Molecular Immunology 48 (2011) 1236–1244

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

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ARTICLE INFO

Article history:
Received 21 January 2011
Accepted 8 March 2011
Available online 6 April 2011

Keywords:
Timothy grass pollen
Allergy
Recombinant IgE
IgA
Epitope characterisation

ABSTRACT

The scarcity of monoclonal human IgE antibodies with specificity for defined allergens is a bottleneck for the molecular characterisation of allergens and their epitopes. Insights into the characteristics of such antibodies may allow for analyses of the molecular basis underlying allergenicity and cross-reactivity, standardisation of allergens as well as improvement of allergy diagnostics and therapeutics. Here we report the generation and application of the first set of authentic human IgG, IgE and IgA antibodies. On the basis of a Phl p 5a specific antibody fragment, a lambda light chain and the IgG1, IgG4, IgE, IgA1, and IgA2 heavy chains, the corresponding human immunoglobulins were constructed and produced in mammalian cells. In parallel, a murine hybridoma line with specificity for Phl p 5a was established, recloned and produced as human chimeric IgE. After purification, immunoreactivity of the antibodies with the allergen was assessed. Applicability in allergy diagnostics was confirmed by establishment of artificial human sera. Functionality of both antibodies was further demonstrated in receptor binding studies and mediator release assays using humanised rat basophil leukaemia cells (RBL-SX38) suggesting the presence of spatially separate epitopes. By using Phl p 5 fusion proteins and recombinant IgE in immunoblotting and mediator release assays we assigned the epitope of the authentic IgE to a looped stretch exclusively present in Phl p 5a. In summary, the Phl p 5-specific antibodies are the first full set of allergy-related antibody isotypes of their kind and represent valuable tools for studies of fundamental mechanisms and structure/function relationships in allergy.

1. Introduction

It is well established that the development of several immune-mediated diseases is linked to circulating concentrations of IgE, the antibody class responsible for allergic hypersensitivity (Ishizaka et al., 1966). IgE antibodies bound to their high affinity receptor (FcεRI) on mast cells and basophils mediate receptor cross-linking by allergens and trigger degranulation and release of proinflammatory mediators responsible for immediate-type hypersensitivity reactions. Long-term exposure to higher concentrations of allergens or therapeutic intervention by specific immunotherapy (SIT) results in a T-helper cell type-1 (Th1) shift in the immune response, leading to an increase in production of allergen-specific Ig, particularly of the IgG4 and IgA2 subclasses (Platts-Mills et al., 1976; Pilette et al., 2007). These antibody isotypes are thought to exert their function by blocking the IgE/allergen interaction (Lichtenstein et al., 1968; Jarolim et al., 1990; Cooke et al., 1935), by recruitment of Fcγ receptors inhibiting FcεR mediated activation (Malbec et al., 2002; Daeron et al., 1995), or by inhibition of IgE-facilitated allergen presentation (van Neerven et al., 1999). Their activity may rely either on a molar excess or on affinity maturation during vaccination (Hantusch et al., 2005). However, the exact interplay of antibodies with their cognate allergens still remains unclear.

50% of patients suffering from type I allergy are sensitised to grass pollen proteins. These allergens are potent elicitors of clinical symptoms, such as rhinitis, conjunctivitis and asthma (Andersson and Lidholm, 2003). Phl p 5 represents one of the major pollen allergens of timothy grass (*Phleum pratense*) and accounts for IgE binding in up to 60% of patients (Vrtala et al., 1993). Two isoforms,

Abbreviations: BCIP, 5-bromo-4-chloro-3-indoyl phosphate; CBD, chitin binding domain; CD, cluster of differentiation; DTT, dithiotreitol; MPBS, PBS supplemented with milk powder; NBT, nitrotetrazolium blue chloride; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RBL, rat basophil leukaemia; TFPA-PEG3-Biotin, tetrafluorophenylazide-(triethyleneglycol)-Biotin.

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doi:10.1016/j.molimm.2011.03.005

Phl p 5a and Phl p 5b exhibit high sequence similarity and differ only slightly in molecular masses and biochemical behaviour (Becker et al., 1995). These isoproteins are composed of two alanine-rich (AR) repeats, which form four alpha-helices each and thereby, group 5 and 6 allergens represent the distinct protein class of 4-helix-bundle allergens (Rajashankar et al., 2002).

Although the interaction of allergens with polyclonal serum-derived IgE has broadly been studied detailed analyses of the role of allergen-specific antibodies in pathophysiology as well as their interplay on a molecular level have been hampered by two critical limitations, the low IgE levels in serum and the lack of monoclonal allergen-specific antibodies of different isotypes. Approaches to generate human allergen-specific IgE-secreting hybridomas from immunised donors have not been successful so far (Zurcher et al., 1995). Furthermore, conventional hybridoma technology is often limited by a low immunogenic potential of allergens (Saarelainen et al., 2002) suggesting that allergens have less preferable binding sites for IgG antibodies compared to IgE antibodies. Additionally, resulting murine antibodies are neither compatible with established human specific assay formats nor recognise authentic IgE epitopes.

