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Mouse/human chimeric IgG1 and IgG4 antibodies directed to the house dust mite allergen Der p 2: use in quantification of allergen specific IgG

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Summary

Background and Objective Chimeric mouse/human monoclonal IgG1 and IgG4 antibodies were developed against the house dust mite allergen Der p 2. These chimeric IgG antibodies, hIgG1-Dp2 A and hIgG4-Dp2 A, have the same binding characteristics as the previously reported chimeric hIgE-Dp2 A and are composed of the heavy chain variable domains and light chains of the original murine monoclonal antibody 2B12, whereas the heavy chain constant domains have been replaced by the human IgG1 or IgG4 heavy chain. The expression level of hIgG1-Dp2 A and hIgG4-Dp2 A was 1 and 3.5 μg/mL, respectively.

Methods and Results Since all IgG in these culture supernatants is allergen-specific, they are useful reference reagents and enable the calculation of the amount of allergen specific IgG1 and IgG4 antibodies in absolute IgG amounts. The results obtained with two panels of sera from patients in immunotherapeutic treatment were evaluated and compared in Der p 2 IgE, IgG1 and IgG4 RAST and with reversed IgG4 RAST using labelled purified Der p 2. Close agreement between the results for the two IgG4 assays was found.

Conclusion With these chimeric reference reagents the quantities of isotype specific antiallergen antibodies can be calculated and compared.

Keywords: RAST, reversed RAST, IgG1, IgG4, IgE, Der p 2, house dust mite, chimeric antibody, immunotherapy


Introduction

The role of allergen specific IgG in allergic inflammation is still unclear. Allergen specific IgG is reported to be predominated by IgG1 and IgG4 antibodies [1–4]. Conflicting results were found in examining the role of IgG in the allergic reaction. Some investigators found evidence for an anaphylactoid role for allergen specific IgG, e.g. IgG4 [5–7]. These results could not be reproduced by others [8]. On the other hand, allergen specific IgG4 might be protective, working as a blocking antibody. Inhibitory effects of allergen specific IgG antibodies, were shown in histamine release experiments [9–14] in skin-prick tests [1,15–17] and in vivo [18].

Prolonged immunization, as in allergen immunotherapy, leads to the development of allergen specific IgG4 antibodies [1,3,19]. Although a correlation of IgG4 titres with clinical outcome of the immunotherapy was not always found [4], allergen specific IgG4 antibodies might effect clinical improvement [19,20–22], and the development of these antibodies is often monitored during immunotherapy. To achieve inhibition of the allergic reaction the amount of allergen specific (blocking) IgG compared to the amount of allergen specific IgE is of interest [17]; the molar ratio IgE/IgG is of importance.
Previously we reported on a chimeric IgE anti-Der p 2 antibody (hIgE-Dp2 A) [23,24] and discussed the use of this antibody for the absolute quantification of allergen specific IgE. We now report on the construction of mouse/human chimeric monoclonal IgG1 and IgG4 antibodies by recombinant DNA techniques from a mouse monoclonal antibody directed to Der p 2, a 14 kDa monomeric allergen [25]. These chimeric antibodies have the binding characteristics of the original mouse monoclonal antibody 2B12 [26], whereas the Fc-domain is derived from human IgG1 or IgG4. More precisely, these chimeric antibodies consist of the heavy chain variable domains and the complete light chains from the mouse hybridoma, whereas the constant domains of the heavy chain are of human origin. Since all IgG in these culture supernatants is allergen specific, they are useful reference reagents and enable the calculation of the absolute amount of allergen specific IgG1 and IgG4 antibodies.

**Materials and methods**

**Hybridoma cell line**

Monoclonal antibody against Der p 2, 2B12, subclone B3 (mouse IgG2b with kappa light chains), has been described previously [26].

**Culture conditions**

The 2B12 hybridoma cells, the heavy chain loss variants and the transfecomas were cultured in IMDM (GIBCO/BRL, Paisley, Scotland) containing 5% heat-inactivated FCS (Bodinco, Alkmaar, The Netherlands), 50 μM 2-mercaptoethanol, penicillin (100 IU/mL), streptomycin (100 μg/mL) and rhIL6 (500 U/mL [27]).

