UDK 577.27:616-022.8/.9
Review
Received: 14 December 2007
Accepted: 20 February 2008

NOVEL CONCEPTS IN IMMUNOLOGY AND THEIR APPLICATION IN CLINICAL ALLERGOLOGY

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Summary

The objective of this review is to present some novel concepts in the field of immunology that help us understand the pathophysiological mechanisms of allergic diseases. The recognition of their heterogeneous nature in combination with animal models and sophisticated *in vitro* immunologic methods should help us translate these findings to clinical practice in order to upgrade the prevention, diagnosis and treatment of allergic diseases.

Keywords: immunology; allergology; *in vitro*

INTRODUCTION

Descriptions of the clinical presentation of allergic diseases have been known for centuries, and according to some sources as early as ancient Egypt, however, the true understanding of the diseases flared up from the beginning of the 20th century with the development of immunology and the work of von Behring, Koch and Bordet. Portier and Richet were the first to perform laboratory studies of allergy with their famous tests of immunization, sensitization and anaphylaxis. The discovery that immunization does not only protect but may also induce hypersensitivity was the cornerstone of research in allergology. With his definition of allergy as a state of altered immunoreactivity, von Pirquet directed further development of allergology as a new clinical discipline.

The current knowledge about innate and cellular immunity, role of B- and T-lymphocytes, role of the thymus, regulation of immunoglobulin genes and T-lymphocyte receptors *via* RAG1 and RAG2 enzyme activities, and the discovery that immunocytes

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secrete soluble factors, cytokines, that are involved in immunoreactions, has been daily enriched with ever new concepts, thus rendering the area of immunology (and allergology perceived as a field of immunology) one of the most dynamic fields of biomedicine.

Currently, more than 350 leukocyte molecules (Cluster of Differentiation, CD) are known, which enable differentiation of various types of immunocytes and 35 interleukins (IL), along with intensive development of new methods for the detection of various factors for the diagnosis and understanding of therapeutic effect variations among patients with allergic diseases.

Like in other basic biomedicine research, there is still a great need for the numerous novel concepts to extrapolate efficiently and meaningfully in daily clinical practice. The key questions of highest relevance in allergology, such as the cause of the disease onset, the pathogenesis and prevention of the disease, have only in part been answered and underlay the development of new therapeutic approaches.

DO WE KNOW WHY ALLERGIES DO OCCUR?

According to the current concept on the development of allergic diseases, these are chronic diseases that occur due to the complex interaction of the environment and individual gene expression at various ages. A great number of genes have been identified to date, whose mutations and/or polymorphisms are associated with atopy and/or allergy, and a modified anti-allergic therapy response; more than 20 have been characterized for asthma alone [1]. The hygiene hypothesis on the effect of microorganisms at an early age (or even *in utero*) to determine the individual's immunoreactivity by acting upon the mechanisms of innate immunity tries to explain the rising incidence of allergies in industrialized countries [2]. Attention has recently been focused on so-called Toll-like receptors and their ligands (e.g., lipopolysaccharide). Genetic studies have only in part confirmed this hypothesis and steered further research to a more complex area requiring additional interdisciplinary studies in the fields of genetics, immunology and epidemiology (such as the GABRIEL project that is under way in Europe since 2006), in order to detect the genes that are truly relevant for allergies, in terms of protection in particular [3,4]. Research efforts have now been focused on the genes that are specifically present in those body compartments where allergic reaction is being manifested (e.g., ADAM33 in respiratory epithelium) [5].

There is still a dilemma whether sensitization already develops *in utero*, thus the recommendation on avoiding allergens during pregnancy, at least in case of children at a high risk of developing allergy (positive family history) being scientifically questionable [6]. The reduced ability of neonatal dendritic cells to secrete IL-12, one of the main cytokines mediating Th1-immunoreaction, is considered crucial for the previously char-

acterized susceptibility of the newborns whose T-lymphocytes are more difficult to activate by the allergen, then predominantly secreting Th2-cytokines (IL-4, IL-5, IL-9, IL-13) [7]. Despite these developments, positive family history remains a major risk factor, since biomarkers of adequate sensitivity are still lacking [8].

