Normal human immunoglobulin G4 is bispecific: it has two different antigen-combining sites

J. SCHUURMAN,* R. VAN REE,* G. J. PERDOK,* H. R. VAN DOORN,† K. Y. TAN* & R. C. AALBERSE* *Central Laboratory of the Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, and †Schieland Hospital, Schiedam, the Netherlands*

SUMMARY

Unlike other immunoglobulin G (IgG) subclasses, IgG4 antibodies in plasma have been reported to be functionally monovalent. In this paper we show that the apparent monovalency of circulating IgG4 is caused by asymmetry of plasma IgG4. A large fraction of plasma IgG4 molecules have two different antigen-binding sites, resulting in bispecificity. Sera from patients with IgG4 antibodies to both house dust mite and grass pollen induced cross-linking of Sepharose-bound grass pollen antigen to radiolabelled house dust mite allergen *Der p* I. This bispecific binding activity was not observed in sera with IgG4 antibodies to either grass pollen or house dust mite exclusively. Depletion of IgG4 antibodies resulted in disappearance of the bispecific activity. By size exclusion chromatography we excluded the possibility that bispecific activity was caused by aggregation of IgG4 antibodies. These results indicate that circulating (polyclonal) IgG4 antibodies have two different antigen-binding sites and therefore are functionally monovalent antibodies.

INTRODUCTION

The interaction of an antibody with an antigen initiates several immunological responses. The consequences of this antibody–antigen interaction are determined by the isotype of the antibody (mediating through the interaction with immunoglobulin receptors) and by the valency of the antibody–antigen interaction. Bivalency of an immunoglobulin G (IgG) antibody is an important aspect of most effector functions.

Human IgG4 antibody is unable to precipitate purified antigens.[1,2] This inability to precipitate allergen was not due to a difference in affinity of the IgG4 antibodies, but was caused by the inability of IgG4 antibodies to cross-link two antigens: human serum IgG4 antibodies are functionally monovalent.[1,2] In addition, IgG4 antibodies react poorly with Fc-receptors compared to the IgG1 antibody and do not efficiently activate complement.[3] Nevertheless, in some individuals chimeric IgG4 antibody was shown to be as potent as IgG1 in antibody-dependent cell-mediated cytotoxicity.[4] This unexpected effect, presumably due to a polymorphism, was also seen in an *in vivo* mouse model of immunotherapy.[5]

Generally, however, IgG4 antibodies are considered to be less efficient in inducing inflammatory responses and may even inhibit the inflammatory effects of other antibodies.[6–11] Prolonged antigenic stimulation with high doses of soluble

protein antigen preferentially induces IgG4 antibodies. IgG4-dominated responses have been reported in allergic patients receiving immunotherapy,[1,12,13] in beekeepers,[1] in patients with chronic parasitic infections, such as schistosomiasis and filariasis,[8–11] and in haemophiliac patients receiving factor VIII.[14] IgG4-mediated inhibition of immune inflammation may be advantageous in some instances, but may be undesirable in infections.

In this report we compared circulating and chimeric IgG4 antibodies. Our results indicate that the apparent monovalency of naturally occurring IgG4 antibodies is due to bispecificity.

MATERIALS AND METHODS

Monoclonal chimeric antibodies and sera
The mouse/human chimeric monoclonal IgG4 antibody (hIgG4-Dp2A) has been described previously.[15] This chimeric antibody is directed against the house dust mite allergen *Der p* II and originates from the mouse hybridoma cell line 2B12.[16] The rabbit hyperimmune antiserum to *Der p* I has been described elsewhere.[17]

The sera and plasma samples used were obtained from the Allergy Diagnostics Department (CLB, Amsterdam, the Netherlands). Nine sera were used in the heterologous binding assay (described below), plasma #741B (obtained by plasmapheresis) and serum #741 were derived from the same patient; plasma #178 (obtained by plasmapheresis) was used in the valency tests. This plasma contained 1400 ng of *Der p* II-specific IgG4 antibodies/ml as was determined by the method described previously.[15] The IgG4 antibodies of this plasma were affinity purified using monoclonal antibody

MH164-4M (CLB) coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden).