Selective medium was supplemented with 500 μg Geneticiin base/mL (Sigma Chemicals Co., St. Louis, MO, USA). Transfectecomas were grown in 1 L rollerbottles (Falcon, Becton Dickinson Labware, New Jersey, USA) in culture medium without G418. The culture supernatants obtained were concentrated (approximately five times) using a hollow fibre dialyser (Hemoflow F5, Fresenius AG, Bad Homburg, Germany).

**Sera**

Sera of mite allergic patients G and H, receiving mite specific immunotherapy (Dermatophagoides pteronyssinus extract, ALK, Denmark) 100 000 SQ per immunization. The sera were drawn before starting the immunotherapy and during therapy.

Mouse/human chimeric IgE, IgG1 and IgG4 monoclonal antibodies (hIgE-Dp2 A, hIgG1-Dp2 A and hIgG4-Dp2 A) derived from monoclonal antibody 2B12

The development of hIgE-Dp2 A was reported previously [23,24]. The chimeric Dp2 A IgG1 and IgG4 antibodies were prepared by combining methods described previously [23,24,28–30]. Briefly: a heavy chain expression plasmid was prepared as follows. RNA was isolated from 10^7 2B12 hybridoma cells, and cDNA was prepared using a RT-H-kit (GIBCO/BRL) in combination with an oligo-dT primer (Boehringer, Mannheim, Germany).

The 2B12 V_H domain was then amplified in a polymerase chain reaction (PCR) using V_H domain specific primers, V_H1FOR [5'TAGGAAGCTT CCTGAGGAGACGGTGACCGTGAGGCGC] and V_H1BACK[5'TSMARCTGCAGSAGTCWGGR] (in which S = C or G, M = A or C, R = A or G and W = A or T [31] with minor modifications), which contained restriction enzyme recognition sequences used in cloning as described below. Restriction sites for HindIII, Bsu36I and PstI are underlined in the primer sequence. Restriction endonucleases were obtained from GIBCO/BRL except for Bsu36I which was obtained from New England Biolabs (Beverly, MA, USA). Plasmid isolations, restriction enzyme digestions and agarose gel electrophoresis were performed as described in Sambrook et al. [32]. DNA sequence analysis of the V_H domain was performed using Sequenase according to the manufacturers suggested conditions (USB, Cleveland, OH, USA).

The heavy chain construct was prepared in the vector pSV-SPORT (GIBCO/BRL) and contained the IgH intron enhancer, IgH promoter, IgH leader sequence, V_H2B12 domain, and intron fragment [29,30]. For IgG1 and IgG4 constructs a human genomic C71- or C74-gene (HindIII-fragments) [29,33] was introduced in the unique HindIII site.

Several heavy chain loss variants (secreting light chains only) were isolated from a population of 2B12 hybridoma cells which was cultured for a period of 6 month during which the cells were transferred 2–3 times per week [30,34]. This population of cells was further enriched for clones producing light chains only by sorting for heavy chain negative clones on a FACS using an antimouse IgG2b FITC-labelled antibody (RM162b-MF, CLB, Amsterdam, The Netherlands). Hybridoma cells producing light chains but not heavy chains were then identified in ELISA [35]. Production of light chains only was confirmed in biosynthetical labelling with ^35^S-methionine and analysis on SDS-PAGE.

Heavy chain plasmid was introduced in a heavy chain loss variant by cotransfection with pSV2-neo [36]. Heavy chain (75 μg) and pSV2-neo (7.5 μg) plasmid DNA were mixed with 10^7 heavy chain loss variant cells in 800 μL iced cold PBS. The suspension was electroporated in a 0.4 cm
vaccine cuvette using a Biorad Gene Pulser with capacity extender (Biorad Laboratories, Richmond, CA) at 960 μF and 250 V. The cells were plated in two 48-wells culture plates (Costar, Cambridge, MA, USA) in culture medium. Selective medium containing 500 μg Gentamicin base/mL was added after two days. Clones were screened for hlgG1 and hlgG4 production and for binding to mite extract coupled to Sepharose in RAST (as described below). Mite-positive clones (transfectedomas) were subcloned by limiting dilution.

