a certain allergen in an extract could be detected by using recombinant allergens which would be useful in determining patients' allergen profiles and when following treatment during the disease (88). Also cross-reactivity of IgE for allergens in tree-pollen, fruit and vegetables has been studied with recombinant proteins (48). Naturally occurring hypoallergenic isoforms of allergens with no or very low IgE binding capacity but with a strong T cell inducing response have been shown to be useful for immunotherapy (56, 88). Production of such recombinant isoforms facilitates high dose treatment in immunotherapy. Another suggested approach for safe treatment is the use of modulated variants of recombinantly produced allergens (88). By exchanging a few single amino acids in the sequence of the major birch-pollen allergen, Bet v 1, a variant with low IgE binding ability but with conserved T cells reactivity was obtained (55).

It has been reported that gene vaccination could be useful as a variant of allergen immunization (167, 179). In this technique plasmid DNA encoding an allergen are used which induces a Th1 response (146). The advantage of this technique is that it seems to be a safer form of immunotherapy since the allergens are produced in the host's cells and are mainly intracellular. Very little allergen is secreted and thereby are risks of anaphylactic reactions diminished (150, 167). This is the case in both rats and mice when immunized with plasmid DNA (166).

Immunotherapy is especially successful in younger patients with seasonal allergy, who are sensitized to only a few allergens (125). In the search for a cure or treatment of the allergic diseases, peptide immunotherapy might be useful. Injecting synthetic peptides representing linear B-cell epitopes could be an alternative when trying to induce tolerance in type I hypersensitive patients. Thus, identification of low affinity IgE-binding epitopes yet with high T cell activating capacity could become useful for immunotherapy. However, in our study (paper V) only B-cell epitopes were studied. It is possible though, that the synthetic peptides 13, 22, and 34 in the  $\alpha_{2u}$ -globulin sequence, which were not recognized by IgE antibodies in any of the sera from the rat allergic patients, might be suitable candidates for immunotherapeutical treatment. However, the T-cell stimulating ability of these peptides needs to be investigated in order to confirm this possibility.

Immunotherapy has been shown to be successful in the treatment of bee venom or yellow jacket venom allergies. Repeated injections of fragments from pepsin-digested bee venom phospholipase A<sub>2</sub> allergen into allergic patients have been

shown to reduce allergen specific IgE and IgG antibody responses (104). In addition, administration of peptides from pepsin-digested ragweed pollen extract has been found to prevent seasonal increases in the concentration of ragweed allergen specific IgE antibodies (103).

To conclude, detailed characterization of protein allergens will undoubtedly become more important not only for diagnostic purposes - *in vitro* allergen tests and standardization of extracts for prick test - but also for immunotherapy. Particularly as the biochemical properties of allergenic proteins probably are of importance for their processing at mucosal surfaces and how the immune system responds to them. It is also possible that certain biochemical or biological properties might predispose proteins to become allergenic especially since IgE cross-reactivity with related as well as unrelated allergens has been observed (48, 168).

## Concluding remarks

The continuing increase in the prevalence of allergic diseases of both environmental and occupational origin calls for extended studies of the nature of allergens. Well characterized and purified allergens allow studies of various aspects of allergy. Laboratory animal allergy (LAA) is classified as an occupational disease. Exposure to rat urine is one of the most common causes of LAA with symptoms such as rhinitis, conjunctivitis, urticaria and asthma.

This thesis deals mainly with the isolation, identification and characterization of some potent allergenic proteins believed to contribute to better understanding of allergic diseases and improved possibilities for diagnosis or treatment of allergies. The following can be concluded:

The chromatographic procedures utilized for isolation and characterization of rat urinary allergens can also be useful for allergens from other sources.

Chemiluminescence and the newly developed luminometer offer high sensitivity and wide measuring ranges for proteins.

IgE binding regions of the major protein  $\alpha_{2u}$ -globulin, corresponding to the allergen Rat n 1.02, were identified using overlapping peptides spanning the whole protein sequence.

# Summary

Bayard C. Purification, characterization and immunological studies of rat urinary proteins causing allergy in humans; *Arbete och Hälsa 1998:15*. National Institute for Working Life, Solna, Sweden.

Rats are among the most frequently used laboratory animals and rat allergy constitutes a common occupational problem. Approximately 20-30% of the personnel engaged in work with laboratory animals show symptoms of allergy. These include rhinitis, conjunctivitis, urticaria and sometimes asthma, symptoms which usually develop during the first three years of exposure. The allergic reaction arises from direct contact with rat urine, or by exposure to airborne dusts originating from dried animal urine. Rat urine contains a complex mixture of proteins some of which are known to be allergenic, for example  $\alpha_{2u}$ -globulin. In urine from fertile male rats  $\alpha_{2u}$ -globulin is one of the most abundant protein.

The primary aim of this thesis was to purify and identify the most potent allergens in rat urine. Knowledge about the biochemical and biological properties of allergenic proteins is of importance for the understanding of allergic diseases. Consequently, two other aims of this thesis were to optimize various assays for evaluation of the allergenicity of the identified allergens and to examine in some detail the IgE binding regions of the major allergen, Rat n 1.02,  $\alpha_{2u}$ -globulin.

Rat urinary proteins were separated and purified by ultrafiltration through centrifugation and by chromatographic methods. The high resolving chromatographic purification procedures used involve molecular size separation by gel filtration followed by charge separation on an ion exchange column. The two purified dominating proteins were identified as different forms of the same parent protein,  $\alpha_{2u}$ -globulin, by amino-acid compositional and sequence analysis as well as mass spectrometry. Electrophoretic methods were also used to analyze the proteins with respect to relative molecular mass by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and isoelectric point was determined by isoelectric focusing. It was further proved that the urinary protein previously named pre-albumin had no amino acid sequence resemblance to prealbumin (transthyretin) present in rat serum. Additionally, the procedures reported here for isolation and studies of allergens can be used for examination of allergens from other sources.

The allergenicity of the rat urinary proteins was studied with Western blotting using IgE antibodies in sera from allergic patients. A detection system utilizing chemiluminescence and a luminometer apparatus gave scanning curves of IgE binding and allowed arbitrary measurement of the amount of IgE bound to a certain urine protein. Specific patterns of IgE binding to the electrophoretically separated rat urinary proteins were shown for each individual. All allergic subjects studied had IgE antibodies binding to the major urinary protein,  $\alpha_{2u}$ -globulin. In most of the sera examined the IgE antibodies specifically binding to  $\alpha_{2u}$ -globulin were more abundant than such binding to other proteins. However, this protein was not always the most dominating allergen.