Radioimmunoassays (RIA)

Mite-extract. *Dermatophagoides pteronyssinus* mites were obtained from Commonwealth Serum Laboratories (Melbourne, Australia), 1·5 g of mites were extracted in phosphate-buffered saline (PBS; 3% w/v) supplemented with Tween-20 (0·1% v/v) and sodium azide (0·1% w/v) for 4 hr. After filtration (black ribbon filter, Schleicher & Schüll, Dassel, Germany), the extract was coupled to 10 g CNBr-activated Sepharose 4B (Pharmacia). Per test, 1·5 mg Sepharose in 250 µl was used, equivalent to 170 KU (0·17 µg) *Der p* II/test. This was also the optimal condition for the two-site assay described below.

Grass pollen. *Phleum pratense* and *Dactylis glomerata*, obtained from Diephuis Laboratories (Groningen, the Netherlands) were extracted as previously described[18] and coupled to CNBr-activated Sepharose. Per test 1 mg Sepharose was used.

Iodination of allergens

Recombinant *Der p* II was used (kindly supplied by G. A. Mueller[19]). *Der p* II was purified by affinity chromatography using monoclonal antibody αDpX.[17]

The *Der p* II was iodinated by the chloramine-T method with carrier free [125]I (Amersham Life Science, Amersham, UK) as was described previously[1] with minor modifications, necessary for iodinating *Der p* II (20 µg in 100 µl PBS): 1-propanol was added to a final concentration of 30% (v/v) prior to labelling. The labelling was performed with 1 mCi of [125]I. Free label was separated from ^{125}I-labelled *Der p* II by dialysis against PBS containing 0·01% (w/v) PEG-4000. *Der p* I, affinity purified with anti-P1[17] from *Dermatophagoides pteronyssinus* mite extract was labelled by the standard chloramine-T method.[1]

Two-site assay: testing the valency of the antibodies

To test whether serum or chimeric antibodies were able to cross-link radiolabelled antigen to immunosorbent bound antigen, a two-site assay was performed. In this assay the amount of capturing antigen coupled to Sepharose has to be optimized in order to avoid bivalent binding of antibodies to adjacent antigens on the immunosorbent. To determine this, the amount of mite extract used for coupling to CNBr-activated Sepharose was titrated.

Sepharose-coupled mite-extract (described above) was incubated with 50 µl serum or chimeric antibody (1–20 ng antibody per test) in a total volume of 300 µl in PBS-AT (PBS supplemented with 0·3% bovine serum albumin, 0·1% Tween-20, 0·05% (w/v) NaN₃). The ability to cross-link was examined by addition of ^{125}I-labelled *Der p* II (in the valency tests) or *Der p* I (in the heterologous cross-linking assay).

Antigen-binding test

For the antigen-binding test 10 µl serum (or serum dilutions) or antibody (1–20 ng antibody/test) was incubated with 1 mg Protein A–Sepharose (Pharmacia) in 500 µl PBS-AT together with ^{125}I-labelled antigen. The results were expressed as the percentage of radioactivity bound relative to the total radioactivity added.

Heterologous cross-linking assay

Sera were tested for bispecific binding activity in the heterologous cross-linking assay. Fifty microlitres of serum (or serum dilutions) were incubated with grass pollen–Sepharose (1 mg/test) in a total volume of 300 µl in PBS-AT. Antibodies bound were detected using radiolabelled mite-allergen *Der p* I. The results were expressed as the percentage of radioactivity bound relative to the total radioactivity added.

Size-exclusion chromatography

Serum #741 (200 µl) was applied to a ACA34 column (Ultrogel LKB, Villeneuve-la-Garenne, France), 1·5-cm diameter and 35-cm length, 62-ml material, equilibrated in PBS-AT. Fractions of 450 µl were collected and analysed for total IgE (180 000 MW), *Der p* I-specific IgG (tested in the antigen-binding test for IgG) and for grass pollen/*Der p* I-bispecific binding activity (tested in the heterologous cross-linking assay). The void volume was determined using Dextran Blue 2000 (Pharmacia). The results were expressed as percentage of starting material.

IgG4 and IgE depletion.