Radio Allergo Sorbent Tests (RAST)
Dermatophagoides pteronyssinus mites (1.5 g, Commonwealth Serum Laboratories (CSL), Melbourne, Australia) were extracted in PBS (3% w/v) supplemented with Tween-20 (0.1% v/v) and sodium azide (0.1% w/v) for 4 h. After filtration (black ribbon filter, Schleicher & Schüll, Dassel, Germany) the extract was coupled to 10 g CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). For IgE-RAST 1.5 mg Sepharose per test (250 μL) was used (about 0.17 μg (170 KU) Der p 2/test). For IgG1 and IgG4 RAST, 3 mg Sepharose per test (in 500 μL) was used.

Der p 2
Recombinant Der p 2 [37] was used. The insert Der p 2 was cloned in the vector pET-21a (Novagen, Madison, WI, USA) and expressed in E. coli BL21-pLysE (Novagen). Der p 2 was purified by affinity chromatography using monoclonal antibody αDpX [38].

Der p 2 (about 60 μg) was coupled to 100 mg CNBr-activated Sepharose 4B. For this IgE, IgG1 and IgG4 RAST 0.5 mg Sepharose per test (in 250 μL, about 300 KU (300 ng) Der p 2/test) was used. Mite-Sepharose and Der p 2-Sepharose were compared using chimeric monoclonal antibody hlgE-Dp2 A [23,24]; hlgE-Dp2 A showed a similar dose response with both Sepharoses (data not shown). Inactivated CNBr-activated Sepharose was used as control Sepharose. Per test 1.5 or 0.5 mg in 250 μL was used.

Per test 50 μL chimeric antibody or human serum dilution was added to Sepharose coupled allergen. Human sera were obtained from the Allergen diagnostics department from the CLB (Amsterdam, The Netherlands).

Detection was performed using 125I-labelled sheep antibodies to human IgE, 125I-labelled anti-IgG1 antibodies (MH161-1 M, CLB) or 125I-labelled anti-IgG4 (MH164-1 M, CLB) antibodies, respectively. The IgE-RAST was described previously [39], the IgG1 and IgG4-RAST were described previously [1,40]. The results were expressed as the amount of radioactive label bound relative to the total amounts of radioactivity added. When the concentration of allergen specific antibodies was calculated the serum dilution resulting in about 20% of binding was used (the steepest part of the dose—response curve).

Mite RIA, detection with antilight chain antibodies
Mite Sepharose (1.5 mg in 250 μL) was incubated with 50 μL antibody dilution. Detection was performed using 125I-labelled rat anti(mouse) kappa light chain antibodies (RM19, CLB) making use of the identical mouse light chain of the chimeric hlgE-Dp2 A, hlgG1-Dp2 A and hlgG4-Dp2 A. The supernatants of the hlgG1-Dp2 A and hlgG4-Dp2 A transfectomas were quantified by this assay using hlgE-Dp2 A [23,24] as a reference reagent.

Reversed RAST
Chimeric antibody dilutions or diluted serum samples (50 μL per incubation) were incubated with 1 mg anti-IgG4-Sepharose (MH164-1 M, CLB, Amsterdam, 1 mg antibody was coupled to 100 mg CNBr-activated Sepharose 4B) in 500 μL. The binding of 125I-recombinant Der p 2 (iodination of Der p 2 is described below) was measured. The results were expressed as the amount of radioactive label bound relative to the total amounts of radioactivity added.