Immunoblotting offered a good overview of the strongly varying individual IgE-binding patterns seen in sera from rat allergic subjects regarding both distribution and intensity of the binding.

Overlapping octapeptides corresponding to the amino acid sequence of the major allergen Rat n 1.02 were synthesized on solid support and screened in parallel to pinpoint the IgE-binding regions of the protein using a modified ELISA procedure. Our results indicate the existence of linear IgE binding epitopes, mainly located towards the N-terminal and C-terminal parts of the protein, as recognized by IgE antibodies in the studied sera. The role of these short amino acid sequences in the allergic reaction and their appropriateness for immunotherapy calls for further investigation.

In conclusion, this thesis has contributed to the isolation, identification and characterization of the allergens involved in a major occupational disease among people who work with laboratory animals, namely rat allergy.

Keywords: allergen, laboratory animal allergy, rat urinary protein, Rat n 1.02,  $\alpha_{2u}$ -globulin, purification, chemiluminescence.

# Sammanfattning

Bayard C. Purification, characterization and immunological studies of rat urinary proteins causing allergy in humans; *Arbete och Hälsa 1998:15*. National Institute for Working Life, Solna, Sweden.

Upp till 30% av personer som hanterar försöksdjur drabbas av allergiska symtom vilka kan relateras till kontakter med djur. Typiska symtom är ökat tårflöde, urtikaria och astma. Dessa utvecklas ofta inom tre år efter första exponeringen. Råttor är ett av de vanligaste försöksdjuren och deras urin innehåller en komplex blandning av olika proteiner. Speciellt hanråttors urin har visat sig innehålla allergiframkallande proteiner som t.ex.  $\alpha_{2u}$ -globulin. Den allergiska reaktionen kan uppkomma genom direkt kontakt med råtturin eller genom inandning av luftburna partiklar i form av damm som härrör från intorkad urin. I urin från unga fertila hanråttor är  $\alpha_{2u}$ -globulin det kvantitativt mest förekommande proteinet.

Studiens syftet var att rena och identifiera de mest potenta allergenerna i råtturin och sedan studera deras allergenicitet med känsliga delvis nya metoder. Dessutom omfattade studien undersökningar av IgE bindande regioner på huvudallergen, Rat n 1.02,  $\alpha_{2u}$ -globulin.

Råtturinproteiner separerades och renades genom ultrafiltrering och högupplösande kromatografiska metoder. Storleksseparation utfördes genom gelfiltrering följt av uppdelning efter laddning på en jonbytarkolonn. Även isoelektrisk fokusering och SDS-polyakrylamidgelelektrofores användes för att analysera proteinerna. Två dominerande proteiner identifierades som olika varianter av samma moderprotein,  $\alpha_{2u}$ -globulin, genom aminosyraanalys och sekvensering samt masspektrometrisk bestämning. Härmed bevisades också att det protein som tidigare kallats pre-albumin inte hade någon aminosyrasekvensiell likhet med prealbumin (transthyretin) i råttserum. De beskrivna separationsmetoderna kan även användas för rening och studier av andra allergener.

IgE antikroppar som finns i sera från råttallergiska patienter studerades med kemiluminiscens och immunoblotting. Individuella reaktivitetsmönster för de elektroforetiskt separerade råtturinproteinerna kunde ses och IgE i alla patientsera band till huvudprotein,  $\alpha_{2u}$ -globulin. Dock var detta protein inte alltid det mest dominanta proteinet som kändes igen i alla sera. Med en specialutvecklad luminometer kunde relativ mängd av IgE som bundit till olika råtturinproteiner mätas i varje sera, samt diagram erhållas som illustrerade relativa mängder bundet IgE.

Överlappande peptider av huvudallergen Rat n 1.02 syntetiserades på fast fas och undersöktes med en modifierad ELISA-metod för att lokalisera IgE bindande regioner. Indikation på linjära IgE bindande epitoper erhöles vid proteinets N- och C-terminala delar. Ytterligare studier av peptidernas roll för den allergiska reaktionen och deras eventuella användbarhet inom immunoterapi bör dock göras.

Sammanfattningsvis kan sägas att denna studie har bidragit till isolering och karakterisering av allergener, vilka ger upphov till yrkesrelaterad råttallergi bland dem som arbetar med laboratoriedjur.

Nyckelord: allergener, laboratoriedjursallergi, råtturinproteiner, Rat n 1.02,  $\alpha_{2u}$ -globulin, rening, kemiluminiscens.

# Acknowledgements

I wish to express my sincere gratitude to everyone in *Lunggruppen*, National Institute for Working Life for support, pleasant times and joyous acclamations during all these years working with this thesis ("... ad infinitum"). In particular I would like to thank:

**Olof Vesterberg**, my supervisor, for introducing me to the world of electrophoresis, for always being enthusiastic about my results and for sharing some of your knowledge.

**Marita Troye-Blomberg**, Department of Immunology, Stockholm University, my co-supervisor, for constructive help when needed and for showing interest in my work.

**Göran Möller**, head of the Department of Immunology, Stockholm University.

**Per Malmberg**, head of the Department of Occupational Medicine, for financial support towards the end of my thesis work and **Kjell Larsson**, head of the *Lung-group*.

**Fernando Acevedo**, lab.mate and co-author, for constructive discussions, for helping me with tricky pictures and for your exuberant enthusiasm.

**Siw Siljerud**, for invaluable and very much appreciated help with running the FPLC and the Phast as well as protein determination and darkroom work (your "favourite" work) and for nice times during the lunch-hours.

**Maud Kaddik**, for nice chats about everything under the sun and for help with paper-work of administrative character whenever I needed it.

**Alexandra Ek and Anne-Sophie Karlsson**, for friendship and jolly times both during and after work.

**Britt-Marie Larsson, Lena Palmberg, Brittis Sundblad, Lotta Müller-Suur, Anne Renström, Ann-Mari Lindqvist, Marie Hedrén and Zhiping Wang**, for nice "girlish" times.

**Helene Saranius**, for doing the ZIA experiments and for nice chats about everything or nothing.

**Birgit Åkerlund**, for help with protein determination and running ZIA experiments.

**Hildegard Moravscik**, for invaluable help with the washing-up.

**Leif Holmquist**, for constructive criticism and help during my early thesis work and when writing paper II.

**Ulla Sundberg**, for helping me type some of the references.

**The "allergy-group"** at the Department of Immunology, Stockholm University, for fruitful discussions.

**Janet Holmén**, for linguistic proof-reading of the thesis. However, improper expressions added as last minute complements are due to me.