The features that characterize an antigen as an allergen are of special importance for proper understanding of the genesis of allergies [9]. Most allergens are parts of proteins with different biologic activities (e.g., proteases, protein carriers, profilins, calcium binding proteins), and also lipids recognized by the immune system as expressed by CD1 molecules [10]. The mechanisms that lead to allergic diseases have been well understood, however, this does not hold for the molecular structures of allergens responsible for their occurrence, which makes their standardization a difficult task. Some allergens can directly influence the development of IgE-mediated immunoreactions (e.g., house dust proteolytic enzymes), whereas others favor their prolonged presence in the environment or the body (e.g., food allergens). The International Association of Immunologic Societies Committee in collaboration with the World Health Organization has been issuing and regularly supplementing the existing nomenclature of allergens (for more detail please refer to the official web site www.allergen.org). More than 40 different allergens have been successfully sequenced, thus allowing for the development of recombinant allergens for diagnostic and therapeutic purposes [11].

THE MECHANISMS OF INNATE AND ACQUIRED IMMUNITY IN ALLERGOLOGY

Along with preserved integrity of the skin and mucosa, various soluble factors and innate immunity cells make the first-line defense against all antigens including allergens.

Allergens can directly activate the complement, an important proinflammatory nonspecific system, by its proteases, or indirectly *via* proteases such as trypsin, thrombin and elastase from mastocytes and other inflammatory cells. Th2-cytokines (IL-4, IL-13) also induce the secretion of C3 in epithelial cells, and a similar action has also been demonstrated for pollutants, tobacco smoke and ozone [12]. Children exposed to environmental tobacco smoke are known to have higher levels of the C3 complement component than unexposed children [13]. Anaphylatoxins (C3a and C5a) have a previously known action in anaphylaxis, and now the expression of their receptors is known to also be modulated by allergens and various pollutants. Interestingly enough, owing to studies in suitable animal models, the action of these anaphylatoxins is currently believed to be variable. So, C5a is considered important for the development of tolerance of dendritic cells in respiratory mucosa only in the conditions when allergic reaction has not yet occurred [14].

In humans, basophils are considered the main cell type responsible for early secretion of Th2-cytokines. Along with the expression of CD40 ligands, they enhance IgE synthesis and are therefore highly relevant for the initiation and maintenance of Th2-immunoreactions. Modern methods of detection of these cells in different body compartments have enabled their role in allergy to identify. The discovery that basophils are activated not only by allergen binding to the allergen-specific IgE on high-affinity IgE receptors (FcåRI) but also by some parasites, lectins and virus superantigens, with involvement of nonspecific IgE [15], is of great interest indeed.

Numerous defects in the mechanisms of innate immunity, including impaired secretion of antimicrobial peptides (surfactant proteins, catelicidin, defensin) and Toll-like receptor gene polymorphism, have been described in allergic diseases. Some of these impairments are primary, i.e. existing even before the onset of allergic disease, whereas others develop as a secondary phenomenon [16,17].

Mastocytes as central executive cells in the early stages of allergic inflammation are among the first to come in contact with the potential allergens [18]. They are found in all organs and vascularized tissues where, upon the allergen, IgE and FcεRI interaction, they release the content of mastocyte granules with preformed factors (histamine, neutral proteases, cytokines, proteoglycans) and initiate the synthesis of these and other factors (prostaglandins D, leukotrienes B4 and C4, cytokines, chemokines). The consequences of the action of these factors (increased vascular permeability and consequential tissue edema, bronchoconstriction, increased mucus production, leukocyte attraction) have been thoroughly investigated. The central role of mastocytes in these pathogenetic events has been confirmed owing to the detection of a suitable animal model (mouse with c-kit gene deletion). These models have also demonstrated that mastocytes play a role in non-allergic (e.g., autoimmune) diseases, i.e. they are involved in the pathogenesis of these diseases *via* numerous mechanisms such as modifying dendritic cell migration and their ability of T-lymphocyte activation; direct action upon T-lymphocytes and modifying their migration to target tissues; secretion of various cytokines and cell contact; possible action on regulatory T-lymphocytes and establishing tolerance)[18]. The true relevance of these results in allergic diseases in humans has not yet been established, especially considering the potential utilization of these concepts in the development of new drugs (c-kit inhibition, new tryptase inhibitors) [19].