Iodination of Der p 2
Der p 2 was iodinated by the chloramine T method with carrier-free 125I (Amersham, UK) as was described for PLA2 [3] with minor modifications: 1-propanol was added to Der p 2 (20 μg in PBS) to a final concentration of 30% (v/v) prior to labelling. The labelling was performed with 1 mCi of 125I. Free label was separated from 125I-recombinant Der p 2 by dialysis to PBS containing 0.01% (w/v) PEG-4000.

Results
Expression of mouse/human chimeric hlgG1-Dp2 A and hlgG4-Dp2 A monoclonal antibodies
Previously, we described mouse/human chimeric IgE antibodies, hlgE-Dp2 A, directed against the house dust mite allergen Der p 2 [23,24]. By the same method also chimeric hlgG1-Dp2 A and hlgG4-Dp2 A were prepared. These isotypic variants have the same antigen binding specificity and differ only in their constant domains of the heavy chain. Culture supernatant dilutions of the transfectedomas were tested in their binding to mite-extract using an IgG1 and IgG4 Der p 2-RAST (Fig. 1). Also the binding of hlgE-Dp2 A is shown in this figure. Radiolabelled anti-IgG1, anti-IgG4 and anti-IgE were used as tracer. As expected when different labelled antibodies are used, the dose response curves of these three assays are not parallel and the maximal binding differs.

Comparison of hlgE-Dp2 A, hlgG1-Dp2 A and hlgG4-Dp2 A
The binding of the chimeric hlgG1-Dp2 A and hlgG4-Dp2 A
Fig. 1. Binding of hIgE-Dp2 A, hIgG1-Dp2 A and hIgG4-Dp2 A in IgE, IgG1 and IgG4 mite-RAST. Serial dilutions of concentrated culture supernatant of hIgE-Dp2 A (○), hIgG1-Dp2 A (■), hIgG4-Dp2 A (▲) were tested in their binding to mite extract coupled to CNBr-activated Sepharose. The amount of antibody bound was detected with \(^{125}\text{I}-\text{antilgE}\) (for hIgE-Dp2 A), \(^{125}\text{I}-\text{antilgG1}\) (for hIgG1-Dp2 A) or \(^{125}\text{I}-\text{antilgG4}\) (for hIgG4-Dp2 A) antibodies. The results were expressed as the amount of radioactive label bound relative to the total amount of label added.

A antibodies to mite-Sepharose was compared with hIgE-Dp2 A using an antibody directed to the light chains of the chimeric antiDer p 2 antibodies. The light chains of the different isotypes of Dp2 A are identical and derived from the original mouse monoclonal antibody 2B12. Through this method the culture supernatants were calibrated using chimeric hIgE-Dp2 A as a reference reagent. Figure 2 shows the binding of the culture supernatant of hIgG1-Dp2 A and hIgG4-Dp2 A to mite-Sepharose. Also the dose response curve of chimeric hIgE-Dp2 A is shown. These dose–response curves were parallel, doubling the antibody concentration resulted in an increase of 14.4% binding of the labelled antilight chain antibodies (expressed as percentage binding of the total amount of label added). The five times concentrated culture supernatant of hIgG1-Dp2 A and hIgG4-Dp2 A transfectedomas contained 5 and 17 \(\mu\)g/mL, respectively.

Calibration of the amount of allergen specific IgE, IgG1 and IgG4 antibodies

Sera of mite allergic patients receiving allergen specific immunotherapy were evaluated for their amount of Der p 2 specific IgE, IgG1 and IgG4 antibodies expressed in ng/mL.

