Allergen specificity of IgG_4-expressing B cells in patients with grass pollen allergy undergoing immunotherapy

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Background: Serum IgG_4 responses to allergen immunotherapy are well documented as blocking allergen binding to receptor-bound IgE on antigen-presenting cells and effector cells, but the molecular characteristics of treatment-induced IgG_4, particularly in relation to expressed antibody, are poorly defined.
Objectives: We aimed to clone and express recombinant IgG_4 from patients receiving grass pollen immunotherapy using single B cells to obtain matched heavy- and light-chain pairs.
Methods: IgG_4^+ B cells were enriched from blood samples taken from 5 patients receiving grass pollen immunotherapy. Matched heavy- and light-chain variable-region sequences were amplified from single IgG_4^+ B cells. Variable regions were cloned and expressed as recombinant IgG_4. Binding analysis of grass pollen–specific IgG_4 was performed by using surface plasmon resonance. Functional assays were used to determine IgE blocking activity. In a separate experiment grass pollen–specific antibodies were depleted from serum samples to determine the proportion of grass pollen–specific IgG_4 within total IgG_4.
Results: Depletion of grass pollen–specific antibodies from serum led to a modest reduction in total IgG_4 levels. Matched heavy- and light-chain sequences were cloned from single IgG_4^+ B cells and expressed as recombinant IgG_4. We identified an IgG_4 that binds with extremely high affinity to the grass pollen allergen Phl p 7. Furthermore, we found that a single specific mAb can block IgE-mediated facilitated allergen presentation, as well as IgE-mediated basophil activation.
Conclusion: Although increases in IgG_4 levels cannot be wholly accounted for within the allergen-specific fraction, allergen immunotherapy might result in the production of high-affinity allergen-specific blocking IgG_4. (J Allergy Clin Immunol 2012;130:663-70.)

Key words: *Immunotherapy, IgG_4, Phl p 7, blocking antibody*

Sensitization to grass pollen affects up to 20% of the general population, causing symptoms that include rhinitis and asthma. IgE antibodies specific for timothy grass *(Phleum pratense)* might be directed toward any of 9 allergenic proteins contained in pollen grains but are most commonly specific to the major allergens Phl p 1 and Phl p 5.[1]

Allergen immunotherapy is the only form of treatment that modulates the immune response to allergen with well-documented effects on both T- and B-cell responses.[2] Treatment with immunotherapy results in long-term reductions in symptoms that are associated with the induction and persistence of blocking antibodies, particularly IgG_4.[3] Blocking antibodies have been shown to inhibit IgE-mediated responses, such as facilitated allergen presentation and basophil histamine release.[4]

Analysis of rearranged immunoglobulin variable-region genes in B cells from atopic subjects has been useful in identifying molecular signatures of allergic disease. For example, a biased use of VH5 genes by IgE^+ B cells has been reported in several studies.[5-8] Although serum antibody responses to allergen immunotherapy have been well characterized, the molecular characteristics of immunotherapy-induced IgG_4 have not been examined. With the exception of a small panel of birch pollen (Bet v 1)–specific IgG_4-producing EBV lines,[9] few monoclonal IgG_4 antibodies have been cloned from samples from patients undergoing immunotherapy. Single-cell RT-PCR has made possible the reconstruction of recombinant antibodies with natural pairings of heavy and light chains. These techniques provide sequence information that can be directly related to the antigen and epitope specificity of the expressed mAbs. These techniques have been applied to infectious diseases and human autoimmune diseases[10-14] but not previously to allergic diseases. Here we report on the construction and expression of recombinant human IgG_4 antibodies obtained from peripheral blood B cells of patients receiving allergen immunotherapy. We have identified a monoclonal IgG_4 antibody specific for the grass pollen allergen Phl p 7 and have defined the binding kinetics of this antibody using surface plasmon resonance (SPR). We have shown that this antibody binds with high affinity and blocks polyclonal IgE responses to grass pollen allergen in functional assays.