**Jörgen Ekström**, Department of Pharmacology, University of Gothenburg, for kindly providing the parotid saliva.

**To all my friends**, for simply being what you are and having patience with me.

**My parents**, for your never ending support, for always being there for me whenever I needed and for keeping my freezer and fridge full.

**My sister and brother**, for being there and for growing up with me and for my brother's generous support.

This work was supported by the Swedish Work Environment Fund and by Stiftelsen Clas Groschinskys minnesfond.

# References

1. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996;383:787-793.
2. Acevedo F, Marin V, Wasserman M. Electrophoretic size separation of proteins treated with sodium dodecyl sulfate in 1 % agarose gels. *Electrophoresis* 1995;16:1394-1400.
3. Acevedo F, Vesterberg O, Bayard C. Visualization and quantification of birch-pollen allergens directly on air-sampling filters. *Allergy* 1998;53:594-601.
4. Agrup G, Belin L, Sjöstedt L, Skerfving S. Allergy to laboratory animals in laboratory technicians and animal keepers. *Br J Ind Med* 1986;43:192-198.
5. Altschul SF. A protein alignment scoring system sensitive at all evolutionary distances. *J Mol Evol* 1993;36:290-300.
6. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403-410.
7. Anderson MC, Bear H, Ohman JL. A comparative study of the allergens of cat urine, serum, saliva and pelt. *J Allergy Clin Immunol* 1985;76:563-569.
8. Anderzén I, Arnetz BB, Söderström T, Söderman E. Stress and sensitization in children: a controlled prospective psychophysiological study of children exposed to international relocation. *J Psychosom Res* 1997;43:259-269.
9. Andrews AT. *Electrophoresis. Theory, techniques, and biochemical and clinical applications.* (2nd ed.) New York: Oxford University press, 1986:452 pp.
10. Antakly T, Pelletier G, Feigelson P.  $\alpha_{2u}$ -Globulin is present in the rat anterior pituitary. *Proc Natl Acad Sci USA* 1983;80:4000-4002.
11. Aoyama K, Ueda A, Manda F, Matsushita T, Ueda T, Yamauchi C. Allergy to laboratory animals: an epidemiological study. *Br J Ind Med* 1992;49:41-47.
12. Appel RD, Bairoch A, Hochstrasser DF. A new generation of information retrieval tools for biologists: the example of the ExPASy WWW server. *Trends Biochem Sci* 1994;19:258-260.
13. Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: coordinators of immune and inflammatory responses. *Annu Rev Biochem* 1990;59:783-836.
14. Arpin C, Déchanet J, Van Kooten C, Merville P, Grouard G, Brière F, Banchereau J, Liu Y-J. Generation of memory B cells and plasma cells in vitro. *Science* 1995;268:720-722.
15. Atassi H, Atassi MZ. Antibody recognition of ragweed allergen Ra3: localization of the full profile of the continuous antigenic sites by synthetic overlapping peptides representing the entire protein chain. *Eur J Immunol* 1986;16:229-235.
16. Atassi H, Atassi Z. Localization of the continuous allergenic sites of ragweed allergen Ra3 by a comprehensive synthetic strategy. *FEBS Lett* 1985;188:96-100.
17. Awdeh ZL, Williamson AR, Askonas BA. One cell-one immunoglobulin. Origin of limited heterogeneity of myeloma proteins. *Biochem J* 1970;116:241-248.
18. Bacchini A, Gaetani E, Cavaggioni A. Pheromone binding proteins of the mouse, *Mus musculus*. *Experientia* 1992;48:419-421.
19. Baldo BAE. Paper symposium: Protein blotting. *Electrophoresis* 1993;14:829-960.
20. Bartholome K, Kissler W, Bear H, Kopiez-Schulte E, Wahn U. Where does cat allergen 1 come from? *J Allergy Clin Immunol* 1985;76:503-506.
21. Bayard C, Holmquist L, Vesterberg O. Purification and identification of allergenic  $\alpha_{2u}$ -globulin species of rat urine. *Biochim Biophys Acta* 1996;1290:129-134.
22. Bendiner E. Baron von Pirquet: The aristocrat who discovered and defined allergy. *Hosp Pract* 1981;16:137-158.

23. Björkstén B. Risk factors in early childhood for the development of atopic diseases. *Allergy* 1994;49:400-407.
24. Bluestone JA. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity* 1995;2:555-559.
25. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol* 1997;15:749-795.
26. Bonini S, Magrini L, Rotiroti G, Ronchetti MP, Onorati P. Genetic and environmental factors in the changing incidence of allergy. *Allergy* 1994;49:6-14.
27. Borghoff SJ, Short BG, Swenberg JA. Biochemical mechanisms and pathobiology of  $\alpha_{2u}$ -globulin nephropathy. *Annu Rev Pharmacol Toxicol* 1990;30:349-367.
28. Bousquet J, Lockey RF, Malling H-J. Allergen immunotherapy: therapeutic vaccines for allergic diseases. WHO position paper. *Allergy* 1998;53:Suppl. 44:1-42.
29. Bowie JU, Lüthy R, Eisenberg D. A method to identify protein sequences that fold into a known three-dimensional structure. *Science* 1991;253:164-170.
30. Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, Kraft D, Scheiner O. Four recombinant isoforms of *Cor a* I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur J Biochem* 1993;212:355-362.
31. Brooks DE. The major androgen-regulated secretory proteins of the rat epididymis bear sequence homology with members of the  $\alpha_{2u}$ -globulin superfamily. *Biochem Int* 1987;14:235-240.
32. Brown PR, Leiternann KM, Ohman JL. Distribution of cat allergen 1 in cat tissues and fluids. *Int Arch Allergy Appl Immunol* 1984;74:67-70.
33. Bryant DH, Boscato LM, Mboloi PN, Stuart MC. Allergy to laboratory animals among animal handlers. *Med J Aust* 1995;163:415-418.
34. Böcskei Z, Groom CR, Flower DR, Wright CE, Phillips SEV, Cavaggioni A, Findlay JBC, North ACT. Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. *Nature* 1992;360:186-188.
35. Carter JM. Epitope mapping of a protein using the Geysen (PEPSCAN) procedure. *Methods Mol Biol* 1994;36:207-223.
36. Chatterjee B, Motwani NM, Roy AK. Synthesis and processing of the dimorphic forms of rat  $\alpha_{2u}$ -globulin. *Biochim Biophys Acta* 1982;698:22-28.
37. Chou PY, Fasman GD. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv Enzymol* 1978;47:45-148.
38. Compton SW, Engelhorn SC. HPHT: High performance hydroxyapatite. *LC* 1983;1:3.
39. Cookson W, Moffatt M. Asthma: An epidemic in the absence of infection. *Science* 1997;275:41-42.
40. Dabrowski AJ, van der Brempt X, Soler M, Seguret N, Lucciani P, Charpin D, Vervloet D. Cat skin as an important source of *Fel d* I allergen. *J Allergy Clin Immunol* 1990;86:462-465.
41. de Vries JE. Novel fundamental approaches to intervening in IgE-mediated allergic diseases. *J Invest Dermatol* 1994;102:141-144.
42. de Vries JE, Zurawski G. Immunoregulatory properties of IL-13: its potential role in atopic disease. *Int Arch Allergy Immunol* 1995;106:175-179.
43. Delespesse G, Sarfati M, Wu CY, Fournier S, Letellier M. The low-affinity receptor for IgE. *Immunol Rev* 1992;125:77-97.
44. Dickson P, Howlett G, Schreiber G. Rat transthyretin (prealbumin). Molecular cloning, nucleotide sequence, and gene expression in liver and brain. *J Biol Chem* 1985;260:8214-8219.
45. Dreborg S, Einarsson R, Longbottom J. The chemistry and standardization of allergens. In: Weir D, ed. *Handbook of Experimental Immunology*. 4 ed. Oxford: Blackwell, 1986: 10.1-10.28. vol 1:Immunochemistry.