During the last decade sophisticated antibody technologies ranging from the generation of antibody fragment libraries to the design of tailor-made immunoglobulins have become tools for dissection of human immune responses. The establishment of combinatorial approaches has enabled the selection of monoclonal antibody fragments from synthetic library formats (Marks et al., 1991; Barbas et al., 1992) or immune repertoires against an almost unlimited panel of target molecules (Griffiths et al., 1994; McCafferty et al., 1990; Smith, 1985). The former approach is particularly attractive for the generation of antibodies against the vast variety of allergenic molecules (Braren et al., 2007). In contrast, the isolation of antibodies from libraries on the basis of lymphoid sources (Clackson et al., 1991; Burton et al., 1991; Barbas et al., 1991) is high in effort due to the evanescent number of IgE-producing cells, but imperative for identifying allergy-related authentic antibodies. Hence, only scarce studies reported cloning of allergen-specific IgE antibody fragments (Steinberger et al., 1996; Persson et al., 2007; Laukkanen et al., 2003; Flicker et al., 2002; Christensen et al., 2010).

The aim of our present work was to gain access to authentic allergen-specific human antibody isotypes allowing insights into the molecular basis of their interaction. Therefore, we employed antibody fragments of varying origin and produced recombinant IgE, IgA and IgG antibodies. On this basis the IgE epitope on the major timothy grass pollen allergen Phl p 5a was assigned and characterised by different techniques. The approach pursued here may facilitate the access to allergen-specific recombinant antibodies and contribute to the elucidation of the complex molecular interactions in allergy.

2. Material and methods

2.1. Expression of recombinant Phl p 5a and Phl p 5b constructs

For expression of recombinant Phl p 5 in *E. coli*, the pMal system (New England Biolabs) was used. Expression clones containing the coding sequence of Phl p 5a and b were kindly provided by Arnd Petersen (Research Center Borstel, Borstel, Germany). Both isoforms were purified from *E. coli* lysates by affinity chromatography using an amylase column and 10 mM maltose in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.5) for elution. Phl p 5 fragments were obtained by introducing particular PCR fragments into the vector pTXB1 (New England Biolabs) and expressed as N-terminal fusion proteins with an intein sequence and a chitin binding domain (CBD). Purification from the *E. coli* cell lysates was performed using chitin beads and elution from the column by addition of 50 mM dithiotreitol (DTT, Sigma–Aldrich) according to the recommendations of the manufacturer.

The oligonucleotides used for generation of the fragments were as follows: F1 a: gatccatatggcaggtaaggcgacgaccgag (Nde I for) and gatcgctcttccgcagccggcgatgatgcggag (Sap I back); F2 a: gatccatatgaagtacaggacgttcgtcgcaacc (Nde I for) and gatcgctcttccgcagccggcgatgatgcggag (Sap I back); F3 a: gatccatatggcaggtaaggcgacgaccgag (Nde I for) and gatc gctcttccgcacttgtaggcggcgtcgagcttg (Sap I back); F4 a: gatccatatgaagtacaggacgttcgtcgcaacc (Nde I for) and gatcgctcttccgcacttgtaggcggcgtcgagcttg (Sap I back); F5 a: gatccatatg aagtacaggacgttcgtcgcaacc (Nde I for) and gatcgctcttccgcactcggctgtcttgtaggcgagc (Sap I back); chimeric F1: gatcccatggcctgttccaacaaggccttcgcggagg (Nco I for) and gatc gcgatcgcacaggaggtgagcgcggccttg (AsiS I back).

2.2. Production of recombinant antibodies

Heterotetrameric IgG and IgE immunoglobulins were produced using recently established vector systems (Braren et al., 2007). The variable regions vH and vL were amplified using oligonucleotides containing restriction sites at the 5′- and 3′-termini of the vH (gatcatttaaatgtgtccagtgtgaggtgcagctggtgg and gatcctcgagacggtgaccagggt) and vL (gatccctgcagggtgccagatgtgagctcacccagtctccatc and gatcgcgatcgcacgtttgatttccacc) by PCR, respectively. IgA2 heavy chain constant regions were amplified from total cDNA of human PBMCs restriction sites were introduced at the 5′- and 3′-termini of the cH (gatcctcgagcgcatccccgaccagcc and gatcggcccagccggcctcaatggtggtgatggtagcaggtgccgtccacc) and lambda cL (gatcgcgatcgcacagcccaaggctgccc and gatctctagactatgaacattctgtaggggc) by PCR, respectively, in a way that a 4x His-Tag is generated at the C-terminal end of the heavy chain. Subsequently, the DNA was introduced into the different expression vectors.

Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbeccoïs modified Eagle medium (DMEM) supplemented with 100 ml/L fetal calf serum, 10,000 IU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Life technologies. HEK-293 cells were transfected by using 2 μg of the particular expression vector DNA complexed with polyethylene imine (Sigma–Aldrich). The secreted immunoglobulins were purified from the culture medium by affinity chromatography using a protein A-medium (SureMAb columns, GE Healthcare) or Ni-NTA-agarose (Qiagen) according to the manufacturers' recommendations.

2.3. Amplification and cloning of FcεRI-IgY Fc and CD64-IgY Fc

The cloning and expression of the soluble IgE Fc receptor FcεRI-IgY Fc has been described elsewhere (Braren et al., 2010). The human CD64 extracellular domains were amplified without the original signal sequence using one PCR primer containing a Pfl23 II site (gatccgtacgtgtgggcaagtggacaccacaaaggc) and another primer containing an Sgs I site (gatcggcgcgccatgaaaccagacaggagttgg) and introduced into pcDNA3.1/zeo providing a rat immunoglobulin leader sequence and avian Fc regions (Greunke et al., 2006).

2.4. Assessment of immunoreactivity in ELISA and immunoblot

For assessment of immunoreactivity in direct ELISA the particular proteins (10 μg/ml diluted with 2% MPBS) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 5% MPBS at RT for 1 h. Thereafter, the recombinant immunoglobulins (1 μg/ml diluted with 2% MPBS) were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and detected with human Ig isotype specific