Two panels of sera, from patient G and H, drawn before starting the immunotherapy and during therapy, were tested for IgE, IgG1 and IgG4 Der p 2-RAST and in reversed RAST for IgG4 with radiolabelled Der p 2. The chimeric hlgE-Dp2 A, hlgG1-Dp2 A and hlgG4-Dp2 A were used as reference reagents. No Der p 2 specific IgG1 was detected (i.e. levels were \(< 30\text{ng/mL}\) in these sera. In Figs 3 and 4 the IgG4 RAST and IgG4 reversed RAST with radiolabelled Der p 2 for two sera of each patient are shown. None of the tested sera showed detectable binding to control Sepharose (i.e. binding \(< 1.4\%\) of added radioactivity, data not shown). The dose–response curves of hlgG4-Dp2 A was compared with the dose–response curves of IgG4-Der p 2 positive sera. In the steepest part of the dose–response curve of hlgG4-Dp2 A, shown in Fig. 3, doubling the antibody concentration led to an increase of 7.6% binding of the anti-IgG4 label (expressed as percentage binding of the total amount of anti-IgG4 label added). The patient sera tested showed an increase of 6.1 ± 1.4 (SD, \(n = 5\))% binding. For the reversed IgG4-RAST with radiolabelled Der p 2, Fig. 4, not all the dose response curves of the sera were parallel with hlgG4-Dp2 A. This might be a result of the few serum dilutions tested in this assay. Doubling the hlgG4-Dp2 A antibody concentration led to an increase of 9.5% binding. When the IgG4 RAST and IgG4 reversed RAST were compared, the reversed IgG4 RAST seemed to be slightly less sensitive than the IgG4 RAST in the
Mouse/human chimeric IgG1 and IgG4 antibodies directed to Der p 2

Fig. 3. Dose–response curves of hIgG4-Dp2 A and Der p 2 positive sera tested in Der p 2 IgG4-RAST. Serial dilutions of hIgG4-Dp2 A (▲) and two sera from patient G drawn at different moment during immunotherapeutical treatment: serum G1 (○) and serum G2 (□) and two sera from patient H, serum H1 (●) and serum H2 (■) were incubated with Der p 2-Sepharose. The binding of the antibodies was determined with 125I-antiIgG4 antibodies. The results were expressed as the amount of 125I-antiIgG4 label bound relative to the total amounts of radioactivity added.

determination of the amount of Der p 2 specific IgG4 (Fig. 5).

Figure 6 shows the different sera of the two patients G and H and their amount of Der p 2 specific IgE and IgG4 antibodies tested in RAST at the different moments after the start of the immunotherapeutic treatment. These Der p 2-RAST data indicated for both sera panels an increase in the amount of Der p 2 specific IgG4 to about 10–100 times the amount of allergen specific IgE during the ongoing immunotherapeutic treatment.

The decrease of the titre of Der p 2 specific IgE in both patients could be an artifact due to competition of the increased amount of Der p 2 specific IgG4 antibodies. However, addition of 1000 ng/mL hlgG4-Dp2 A did not influence the binding of hlgE-Dp2 A to mite-Sepharose. The binding of 1.75, 3.5 or 7 ng/mL hlgE-Dp2 A to mite-extract coupled to Sepharose was 5.1%, 8.0% or 12.5%, respectively (detected with radiolabelled antiIgE antibodies). After addition of 1000 ng/mL hlgG4 the binding was 4.8%, 8.0% or 13.5%, respectively.

Discussion

Immunotherapy is known to induce a rise in allergen specific IgG4 antibodies. This increase is often monitored during immunotherapy and may be linked to clinical improvement [19–22]. The scope of this paper is to show that using a reference chimeric antibody reagent the amount of allergen specific antibodies can be calculated. Usually the

Fig. 4. Dose–response curves of hlgG4-Dp2 A and Der p 2 positive sera tested in reversed IgG4-RAST with radiolabelled Der p 2. Serial dilutions of hlgG4-Dp2 A (▲) and two sera from patient G, serum G1 (○) and serum G2 (□) and two sera from patient H, serum H1 (●) and serum H2 (■) were incubated with antiIgG4-Sepharose. The binding of Der p 2 specific IgG4 antibodies was determined with 125I-Der p 2. The results were expressed as the amount of 125I-Der p 2 label bound relative to the total amounts of radioactivity added.