Serum #741 was depleted for IgG4 and IgE antibodies. Anti-IgG4 monoclonal antibody MH164-4M (CLB), 10 mg, was coupled to 1 g CNBr-activated Sepharose (Pharmacia). For the depletion of IgE antibodies polyclonal sheep antihuman IgE serum coupled to Sepharose (10 ml antiserum was coupled to 20 g CNBr-activated Sepharose) was used. As a control for the depletion experiment, serum was incubated with CNBr-activated Sepharose that had been quenched by the addition of glycine. For depletion of 40 µl serum #741, 16 mg anti-IgG4, anti-IgE, or inactivated CNBr-activated Sepharose was used. The depletion was performed by overnight incubation (end over end at room temperature) in a final volume of 500 µl PBS-AT.

RESULTS

Valency of natural and chimeric IgG4 antibodies directed to the house dust mite allergen *Der p* II

The amount of *Der p* II-specific IgG antibodies (or, more accurately, $IgG_{124} = IgG1 + IgG2 + IgG4$ antibodies) in plasma #178 was determined in the antigen-binding test for *Der p* II, using as solid phase either Sepharose-coupled Staphylococcal protein A or Sepharose-coupled anti-IgG4; chimeric IgG4 antibody was used as a reference (Fig. 1). From the data presented in Fig. 1 the contribution of IgG4 antibody relative to IgG_{124} antibody in this serum can be calculated to be 56%. Next, the bivalent reactivity of naturally occurring IgG4 antibodies (purified from plasma #178) was compared to the bivalent reactivity of the mouse/human chimeric IgG4 monoclonal antibody directed to the house dust mite allergen *Der p* II. Bivalent reactivity was measured as the ability to cross-link radiolabelled *Der p* II and Sepharose-bound *Der p* II (Fig. 2). Chimeric IgG4 induced cross-linking of radiolabelled *Der p* II to Sepharose-bound *Der p* II. In contrast, neither plasma #178 nor affinity purified plasma #178-derived IgG4 antibodies induced significant binding in this assay. Chimeric IgG1 and IgG4 antibodies were equally effective in cross-linking (not shown). These observations confirmed that naturally occurring (polyclonal) IgG4 antibodies behave as mono-
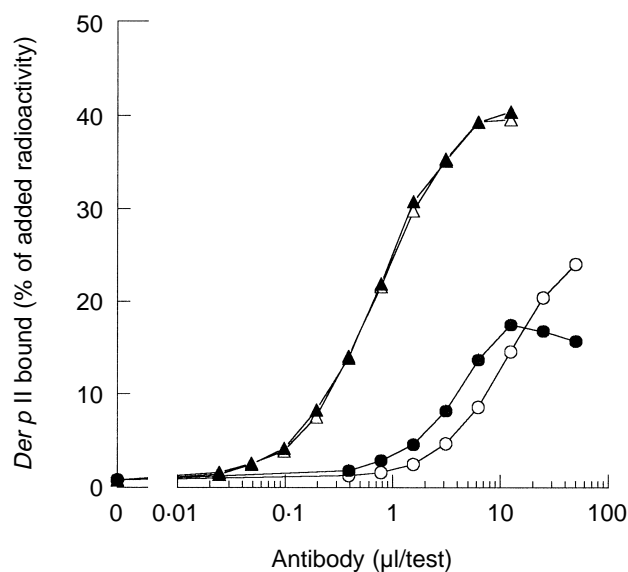
Figure 1. The relative contribution of IgG4 to the total IgG$_{124}$ antibody activity with respect to the binding of ^{125}I-labelled *Der p* II of plasma #178 was deduced from the comparison between Sepharose-coupled anti-IgG4 (○) and Sepharose-coupled Staphylococcal Protein A (●); the results obtained with the chimeric IgG4 antibody, 17 µg/ml, are also shown (anti-IgG4, △, Protein A, ▲).

valent reagents towards purified *Der p* II in contrast to the bivalent behaviour of chimeric IgG4 antibodies.