46. Dubray G, Bezard G. A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. *Anal Biochem* 1982;119:325-329.
47. Dugas B, Mencia-Huerta JM, Braquet P. The low affinity receptor for IgE (CD23) in human: a multifunctional receptor/cytokine? *Eur Cytokine Netw* 1992;3:35-41.
48. Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, Kraft D, Scheiner O. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* 1995;95:962-969.
49. Eggleston PA, Newill CA, Ansari AA, Pustelnik A, Lou S-R, Evans R, Marsh DG, Longbottom JL, Corn M. Task-related variation in airborne concentrations of laboratory animal allergens: Studies with *Rat n I*. *J Allergy Clin Immunol* 1989;84:347-352.
50. Eggleston PA, Wood RA. Management of allergies to animals. *Allergy Proc* 1992;13:289-292.
51. Ekström RC, Hoekstra WG. Investigation of putative androgen-like activity of  $\alpha_2\mu$ -globulin in castrated and estrogen-treated male rats. *Proc Soc Exp Biol Med* 1984;175:491-496.
52. Elsayed S, Apold J. Immunochemical analysis of cod fish allergen M: locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* 1983;38:449-459.
53. Elsayed S, Holen E, Dybendal T. Synthetic allergenic epitopes from the amino-terminal regions of the major allergens of hazel and birch pollen. *Int Arch Allergy Appl Immunol* 1989;89:410-415.
54. Eriksson E, Terenius L, Mattson K, Stagh T, Wiklund L. Använda försöksdjur i Sverige. *CFN:s Skriftserie* 1998;13-37.
55. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger H, Scheiner O. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998;12:231-242.
56. Ferreira F, Hirtlenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger H-J, Ebner C. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996;183:599-609.
57. Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, Steiner R, Bohle B, Sperr WR, Valent P, Kungl AJ, Breitenbach M, Kraft D, Scheiner O. Purification and characterization of recombinant Bet v I, the major birch pollen allergen. *J Biol Chem* 1993;268:19574-19580.
58. Firestein GS, Roeder WD, Laxer JA, Townsend KS, Weaver CT, Hom JT, Linton J, Torbett BE, Glasebrook AL. A new murine CD4<sup>+</sup> T cell subset with an unrestricted cytokine profile. *J Immunol* 1989;143:518-525.
59. Flower DR. The lipocalin protein family: structure and function. *Biochem J* 1996;318:1-14.
60. Flower DR, North ACT, Attwood TK. Structure and sequence relationships in the lipocalins and related proteins. *Protein Science* 1993;2:753-761.
61. Ford AW, Alterman L, Kemeny DM. The allergens of dog. I. Identification using crossed radio-immunoelectrophoresis. *Clin Exp Allergy* 1989;19:183-190.
62. Gao F, Endo H, Yamamoto M. Length heterogeneity in rat salivary gland  $\alpha_2\mu$  globulin mRNAs: multiple splice-acceptors and polyadenylation sites. *Nucleic Acids Res* 1989;17:4629-4636.
63. Gascan H, Gauchat J-F, Roncarolo M-G, Yssel H, Spits H, de Vries JE. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4<sup>+</sup> T cell clones. *J Exp Med* 1991;173:747-750.