METHODS

Patient data

Five patients (Table I) receiving subcutaneous immunotherapy with an alum-absorbed 6-grass mix (Alutard; ALK-Abelló, Hørsholm, Denmark)

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Abbreviations used
CDR: Complementarity-determining region
FWR: Framework region
HBS: HEPES-buffered saline
ISU: ISAC standardized units
SPR: Surface plasmon resonance

were recruited at the Royal Brompton Hospital (London, United Kingdom). The study was approved by the London and City Research Ethics Committee, and blood samples were obtained after obtaining written informed consent. Levels of serum IgE and IgG_4 specific for the grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 7 were determined by using ELISA (see Fig E1 in this article's Online Repository at www.jacionline.org) with 10 μg/mL allergen, as described below.

Enrichment of IgG_4 memory B cells

PBMCs were isolated from blood by mean of density gradient centrifugation over Ficoll-Paque Plus medium (GE Healthcare, Fairfield, Conn). Switched memory B cells were isolated from PBMCs by means of negative selection (switched memory B-cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). IgG_4 memory B cells were enriched by using magnetic depletion of non-IgG_4 isotypes with biotin-labeled antibodies specific for IgG_1 (clone 8c/6-39), IgG_2 (clone HP-6014), IgG_3 (clone HP-6050; all from Sigma-Aldrich, St Louis, Mo), and IgA (clone IS11-8E10, Miltenyi Biotec) and anti-biotin microbeads (Miltenyi Biotec). Enriched IgG_4 memory B cells were diluted to 1 cell per microliter in PBS, and single cells were identified by means of light microscopy. In one sample (SCIT105) single cells were isolated by means of a cell sorter (FACSAria II; BD Biosciences, San Jose, Calif) after enrichment of IgG_4 B cells, as described. Cells were stored frozen at −80°C.

Single-cell RT-PCR

Single IgG_4-enriched B cells were thawed on ice and lysed for 15 minutes in buffer containing 0.4% Igepal (Sigma-Aldrich), 60 μmol/L deoxyribonucleotide triphosphates (Bioline, London, United Kingdom), 2.5 μmol/L dithiothreitol, and 8 units of RNase OUT (Invitrogen, Carlsbad, Calif). cDNA was generated with Superscript III reverse transcriptase by using 5 μg/mL each of oligo (dT) and random primers (both Invitrogen) for 10 minutes at 37°C, 45 minutes at 42°C, and 10 minutes at 50°C.

Heavy- and light-chain variable regions (V-regions) were amplified from single-cell cDNA by means of nested PCR (see Fig E2 in this article's Online Repository at www.jacionline.org) in a 25-μL reaction by using Platinum Taq polymerase (Invitrogen) and 2.5 μL of cDNA (first round) or 2.5 μL of first-round PCR product (second round). A mixture of forward primers annealing in the leader region (round 1) or framework region (FWR; round 2) were used to cover all known V-region genes (see Table E1 in this article's Online Repository at www.jacionline.org). Reverse primers were specific for IgG_4, Igκ, or Igλ constant regions (see Table E1). PCR products were purified (PCR purification kit; Qiagen, Hilden, Germany) and sequenced by using the appropriate reverse primer (Eurofins MWG, Ebersberg, Germany). Sequences (excluding primer annealing regions) were analyzed with IMGT V-Quest.[15] The percentage frequency of replacement mutations in both the complementarity-determining regions (CDRs) and FWRs were compared with the expected frequency (based on germline sequences) by using the focused binomial test by Hershberg et al.[16]

Antibody cloning

First-round PCR products were used as a template for a second PCR by using V-region– or J-region–specific primers (see Table E1) containing restriction enzyme target sequences. PCR products were digested with the corresponding restriction enzymes (New England Biolabs, Ipswich, Mass) and purified by using agarose gel (1%) electrophoresis and gel extraction (Gel Purification Kit, Qiagen). Purified, restriction site–modified PCR products were ligated into modified pTT3 vectors[17] containing a human V-region leader sequence (VH1-02, VκA26, Vλ8a) and either IgG_4 (pIgγ4), Igκ (pIgκ), or Igλ (pIgλ) constant regions (see Fig E2).[18] Ligated vectors were transformed into competent *Escherichia coli* (XL-2 Blue; Stratagene, La Jolla, Calif), and LB-broth containing 0.1 mg/mL ampicillin (Sigma-Aldrich) was inoculated with single colonies and incubated overnight at 37°C. Plasmid DNA was purified (Qiagen) and sequenced with vector-specific primers (see Table E1). Sequences were aligned to the original nested PCR product (ClustalW, www.ebi.ac.uk) to ensure no base mutations had been introduced.