Allergen-specific immunotherapy results in bispecific immunoglobulins

Sera from patients receiving therapeutic injections with both house dust mite and grass pollen extract were tested in a heterologous cross-linking assay. In this heterologous cross-linking assay the binding of purified radiolabelled house dust mite allergen *Der p* I to Sepharose-coupled grass pollen was measured (Table 1). All sera with IgG4 antibodies to both house dust mite allergen *Der p* I and to grass pollen allergen were able to cross-link mite-allergen *Der p* I to Sepharose-coupled grass pollen; binding ranging from 4·6 to 34·1% of added radioactivity. Sera of patients with IgG (IgG4) antibodies directed exclusively to house dust mite (represented by *Der p* I) or grass pollen (represented by *Lol p* I), were negative in this test (less than 2% binding of the amount of radioactivity added; the binding was indistinguishable from the binding of *Der p* I to control Sepharose to which an irrelevant ligand, glycine, had been coupled).

A fresh plasma sample from the patient that donated serum #741 was obtained, coded #741B. This sample showed similar results: 44·2% binding to Sepharose-coupled grass pollen and 0·8% binding to control Sepharose (binding expressed as percentage of labelled *Der p* I added).

To test whether a *Der p* I contamination of the allergosorbent (Sepharose-coupled grass pollen) was the cause of the bispecific binding of these sera, hyperimmune rabbit antiserum to *Der p* I was incubated with Sepharose-coupled grass pollen (Fig. 3). Although *Der p* I-binding activity of this hyperimmune rabbit antiserum exceeded the binding of plasma #741B,
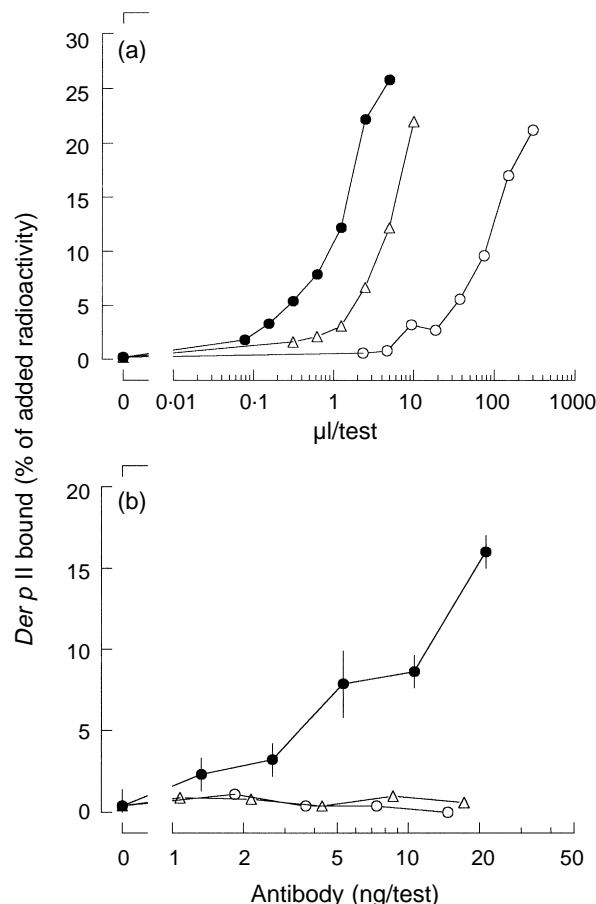
Figure 2. The functional valency of chimeric IgG4, plasma #178 and affinity-purified IgG4 from plasma #178. (a) Binding of ^{125}I-labelled *Der p* II to serial dilutions of chimeric IgG4 (17 µg/ml, ●), plasma #178 (△) and affinity purified IgG4 from plasma #178 (○) to Sepharose-coupled Staphylococcal Protein A were tested and used to determine the amount of *Der p* II-specific IgG$_{124}$ in the plasma. (b) The cross-linking of Sepharose-coupled mite-extract to ^{125}I-labelled *Der p* II by chimeric IgG4 (●), plasma #178 (△) or affinity-purified IgG4 from plasma #178 (○) was expressed as the amount of radioactivity bound relative to the amount of radioactivity added.

the hyperimmune rabbit antiserum induced less than 1% binding of labelled *Der p* I to grass pollen–Sepharose.

Size fractionation of bispecific binding activity

Serum #741 was subjected to gel filtration to exclude the involvement of antibody aggregates. Size-exclusion chromatography was performed using ACA34. Fractions were tested for total IgE, *Der p* I-specific IgG and the ability to cross-link Sepharose-coupled grass pollen to *Der p* I (Fig. 4). *Der p* I-specific IgG and the bispecific binding activity were detected in the same fractions, eluting just behind IgE. This indicates that the heterologous cross-linking activity was due to antibodies with the size of monomeric IgG.