64. Gavel Y, von Heijne G. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. *Protein Eng* 1990;3:433-442.
65. Gell PGH, Coombs RRA. The classification of allergic reactions underlying disease. In: Gell PGH, Coombs RRA, ed. *Clinical aspects of immunology*. Oxford: Blackwell scientific publications, 1962: 575-596 chap. 13.
66. Godovac-Zimmermann J. The structural motif of  $\beta$ -lactoglobulin and retinol-binding protein: a basic framework for binding and transport of small hydrophobic molecules? *Trends Biochem Sci* 1988;13:64-66.
67. Gordon S, Tee RD, Newman Taylor AJ. Analysis of rat urine proteins and allergens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. *J Allergy Clin Immunol* 1993;92:298-305.
68. Groot Hd, Goei KGH, Swieten Pv, Aalberse RC. Affinity purification of a major and a minor allergen from dog extract: serologic activity of affinity-purified *Can f1*-depleted extract. *J Allergy Clin Immunol* 1991;87:1056-1065.
69. Gross NJ. Allergy to laboratory animals: epidemiologic, clinical, and physiologic aspects, and a trial of cromolyn in its management. *J Allergy Clin Immunol* 1980;66:158-165.
70. Gubits RM, Lynch KR, Kulkarni AB, Dolan KP, Gresik EW, Hollander P, Feigelson P. Differential regulation of  $\alpha$ 2u globulin gene expression in liver, lachrymal gland, and salivary gland. *J Biol Chem* 1984;259:12803-12809.
71. Guruprasad K, Reddy BV, Pandit MW. Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. *Protein Eng* 1990;4:155-161.
72. Haars LJ, Pitot HC. Hormonal and developmental regulation of glycosylated  $\alpha$ 2u-globulin synthesis. *Arch Biochem Biophys* 1980;201:556-563.
73. Hard GC, Sevin Rodgers I, Baetcke KP, Richards WL, McGaughy RE, Valcovic LR. Hazard evaluation of chemicals that cause accumulation of  $\alpha$ 2u-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. *Environ Health Perspect* 1993;99:313-349.
74. Hellman U. Isolation of peptides for microsequencing by in-gel proteolytic digestion. In: Kamp RM, Choli-Papadopoulou T, Wittmann-Liebold B, ed. *Protein structure analysis. Preparation, characterization, and microsequencing*. Heidelberg: Springer-Verlag, 1997: 97-104.
75. Hellman U, Wernstedt C, Gónez J, Heldin C-H. Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal Biochem* 1995;224:451-455.
76. Hollander A. *Laboratory animal allergy. Allergen exposure assessment and epidemiological study of risk factors, Thesis*. Landbouw Universiteit, 1997.
77. Hollander A, van Run P, Spithoven J, Heederik D, Doekes G. Exposure of laboratory animal workers to airborne rat and mouse urinary allergens. *Clin Exp Allergy* 1997;27:617-626.
78. Holmquist L, Agrup G, Anundi H, Vesterberg O. High performance concentration and gel filtration of rat urinary protein allergens. *J Biochem Biophys Meth* 1990;20:303-316.
79. Holmquist L, Vesterberg O. Quantification of apolipoprotein D in human urine by zone immunoelectrophoresis assay: a methodological and clinical study. *J Biochem Biophys Methods* 1991;23:315-327.
80. Holt PG, Sly PD, Björkstén B. Atopic versus infectious diseases in childhood: a question of balance? *Pediatr Allergy Immunol* 1997;8:53-58.
81. Huwe JK, Larsen GL, Castellino S. An investigation of the binding site of  $\alpha$ 2u-globulin using isotopically labeled ligands and inverse nuclear magnetic resonance techniques. *Chem Res Toxicol* 1996;9:215-222.

82. Jabara HH, Fu SM, Geha RS, Vercelli D. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J Exp Med* 1990;172:1861-1864.
83. June CH, Bluestone JA, Nadler LM, Thompson CB. The B7 and CD28 receptor families. *Immunol Today* 1994;15:321-331.
84. Kaplan HA, Welply J, Lennarz W. Oligosaccharyl transferase: the central enzyme in the pathway of glycoprotein assembly. *Biochim Biophys Acta* 1987;906:161-173.
85. Karlin S, Altschul SF. Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. *Proc Natl Acad Sci USA* 1990;87:2264-2268.
86. King TP, Hoffman D, Löwenstein H, Marsh DG, Platts-Mills TAE, Thomas W. Allergen nomenclature. *Allergy* 1995;50:765-774.
87. Kirkwood T. Predicting the stability of biological standards and products. *Biometrics* 1977;33:736-742.
88. Kraft D, Ferreira F, Ebner C, Valenta R, Breiteneder H, Susani M, Breitenbach M, Scheiner O. Recombinant allergens: the future of the diagnosis and treatment of atopic allergy. *Allergy* 1998;53:62-66.
89. Kurtz DT, Sippel AE, Feigelson P. Effect of thyroid hormones on the level of the hepatic mRNA for  $\alpha_{2u}$ -globulin. *Biochemistry* 1976;15:1031-1036.
90. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.
91. Laffer S, Spitzauer S, Susani M, Pairleitner H, Schweiger C, Grönlund H, Menz G, Pauli G, Ishii T, Nolte H, Ebner C, Sehon AH, Kraft D, Eichler HG, Valenta R. Comparison of recombinant timothy grass pollen allergens with natural extract for diagnosis of grass pollen allergy in different populations. *J Allergy Clin Immunol* 1996;98:652-658.
92. Lane SE, Neuhaus OW. Comparison of some sex-dependent proteins in the adult male rat. *Biochim Biophys Acta* 1972;263:424-432.
93. Lane SE, Neuhaus OW. Further studies on the isolation and characterization of a sex-dependent protein from the urine of male rats. *Biochim Biophys Acta* 1972;257:461-470.
94. Lehman-McKeeman L, Rivera-Torres M, Caudill D. Lysosomal degradation of  $\alpha_{2u}$ -globulin-xenobiotic conjugates. *Toxicol Appl Pharmacol* 1990;103:539-548.
95. Lehman-McKeeman LD, Caudill D. Quantitation of urinary  $\alpha_{2u}$ -globulin and albumin by reverse-phase high performance liquid chromatography. *J Pharmacol Methods* 1991;26:239-247.
96. Lehman-McKeeman LD, Caudill D.  $\alpha_{2u}$ -globulin is the only member of the lipocalin protein superfamily that binds to hyaline droplet inducing agents. *Toxicol Appl Pharmacol* 1992;116:170-176.
97. Lehman-McKeeman LD, Caudill D, Rodriguez PA, Eddy C. 2-sec-butyl-4,5-dihydrothiazole is a ligand for mouse urinary protein and rat alpha 2u-globulin: physiological and toxicological relevance. *Toxicol Appl Pharmacol* 1998;149:32-40.
98. Lehrer SB, Horner WE, Reese G. Why are some proteins allergenic? Implications for biotechnology. *Crit Rev Food Sci Nutr* 1996;36:553-564.
99. Lennarz W. Membrane Biogenesis. In: Op den Kamp JAF, ed. Berlin: Springer Verlag, *NATO ASI Series* 1988;H:16:287-306.
100. Levy F, Kristofic C, Heusser C, Brinkmann V. Role of IL-13 in CD4 T cell-dependent IgE production in atopy. *Int Arch Allergy Immunol* 1997;112:49-58.
101. Liebers V, Sander I, van Kampen V, Raulf-Heimsoth M, Rozynek P, Baur X. Overview on denominated allergens. *Clin Exp Allergy* 1996;26:494-516.
102. Lilja G, Wickman M. Allergy - atopy - hypersensitivity - a matter of definition. *Allergy* 1998;53:1011-1012.