Fig. 5. Comparison of the Der p 2 IgG4-RAST and the reversed IgG4-RAST with radiolabelled Der p 2. The amount of Der p 2 specific IgG4 determined in RAST was compared to the amount determined in the reversed IgG4-RAST with radiolabelled Der p 2 (binding to anti-IgG4 coupled to Sepharose). ○, patient #G; ●, patient #H.
Fig. 6. Calibration of the amount of allergen specific IgE, IgG1 and IgG4 antibodies using Der p 2-RAST. Panels of sera of patient G (a) and H (b), taken prior to the start and during the immunotherapy were tested on their amount of Der p 2 specific IgE (○) and IgG4 (△) antibodies in Der p 2-RAST using hlgE-Dp2 A, hlgG1-Dp2 A and hlgG4-Dp2A as a reference reagents. The amount of Der p 2 specific IgG1 in these sera was under the limit of detection and is therefore not shown in this figure. The amount of antibodies is expressed in ng/mL.

amount of allergen specific IgG is expressed in arbitrary units. Expression of the amount of allergen specific IgG, as well as that of allergen specific IgE, in absolute amounts of protein was illustrated by using hlgG1-Dp2 A and hlgG4-Dp2 A as reference reagent. Two panels of sera collected during immunotherapeutical treatment were examined. These sera contained low amounts of Der p 2 specific IgG1 antibodies (all below the limit of detection). This was unexpected since allergen specific IgG antibodies are reported to be predominantly of the IgG1 and IgG4 subclass [1–4]. However, sera of only two patients were tested for Der p 2 specificity. Also patients treated with dog-dander immunotherapy showed a failure of the IgG1 specific response [16].

The calibration of Der p 2 specific IgG4 antibodies in these sera was performed in two different assays. The two assays used, the Der p 2-RAST (Fig. 3) and reversed RAST with radiolabelled Der p 2 (Fig. 4) using hlgG4-Dp2 A as reference reagent, were comparable for IgG4 calibration (Fig. 5): the amount of Der p 2 specific IgG4 measured was almost similar in both assays. Comparing the hlgG4-Dp2 A dose response curves in these two assays, the reversed RAST was slightly more sensitive than the Der p 2 RAST (data not shown). However, this is not true for every allergen and is dependent of the quality of labelled tracer also. The use of reversed RAST in the measuring allergen specific IgG antibodies has some disadvantages compared to RAST. The reversed RAST is more sensitive to allergen-antibody avidity differences, purified and labelled allergens are necessary. For each allergen another allergen specific reference reagent is needed. Testing of allergen specific IgG in RAST is less dependent on avidity, however, allergen excess conditions are necessary for a realistic quantification (also discussed in [24]). The efficiency of the allergosorbent to extract allergen specific antibodies from the serum sample is influenced by the avidity of the antibody–allergen interaction. When the amount of allergosorbent is added in excess these complications can be circumvented. Re-testing of the serum sample after the first incubation, or testing of the variability in binding when a twofold variation of allergosorbent dose is used will give information about the amount of allergosorbent needed. Furthermore testing in RAST enables the calibration of all the allergen specific assays with one reference reagent and one tracer (directed to the heavy chain).

The role of allergen specific IgG4 in the clinical improvement of allergic patients remains unclear. For a blocking effect of allergen specific IgG antibodies in the early phase of the allergic reaction, as was shown in a decrease in skin reactivity [17], the molar ratio of allergen specific IgE to IgG antibodies is of interest. To properly compare the amounts of allergen specific IgE and IgG the raw data (percentage binding) can not be compared (as is shown in Fig. 1).

Although the sera tested and discussed in this paper are limited, the usage of these antibodies as a reference reagent is demonstrated. Like hlgG-Dp2 A [24], chimeric hlgG1-Dp2 A and hlgG4-Dp2 A antibodies have several advantages in their use as reference serum compared to serum antibodies. The production by a hybridoma-like cell line provides unlimited access to well defined antibodies. The only immunoglobulin present in the culture supernatant is allergen specific IgG1 or IgG4. Some light chain overexpression was observed; however, these light chains did

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not bind to the absorbent (data not shown). This panel of chimeric antibodies enables the quantification and comparison of isotype specific, antiallergen antibodies in sera.

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