IgG subclass of bispecific binding activity: IgG4

To investigate whether IgG4 antibodies were responsible for the bispecific binding measured in the heterologous binding

Table 1. Sera from patients receiving immunotherapy treatment were tested in a heterologous binding test

Serum	Heterologous binding test*	Negative control*	Der p I		Lol p I	
			IgG†	IgG4†	IgG†	IgG4†
#701	0·7	0·4	0	0	89	54
#719	1·2	0·9	50	39	0	0
#748	1·3	NT	179	162	7	2
#831	4·6	0·4	92	11	52	16
#822	5·0	0·2	66	45	57	25
#766	7·9	0·5	64	11	152	16
#797	8·8	0·7	64	64	81	41
#614	10·7	0·4	58	47	96	50
#741	34·1	0·4	76	62	249	59

Bispecific binding, the binding to grass pollen–Sepharose and glycine-inactivated Sepharose (control Sepharose) of 1 : 10 diluted serum was detected by radiolabelled *Der p* I; NT = not tested. *The results were expressed as the amount of radioactivity bound relative to the amount of radioactivity added. No correction for background binding was applied. †IgG and IgG4 antibodies to the house dust mite allergen *Der p* I and the grass pollen allergen *Lol p* I are expressed as arbitrary antibody units/ml (using a previously described method[27]).
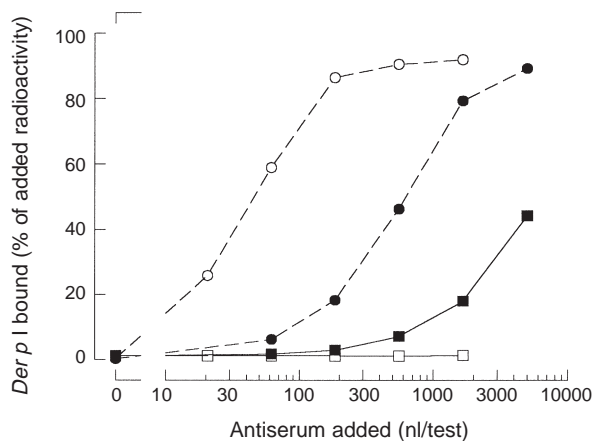

Figure 3. The bispecific binding is not caused by *Der p* I contamination of the grass pollen–Sepharose. Serial dilutions of rabbit polyclonal antiserum to *Der p* I (○, □) and plasma #741B (●, ■) were incubated with protein A–Sepharose (○, ●) or with grass pollen–Sepharose (□, ■). The binding was detected using radiolabelled *Der p* I. The results were expressed as the amount of radioactivity bound relative to the amount of radioactivity added.

assay, serum #741 was depleted for its IgG4, using anti-IgG4 antibodies coupled to CNBr-activated Sepharose. As controls, the serum was also depleted for IgE antibodies (using anti-IgE–Sepharose) and was incubated with control Sepharose (glycine-inactivated Sepharose). After depletion, the three samples of manipulated serum #741 were tested in the antigen-binding test for *Der p* I using protein A–Sepharose, and in the grass pollen/*Der p* I heterologous cross-linking assay (Fig. 5). Binding of *Der p* I in the antigen-binding test was decreased ($\approx$ 10-fold) after depletion of IgG4 antibodies; depletion of IgE, or incubation of the serum with control Sepharose (glycine-inactivated Sepharose), did not decrease *Der p* I binding. By the removal of IgG4, the bispecific binding activity disappeared. This bispecific binding activity remained when IgE was removed, or when the sample was incubated with
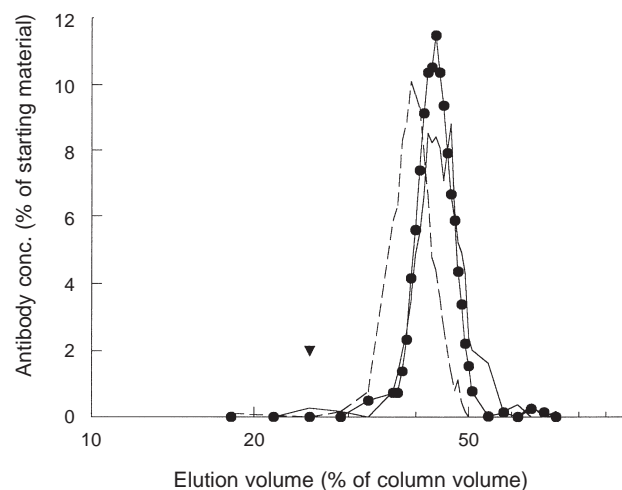