103. Litwin A, Pesce AJ, Fisher T, Michael M, Michael JG. Regulation of the human immune response to ragweed pollen by immunotherapy. A controlled trial comparing the effect of immunosuppressive peptic fragments of short ragweed with standard treatment. *Clin Exp Allergy* 1991;21:457-465.
104. Litwin A, Pesce AJ, Michael JG. Regulation of the immune response to allergens by immunosuppressive allergenic fragments. 1. Peptic fragments of honey bee venom phospholipase A2. *Int Arch Allergy Appl Immunol* 1988;87:361-366.
105. Longbottom JL. Purification and characterization of allergens from the urines of mice and rats. In: Oehling A, Glazer I, Mathov E, Arbesman C, ed. *Advances in Allergology and Immunology*. Oxford: Pergamon Press, 1980: 483-490.
106. Longbottom JL. Characterization of allergies from the urines of experimental animals. In: Kerr JW, Ganderton MA, ed. *Proceedings of invited symposia/XI International Congress of Allergology and Clinical Immunology*. London: Macmillan Press, 1983: 525-529.
107. Longbottom JL. Crossed radio-immunoelectrophoresis as a method of identifying isoallergens: Identification of isoallergens in rat urine extracts. *J Immunol Meth* 1984;68:297-304.
108. Longbottom JL. Occupational allergy due to animal allergens. *Clinics in Immunology and Allergy* 1984;4:19-36.
109. Lutsky I, Neuman I. Laboratory animal dander allergy: I. An occupational disease. *Ann Allergy* 1975;35:201-205.
110. MacInnes JI, Nozik ES, Kurtz DT. Tissue-specific expression of the rat alpha<sub>2u</sub> globulin gene family. *Mol Cell Biol* 1986;6:3563-3567.
111. Macritchie F. Proteins at interfaces. *Adv Protein Chem* 1978;32:283-326.
112. Manetti R, Parronchi P, Giudizi MG, Piccinni M-P, Maggi E, Trinchieri G, Romagnani S. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 1993;177:1199-1204.
113. Margolis J, Kenrick KG. Polyacrylamide gel electrophoresis in a continuous molecular sieve gradient. *Anal Biochem* 1968;25:347-362.
114. Marshall R. Glycoproteins. *Annu Rev Biochem* 1972;41:673-702.
115. Marshall T. Detection of protein in polyacrylamide gels using an improved silver stain. *Anal Biochem* 1984;136:340-346.
116. Marshall T. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of serum after protein denaturation in the presence or absence of 2-mercaptoethanol. *Clin Chem* 1984;30:475-479.
117. Marshall T, Gaston SJS, Williams KM. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis without a stacking gel: Use for high sample throughput. *Electrophoresis* 1989;10:63-65.
118. Mazur G, Baur X, Modrow S, Becker W. A common epitope on major allergens from non-biting midges (chironomidae). *Mol Immunol* 1988;25:1005-1010.
119. McWilliam AS, Tree P, Gordon S. Carbohydrate recognition receptors of the macrophage and their regulation. In: van Furth R, ed. *Mononuclear phagocytes. Biology of monocytes and macrophages*. Dordrecht: Kluwer Academic Publishers, 1992:224-232.
120. Menz G, Dolecek C, Schönheit-Kenn U, Ferreira F, Moser M, Schneider T, Suter M, Bolitz-Nitulescu G, Ebner C, Kraft D, Valenta R. Serological and skin-test diagnosis of birch pollen allergy with recombinant *Bet v* I, the major birch pollen allergen. *Clin Exp Allergy* 1996;26:50-60.
121. Metzger H. The receptor with high affinity for IgE. *Immunol Rev* 1992;125:37-48.
122. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol* 1993;11:165-190.

123. Mosmann TR, Chervinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T-cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348-2357.
124. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996;17:138-146.
125. Muller UR. The value of immunotherapy in treatment of IgE-induced allergic diseases. *Ther Umsch* 1994;51:67-76.
126. Murty CVR, Mancini MA, Chatterjee B, Roy AK. Changes in transcriptional activity and matrix association of  $\alpha_{2u}$ -globulin gene family in the rat liver during maturation and aging. *Biochim Biophys Acta* 1988;949:27-34.
127. Neuhaus O. Regulation of urinary excretion of alpha-2u-globulin during circadian rhythm. In: Bianchi C, Bocci V, Carone F, Rabkin R, ed. *Kidney. Proteins and drugs. Contrib Nephrol*. Basel: Karger, 1990: 82-87. vol 83.
128. Neuhaus O, Wayne F. Age-dependent changes in the excretion of urinary proteins by the rat. *Nephron* 1978;22:570-576.
129. Neuhaus OW, Lerseth DS. Dietary control of the renal reabsorption and excretion of  $\alpha_{2u}$ -globulin. *Kidney Int* 1979;16:409-415.
130. Newman-Taylor AN, Longbottom JL, Pepys J. Respiratory allergy to urine proteins of rats and mice. *Lancet* 1977;2:847-849.
131. Nieuwenhuijsen MJ, Gordon S, Tee RD, Venables KM, McDonald JC, Newman Taylor AJ. Exposure to dust and rat urinary aeroallergens in research establishments. *Occup Environ Med* 1994;51:593-596.
132. North ACT. Three-dimensional arrangement of conserved amino acid residues in a superfamily of specific ligand binding proteins. *Int J Biol Macromol* 1989;11:56-58.
133. O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998;8:275-283.
134. Olson MJ, Johnson JT, Reidy CA. A comparison of male rat and human urinary proteins: Implications for human resistance to hyaline droplet nephropathy. *Toxicol Appl Pharmacol* 1990;102:524-536.
135. Paul WE. Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* 1991;77:1859-1870.
136. Pauli G, Oster JP, Deviller P, Heiss S, Bessot JC, Susani M, Ferreira F, Kraft D, Valenta R. Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: Diagnostic value for birch pollen and associated allergies. *J Allergy Clin Immunol* 1996;97:1100-1109.
137. Peitsch MC, Boguski MS. The first lipocalin with enzymatic activity. *Trends Biochem Sci* 1991;16:363.
138. Pellequer JL, Westhof E, Van Regenmortel MHV. Predicting location of continuous epitopes in proteins from their primary structures. *Methods Enzymol* 1991;203:176-201.
139. Pène J, Rousset F, Brière F, Chrétien I, Bonnefoy J-Y, Spits H, Yokota T, Arai N, Arai K-I, Banchereau J. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons  $\gamma$  and  $\alpha$  and prostaglandin  $E_2$ . *Proc Natl Acad Sci USA* 1988;85:6880-6884.
140. Pervaiz S, Brew K. Homology and structure-function correlations between  $\alpha_1$ -acid glycoprotein and serum retinol-binding protein and its relatives. *FASEB J* 1987;1:209-214.
141. Pless DD, Lennarz WJ. Enzymatic conversion of proteins to glycoproteins. *Proc Natl Acad Sci USA* 1977;74:134-138.
142. Price JA, Longbottom JL. Allergy to rabbits. II. Identification and characterization of a major rabbit allergen. *Allergy* 1988;43:39-48.
143. Price JA, Longbottom JL. ELISA method for measurement of airborne levels of major laboratory animal allergens. *Clinical Allergy* 1988;18:95-107.