Human embryonic kidney cells (HEK293E; ATCC no. CRL-1537) were transfected with a 4:1 ratio of polyethylenimine (Sigma-Aldrich) to DNA and a 2:1 ratio of pIgκ/λ to pIgγ4. Cells were cultured for 10 days in Dulbecco modified Eagle medium supplemented with 10% FCS, 2 mmol/L L-glutamine, 10 U of penicillin/streptomycin, and 250 μg/mL Geneticin (all from Invitrogen). Culture supernatants were harvested, centrifuged to remove cell debris, and filtered through 0.22 μM syringe filters.

IgG_4 ELISA and microarray immunoassay

Antibody expression was confirmed by means of ELISA of cell-free supernatants. ELISA plates (Maxisorp, Nunc) were coated with 2 μg/mL anti-human–IgG_4 (clone JDC-14, BD Biosciences). Plates were blocked with PBS and 1% BSA, and 50-μL supernatants were incubated in duplicates for 16 hours at 4°C. Plates were incubated with 1 μg/mL biotin-conjugated anti-human IgG_4 (clone G17-4, BD Biosciences), followed by streptavidin–horseradish peroxidase (R&D Systems, Minneapolis, Minn). IgG_4 was detected with TMB (R&D Systems) by measuring absorbance at 450 nm with human IgG_4 (Sigma-Aldrich) to generate a standard curve. All antibodies were expressed at 0.5 to 12 μg/mL.

Antibody specificity was tested by using a commercially available allergen microarray immunoassay (ISAC; Phadia, Uppsala, Sweden). Allergen chips were washed and incubated with 20 μL of undiluted cell-free supernatant for 2 hours at room temperature. Specific IgG_4 was detected with a fluorescently labeled anti-human IgG_4 antibody. Slides were analyzed with a biochip scanner, and results were evaluated with MIA software (Phadia).

Depletion of allergen-specific IgG_4

Phleum pratense (0.5 mg; ALK-Abelló) or BSA (Sigma-Aldrich) were immobilized with amine-reactive Sepharose beads (GE Healthcare UK, Hertfordshire, United Kingdom) by means of overnight incubation at 4°C. Unreacted sites were blocked with 0.5 mol/L ethanolamine, followed by extensive washing in PBS to remove unbound allergen. Serum samples were diluted in PBS to normalize the total IgG_4 concentrations to approximately 500 μg/mL. Diluted sera were incubated with 50 μL of *P pratense*– or BSA-coupled beads for 4 hours at room temperature with constant gentle rotation. Specific antibodies were depleted from serum by means of centrifugation. Total IgG_4 levels were measured with ELISA, as described, and specific IgG_4 levels were measured by using a modified protocol in which ELISA plates were coated with 10 μg/mL grass pollen extract (*P pratense*, ALK-Abelló) instead of anti-IgG_4. Specific depletion of grass pollen–specific antibody was confirmed by using serial dilutions of a monoclonal grass pollen–specific IgG_4 antibody incubated with either grass pollen or BSA-coupled Sepharose beads (data not shown).

SPR

Recombinant IgG_4 (102.1F10) was purified on a protein G column (GE Healthcare) according to the manufacturer's instructions. Purified IgG_4 was dialyzed into HEPES-buffered saline (HBS) and concentrated to 0.5 mg/mL (Amicon-Ultra; Millipore, Temecula, Calif). SPR assays were performed with a Biacore T100 instrument (Biacore, Piscataway, NJ). IgG_4 was immobilized on a CM5 chip (Biacore) by using an amine coupling kit (Biacore). A second surface was prepared with purified polyclonal human IgG_4 (Sigma-Aldrich) as a reference control. Recombinant Phl p 7 allergen (Biomay, Vienna, Austria) was diluted in HBS, 2 mmol/L $CaCl_2$, and 2 mmol/L