Figure 4. Gel filtration of serum #741. Serum #741, 200 µl was fractionated on an ACA 34 column. The fractions were tested for total amounts of IgE (180 000 MW, broken line), *Der p* II-specific IgG (solid line) and for the binding in the heterologous binding assay (●). The void volume is indicated by ▼. The results are expressed as percentage of starting material.

glycine-inactivated Sepharose. These observations demonstrate that IgG4 antibodies cause the bispecific binding of allergen in this serum.

DISCUSSION

Our results indicated the presence of bispecific antibodies in sera from allergic patients receiving immunotherapy (Table 1). We considered four trivial explanations of the apparent bispecificity:

1 Contamination of the grass pollen–Sepharose with allergen *Der p* I;
2 Cross-reactivity of a grass pollen allergen with the house dust mite allergen *Der p* I;
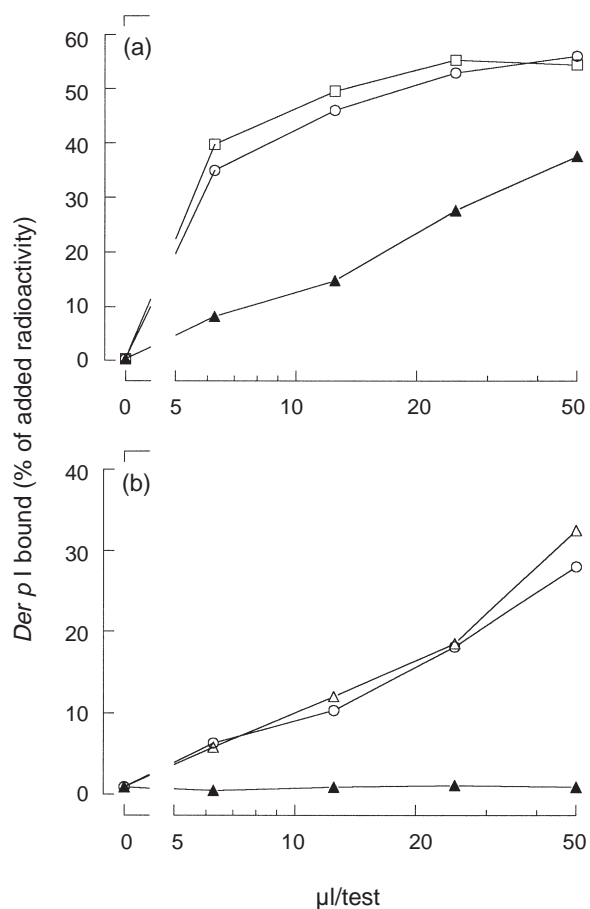
Figure 5. Depletion of IgG4 antibodies from serum #741. Serum #741 was depleted for IgG4 (▲). As control, a sample was also depleted for IgE (□) and incubated with control Sepharose (○). The three samples were tested for the amount of *Der p* I-specific IgG (a) and for the binding activity in the heterologous binding assay (b).

3 Antibody aggregation;
4 Non-immune binding of IgG4 to Sepharose-coupled grass pollen.

First, the bispecific binding was not caused by *Der p* I contamination of the grass pollen–Sepharose. This was tested using a hyperimmune rabbit antiserum (Fig. 3).