144. Punnonen J, Aversa G, Cocks BG, McKenzie ANJ, Menon S, Zurawski G, de Wall Malefyt R, de Vries JE. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993;90:3730-3734.
145. Rapaport H. Clemens von Pirquet and allergy. *Ann Allergy* 1973;31:467-475.
146. Raz E, Tighe H, Sato Y, Corr M, Dudler JA, Roman M, Swain SL, Spiegelberg HL, Carson DA. Preferential induction of a Th<sub>1</sub> immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci USA* 1996;93:5141-5145.
147. Renström A. *Allergy to laboratory animals. Risk factors for development of allergy and methods for measuring airborne rodent allergens. Thesis.* National Institute for Working Life, Karolinska Institute, 1997.
148. Renström A, Larsson PH, Malmberg P, Bayard C. A new amplified monoclonal rat allergen assay used for evaluation of ventilation improvements in animal rooms. *J Allergy Clin Immunol* 1997;100:649-655.
149. Romagnani S. Lymphokine production by human T cells in disease states. *Annu Rev Immunol* 1994;12:227-257.
150. Roman M, Spiegelberg HL, Broide D, Raz E. Gene immunization for allergic disorders. *Springer Semin Immunopathol* 1997;19:223-232.
151. Roy AK, Nath TS, Motwani NM, Chatterjee B. Age-dependent regulation of the polymorphic forms of  $\alpha_{2u}$ -globulin. *J Biol Chem* 1983;258:10123-10127.
152. Roy AK, Neuhaus OW. Identification of rat urinary proteins by zone and immunoelectrophoresis. *Proc Soc Exp Biol Med* 1966;121:894-899.
153. Roy AK, Raber DL. Immunofluorescent localization of  $\alpha_{2u}$ -globulin in the hepatic and renal tissues of rat. *J Histochem Cytochem* 1972;20:89-96.
154. Roy AK, Schiop MJ, Dowbenko DJ. Regulation of the hepatic synthesis of  $\alpha_{2u}$ -globulin and its corresponding messenger RNA in maturing rats. *FEBS Lett* 1976;70:137-140.
155. Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8<sup>+</sup> T cells into cytotoxic CD8<sup>+</sup> T cells secreting Th1 or Th2 cytokines. *Immunity* 1995;2:271-279.
156. Sansom C, North A, Sawyer L. Structural analysis and classification of lipocalins and related proteins using a profile-search method. *Biochim Biophys Acta* 1994;1208:247-255.
157. Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995;50:384-391.
158. Schou C, Svendsen UG, Løwenstein H. Purification and characterization of the major dog allergen *Can f* I. *Clin Exp Allergy* 1991;21:321-328.
159. Scott P. IL-12: Initiation cytokine for cell-mediated immunity. *Science* 1993;260:496-497.
160. Seder RA, Boulay JL, Finkelman F, Barbier S, Ben-Sasson SZ, Le Gross G, Paul WE. CD8<sup>+</sup> T cells can be primed in vitro to produce IL-4. *J Immunol* 1992;148:1652-1656.
161. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4<sup>+</sup> T cells. *Annu Rev Immunol* 1994;12:635-673.
162. Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. *Science* 1997;275:77-79.
163. Sivaprasadarao A, Boudjelal M, Findlay JB. Lipocalin structure and function. *Biochem Soc Trans* 1993;21:619-622.
164. Sjöstedt L. *Laboratory animal allergy. Long-term incidence. Effects on lung function. Individual risk factors. Thesis.* Lund University, 1996.
165. Song Z, Casolaro V, Chen R, Georas SN, Monos D, Ono SJ. Polymorphic nucleotides within the human IL-4 promoter that mediate overexpression of the gene. *J Immunol* 1996;156:424-429.