Numerous studies of the role of eosinophils have resulted in some new concepts on the role of these cells in various diseases including allergies [20-22]. Currently, eosinophils are considered multifunctional leukocytes that are involved not only in the initiation but also in the modulation of innate and acquired immunity. The so-called secondary granules of eosinophils contain many factors (eosinophil peroxidase, major basic protein, eosinophilic cationic protein and eosinophilic neurotoxin) that primarily

act as cytotoxic molecules (in asthma, they act on the respiratory epithelium cells), while eosinophilic cationic protein and eosinophilic neurotoxin also act as ribonucleases. Eosinophils are activated by various stimuli (nonspecific tissue damage, infection, transplant, allergens and tumor cells). Besides preformed factors, they can also secrete cytokines, chemokines, lipid mediators and neuromodulators. In addition, eosinophils present antigens/allergens to T-lymphocytes and influence their polarization by releasing indoleamine 2,3-dioxygenase, an enzyme important for oxidative metabolism of tryptophan, from which kynurenine, an important regulator of Th1/Th2 balance, is subsequently formed [23]. The major cationic protein is also involved in the development of bronchial hyperreactivity by acting upon vagal muscarinic M2-receptors [20]. By releasing great amounts of leukotrienes, eosinophils increase vascular permeability and bronchospasm. Clinical trials have shown that pulmonary eosinophilia in asthma patients is associated with chronic coughing but not with bronchial hyperreactivity. The administration of humanized anti-IL-5 in asthma patients has only in part proved useful in blocking the major cytokine responsible for the formation of eosinophils, thus posing new questions on the role of other molecules (e.g., eotaxin) in the genesis of pulmonary eosinophilia [24,25].

Recent studies have also been focused on the subgroup of CD1-associated NK-cells expressing T-lymphocyte receptor on their surface (iNKT-cells). Studies in animals without these NK-cells have suggested that they may have a major role in shifting immunoreactions toward Th2. These cells were found in a number of patients with allergic asthma [26].

Studies initiated in the 1980s resulted in a changed opinion about differences in the allergen-specific effector helper T-lymphocytes that predominantly release Th2-interleukins (IL-4, IL-5, IL-9, IL-13) responsible for the onset and numerous manifestations of allergic disease. So, IL-10 and interferon-γ (IFN-γ) were considered as modulating cytokines enabling allergen tolerance in healthy individuals. Based on these concepts, many studies were performed measuring cytokines by various techniques and in various analytical specimens (e.g., peripheral blood, serum, skin and nasal mucosa biopsy specimens, bronchoalveolar lavage, induced sputum, etc.). However, recent studies have indicated that Th1-lymphocytes, i.e. those that predominantly release IFN-γ, can also lead to severe allergic inflammation. With the discovery of Th17, a novel subgroup of inflammatory T-lymphocytes, attempts have been made to link the previously described phenomenon of granulocyte accumulation with consequential inflammation at the site of allergic reaction (e.g., in the airways) [27]. These findings point to the need of simultaneous determination of different cytokines, primarily at the site of allergic inflammation, rather than in peripheral blood or serum samples, as mostly done to date [28].

Although B-lymphocytes have long been considered to play passive role in immunoreactions including allergies, they are not simply the cells that release IgE upon ap-

propriate activation by the allergen, cytokines and contact with allergen-specific T-lymphocytes. Naïve B-lymphocytes can induce the formation of regulatory T-lymphocytes, which play an important role in the onset of allergies and probably also in the restitution of immunotolerance following successful hyposensitization[29]. It has recently been demonstrated on a mouse model that IgE+ cells are mostly found beyond germinal centers that represent special compartments within the secondary lymphatic tissue, formed upon contact with the antigen. In these centers, the formation of B-lymphocytes that will release immunoglobulins or so-called memory B-lymphocytes is enabled by somatic hypermutation and maturation of immunoglobulin genes and rebinding of immunoglobulin classes. Another source of IgE-secreting plasma cells is created by the additional sequential rebinding of B-lymphocytes that release IgG1 under the action of the cytokine IL-4 (IL-21 has an opposite action). This may explain the persistence of IgE seen in allergic diseases[30].

It remains to be investigated whether STAT3, its mutation resulting in the occurrence of primary immunodeficiency of hyper-IgE syndrome, plays a role in allergic diseases, and if so, what its role is [31].