Second, if the observed bispecificity of IgG4 was due to cross-reactivity, the bispecific sera would show higher binding to Sepharose-coupled mite extract than to Sepharose-coupled grass pollen extract. Even if 100% of the antibodies that reacted with radiolabelled *Der p* I were cross-reactive with grass pollen extract, the cross-reactive binding of these *Der p* I-reactive antibodies could not be higher to grass pollen than to the homologous antigen, in this case: to Sepharose-coupled *Der p* I (or to Sepharose-coupled mite extract). However, we observed that the *Der p* I-binding activity of plasma #741B was approximately threefold lower to optimally coupled mite-extract than to Sepharose-coupled grass pollen (data not shown). This excludes cross-reactivity as explanation for the observed bispecificity. Furthermore, no grass pollen allergen has been described to be cross-reactive with the house dust mite allergen *Der p* I; IgE reactivity is absent in the majority of mite allergic sera. The bispecific binding described in this paper was not caused by, but could easily be mistaken for, cross-reactivity of allergens. In addition, in sera from patients treated for grass pollen allergy, bispecific antibodies to two non-homologous grass pollen allergens, *Lol p* I and *Lol p* V, were observed (R. van Ree, manuscript in preparation). Bispecific antibodies were also shown in sera from patients receiving immunotherapy for other allergen combinations than grass pollen and house dust mite and were measured to diphtheria and tetanus toxoid (data not shown).

Third, the gel filtration experiment indicated that bispecific antibodies were monomeric IgG molecules, and not antibody aggregates (Fig. 4).

Fourth, the absence of binding of serum #748 (with extremely high levels of *Der p* I-specific IgG4 but with negligible levels of IgG4 to grass pollen) in the heterologous cross-linking assay, excluded non-immune binding of the antibodies to the grass pollen as explanation. The absence of binding of the tested sera to control Sepharose (Table 1) also showed that the binding in the heterologous two-site assay was not caused by a non-specific interaction with Sepharose.

Depletion of the IgG4 antibodies from the serum clearly showed that the bispecific binding was largely, if not completely, caused by IgG4 antibodies (Fig. 5b). A possible explanation of the bispecificity of IgG4 antibodies can be found in some structural features of IgG4. IgG4 half-molecules (containing one heavy and one light chain when analysed on non-reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis) were noted by several investigators.[20–23] These half-molecules are virtually absent in IgG4 with a mutation of a single amino acid (serine 241 to proline, amino acid numbering according to Kabat *et al.*[24]) in the hinge region in IgG4[25] resulting in a closer resemblance to the IgG1 hinge region. This incomplete formation of interheavy chain disulphide bonds might make IgG4 antibodies susceptible to exchange of half-molecules. Exchange in a pool of polyclonal IgG4 would result in bispecific antibodies that behave as monovalent antibodies towards a single antigen. In contrast, monoclonal chimeric IgG4 antibodies would remain bivalent. The bivalency of monoclonal chimeric IgG4 antibodies was found both in electron microscopic analysis[26] and in radio-immunoassay (shown in Fig. 2).

The simplest explanation of the bispecificity of IgG4 is the above-mentioned postsecretion exchange of IgG4 half-molecules. An alternative, but less likely explanation is failure of allelic exclusion. This explanation would require an unprecedented phase in the development of IgG4-producing B cells, but would be in line with the observation that *in vitro* incubation of mixtures of chimeric antibodies did not result in bispecific reactions. Also, *in vitro* culturing of a mixture of the nitrophenyl (NP) and *Der p* II-specific IgG4 hybridoma cell lines did not induce bispecific antibodies (not shown).

The postulated physiological mechanism of generation of bivalency *in vivo*, by IgG4 half-molecule exchange, needs further study.

The presence of bispecific IgG4 antibodies creates two new questions:

1 What is the mechanism of this half-molecule exchange?
2 Is there any (patho-)physiological significance of this bispecificity, for instance in regard to helminth infections and to allergen-specific immunotherapy, or is the generation

of bispecific antibodies a mechanism to generate monovalent antibodies. Monovalent antibodies might be beneficial to inhibit continuous immune-inflammation.

Our data indicate that the apparent monovalency of circulating IgG4 antibodies is owing to bispecificity. This indicates that results obtained with IgG4 antibodies have to be analysed with care. The hypotheses proposed, an intracellular failure of allelic exclusion or an extracellular mechanism involving the exchange of IgG4 half-molecules would explain three observations: first, the apparent monovalency of polyclonal IgG4; second, the difference in valency between polyclonal and monoclonal IgG4, and third, the bispecific activity of IgG4 in selected sera.

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