166. Spiegelberg HL, Orozco EM, Roman M, Raz E. DNA immunization: a novel approach to allergen-specific immunotherapy. *Allergy* 1997;52:964-970.
167. Spiegelberg HL, Tighe H, Roman M, Broide D, Raz E. Inhibition of IgE formation and allergic inflammation by allergen gene immunization and by CpG motif immunostimulatory oligodeoxynucleotides. *Allergy* 1998;53:93-97.
168. Spitzauer S, Pandjaitan B, Söregi G, Mühl S, Ebner C, Kraft D, Valenta R, Rumpold H. IgE cross-reactivities against albumins in patients allergic to animals. *J Allergy Clin Immunol* 1995;96:951-959.
169. Spitzauer S, Rumpold H, Ebner C, Schweiger C, Valenta R, Gabl F, Anrather J, Breitenbach M, Scheiner O, Kraft D. Allergen profiles of dog hair and dander, body fluids and tissues as defined by immunoblotting. *Int Arch Allergy Appl Immunol* 1991;94:346-348.
170. Stewart GA, Thompson PJ. The biochemistry of common aeroallergens. *Clin Exp Allergy* 1996;26:1020-1044.
171. Stewart GA, Thompson PJ. Letter to the Editors. Re: Nomenclature of rat allergens. Reply to Gordon S, Jones MG, Tee RD, Newman Taylor AJ. *Clin Exp Allergy* 1997;27:714-715.
172. Stewart GA, Thompson PJ, McWilliam AS. Biochemical properties of aeroallergens: contributory factors in allergic sensitization? *Pediatr Allergy Immunol* 1993;4:163-172.
173. Street NE, Schumacher JH, Fong AT, Bass H, Fiorentino DF, Leverah JA, Mosmann TR. Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. *J Immunol* 1990;144:1629-1639.
174. Suresh Kumar T, Prasad V, Pandit M. Origin of multiple bands of proteins on sodium dodecyl sulphate-polyacrylamide gel electrophoresis-intermolecular disulphide cross-linking due to the presence of oxidizing components in the reducing agent. *J Biochem Biophys Methods* 1994;28:243-247.
175. Swenberg J, Short B, Borghoff S, Strasser J, Charbonneau M. The comparative pathobiology of  $\alpha_{2u}$ -globulin nephropathy. *Toxicol Appl Pharmacol* 1989;97:35-46.
176. Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breiteneder H, Pittenauer E, Schmid E, Vicente O, Heberle-Bors E, Ahorn H, Breitenbach M. Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* 1995;270:2607-2613.
177. Takatsu K, Takaki S, Hitoshi Y. Interleukin-5 and its receptor system: implications in the immune system and inflammation. *Adv Immunol* 1994;57:145-190.
178. Takenaka H, Zhang K, Diaz-Sanchez D, Tsien A, Saxon A. Enhanced human IgE production results from exposure to the aromatic hydrocarbons from diesel exhaust: Direct effects on B-cell IgE production. *J Allergy Clin Immunol* 1995;95:103-115.
179. Tighe H, Corr M, Roman M, Raz E. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol Today* 1998;19:89-97.
180. Tovey ER, Baldo BA. Characterisation of allergens by protein blotting. *Electrophoresis* 1987;8:452-463.
181. Trifilieff E, Dubs MC, Van Regenmortel MH. Antigenic cross-reactivity potential of synthetic peptides immobilized on polyethylene rods. *Mol Immunol* 1991;28:889-896.
182. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995;13:251-276.
183. Unterman RD, Lynch KR, Nakhasi HL, Dolan KP, Hamilton JW, Cohn DV, Feigelson P. Cloning and sequence of several  $\alpha_{2u}$ -globulin cDNAs. *Proc Natl Acad Sci USA* 1981;78:3478-3482.
184. Valenta R, Duchene M, Vrtala S, Birkner T, Ebner C, Hirschwehr R, Breitenbach M, Rumpold H, Scheiner O, Kraft D. Recombinant allergens for immunoblot diagnosis of tree-pollen allergy. *J Allergy Clin Immunol* 1991;88:889-894.

185. Valenta R, Kraft D. Recombinant allergens for diagnosis and therapy of allergic diseases. *Curr Opin Immunol* 1995;7:751-756.
186. Valenta R, Vrtala S, Ebner C, Kraft D, Scheiner O. Diagnosis of grass pollen allergy with recombinant timothy grass (*Phleum pratense*) pollen allergens. *Int Arch Allergy Immunol* 1992;97:287-294.
187. Valenta R, Vrtala S, Laffer S, Steinberger P, Ball T, Ferreira F, Scheiner O, Kraft D. B-cell epitopes of allergens determined by recombinant techniques; use for diagnosis and therapy of type I allergy. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1994;87:235-246.
188. van der Heijden FL, van Neerven RJ, van Katwijk M, Bos JD, Kapsenberg ML. Serum-IgE-facilitated allergen presentation in atopic disease. *J Immunol* 1993;150:3643-3650.
189. van der Zee JS, de Groot H, van Swieten P, Jansen HM, Aalberse RC. Discrepancies between the skin test and IgE antibody assays: study of histamine release, complement activation in vitro, and occurrence of allergen-specific IgG. *J Allergy Clin Immunol* 1988;82:270-281.
190. van Milligen FJ, van't Hof W, van den Berg M, Aalberse RC. IgE epitopes on the cat (*Felis domesticus*) major allergen Fel d I: A study with overlapping synthetic peptides. *J Allergy Clin Immunol* 1994;93:34-43.
191. van Ree R. Analytic aspects of the standardization of allergenic extracts. *Allergy* 1997;52:795-805.
192. Vandoren G, Mertens B, Heyns W, Van Baelen H, Rombauts W, Verhoeven G. Different forms of  $\alpha_2\mu$ -globulin in male and female rat urine. *Eur J Biochem* 1983;134:175-181.
193. Vercelli D, De Monte L, Monticelli S, Di Bartolo C, Agresti A. To E or not to E? Can an IL-4-induced B cell choose between IgE and IgG4? *Int Arch Allergy Immunol* 1998;116:1-4.
194. Vesterberg O. Quantification of proteins with a new sensitive method- zone immunoelectrophoresis assay. *Hoppe-Seyler's Z Physiol Chem* 1980;361:617-624.
195. Vesterberg O. A new luminometer for sensitive quantification by chemiluminescence of specific proteins in microtitre plates and on blot membranes. *J Biochem Biophys Methods* 1995;30:301-314.
196. Vesterberg O, Gramstrup-Christensen B. Sensitive silver staining of proteins after isoelectric focusing in agarose gels. *Electrophoresis* 1984;5:282-285.
197. Vesterberg O, Svensson H. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. *Acta Chemica Scand* 1966;20:820-834.
198. Walls AF, Longbottom JL. Quantitative immunoelectrophoretic analysis of rat allergen extracts. I. Antigenic characterisation of fur, urine, saliva and other rat-derived materials. *Allergy* 1983;38:419-431.
199. Walls AF, Longbottom JL. Comparison of rat fur, urine, saliva, and other rat allergen extracts by skin testing, RAST, and RAST inhibition. *J Allergy Clin Immunol* 1985;75:242-251.
200. Walls AF, Newman Taylor AJ, Longbottom JL. Allergy to guinea pigs: I. Allergenic activities of extracts derived from the pelt, saliva, urine and other sources. *Clin Allergy* 1985;15:241-251.
201. Walsh B, Howden M. A method for the detection of IgE binding sequences of allergens based on a modification of epitope mapping. *J Immunol Methods* 1989;121:275-280.
202. Weaver RN, Gray JE, Schultz JR. Urinary proteins in Sprague-Dawley rats with chronic progressive nephrosis. *Lab Anim Sci* 1975;25:705-710.
203. Wichmann HE. Possible explanation for the different trends of asthma and allergy in east and west Germany. *Clin Exp Allergy* 1996;26:621-623.
204. Wright HT. Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit Rev Biochem Mol Biol* 1991;26:1-52.

205. Yamamoto M, Gao F, Furuichi M, Ichiyoshi Y, Endo H. A LINE 1 sequence interrupts the rat  $\alpha_2$  globulin gene. *Biochim Biophys Acta* 1989;1008:322-328.
206. Åkerström B, Lögdberg L. An intriguing member of the lipocalin protein family:  $\alpha_1$ -microglobulin. *Trends Biochem Sci* 1990;15:240-243.