Regulatory T-lymphocytes are a T-lymphocyte population which is important for the establishment of peripheral immunotolerance, and are of special relevance in allergies, autoimmune disorders, tumors, infections and transplantation medicine for the control of local immunoreaction [32]. In fact, this T-lymphocyte population has a suppressor effect on other cells of the immune system, thus contributing to local modulation of the immune reaction. There are two main subpopulations of regulatory T-lymphocytes, i.e. natural regulatory T-lymphocytes as a separate line of T-lymphocytes developing in the thymus, and inducible regulatory T-lymphocytes developing in the periphery upon contact with the allergen and acting primarily *via* cytokines (IL-10 and TGF-β). The role of regulatory T-lymphocytes has also been intensively investigated in the field of allergies and asthma [33]. The ability of regulatory T-lymphocytes to suppress the allergen induced proliferation of effector T-lymphocytes during pollination season is lower in atopy patients than in healthy individuals. Similarly, they also play a major role in other forms of allergy, e.g., in contact hypersensitivity and UV activity. Furthermore, regulatory cells are involved in the development of tolerance to food allergens. Children allergic to cow's milk have a lower level and reduced functional ability of regulatory T-lymphocytes. After several-month abstinence from milk consumption, the children that had developed milk tolerance and "overcome allergy" showed elevated levels of regulatory T-lymphocytes in the circulation. Functional modulation of the existing and/or formation of new regulatory T-lymphocytes have been described as one of the mechanisms of efficient hyposensitization [34].

HOW TO PREVENT THE ONSET/EXACERBATIO OF ALLERGIC DISEASES?

By carefully designed clinical studies, efforts have been invested to reconsider the existing and develop novel procedures for the prevention of allergic diseases. As mentioned above, recommendations on avoiding allergens during pregnancy, at least in children at a high risk of developing allergies (positive family history), have been shown to be scientifically questionable [6]. Inadequate preservation of the skin and mucosa integrity contributes significantly to the occurrence of allergic diseases because the effects of air pollutants, tobacco smoke and ozone on the allergens, inflammatory mediators and cells involved in allergic reactions are at least partially known. Atopic dermatitis in early infancy begins as a disease in the pathogenesis of which IgE is not involved. A change in the skin barrier enables gradual sensitization to allergens, and then also to own antigens, so at long term the disease can be considered an autoimmune disorder as well. The phenotype and function of dendritic cells of the skin are modified by the calcineurin inhibitors, thus they have been postulated to help in establishing permanent tolerance [35].

Great attention has lately been paid to different viruses, primarily in asthma exacerbation (rhinoviruses, influenza virus), and also in the genesis of asthma (respiratory syncytial virus, metapneumovirus) [36]. The prospective studies published to date have demonstrated that bronchiolitis caused by respiratory syncytical virus can increase the risk of wheeziness, and in some studies of developing asthma later in life. However, it should be borne in mind that only a part of children develop severe infection (i.e. bronchiolitis), which is now primarily related to polymorphisms of the genes involved in nonspecific immunity [37]. Taking in consideration current trends in allergic diseases, which also point to the role of innate immunity in the development of allergen tolerance, additional studies are expected to elucidate whether a more severe picture of viral infections can in fact identify the children with predisposition to asthma, or may even lead to its development.

NOVEL APPROACHES IN THE MANAGEMENT OF ALLERGIC DISEASES

In addition to the known omalizumab, a humanized monoclonal antibody against IgE, another biological, etanercept, a soluble TNF-α receptor, has also been tested in asthma patients. The agent proved efficacious in patients with severe asthma and predominant Th1-phenotype but not in those with allergen-mediated inflammation, bronchial hyperreactivity and mild/moderate clinical picture [38].

Highly promising is DNA therapy, i.e. administration of immunostimulatory nonmethylated DNA to enhance innate immunity and Th-1 immunoreaction. Also, anti-

sense oligonucleotides could be used for target blocking of respective RNA [39]. The anti-inflammatory activity of antibiotics (macrolides), chemokine antagonists (e.g., eotaxin), transcription factor antagonists (e.g., NF-κB), and the activity of various factors on mucus production (e.g., aerosol surfactants) have been investigated [3,40,41]. Development of new respiratory virus vaccines (respiratory syncytial virus in particular) should help control the effect of viral infections in the onset and/or exacerbations of asthma[42].

Continuing research into the immunopathogenesis of allergic diseases along with new discoveries in the fields of genomics, proteomics, pharmacogenetics and molecular pharmacology is expected to result in the advent of novel drugs, thus upgrading the control of these disorders.

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Sažetak

Suvremene spoznaje u imunologiji i njihova primjena u kliničkoj alergologiji

U ovom revijskom prikazu namjera je prikazati neke nove spoznaje iz područja imunologije koja su pomogla razumijevanju patofizioloških mehanizama alergijskih bolesti. Prepoznavanjem složene naravi tih bolesti i uporabom životinjskih modela te suvremenih imunoloških metoda istraživanja *in vitro* nastoji se iste rabiti u rutinskom kliničkom radu u svrhu uspješnije prevencije, dijagnostike i liječenja alergijskih bolesti.

Ključne riječi: imunologija; alergologija; *in vitro*