

SEROLOGIC ASPECTS OF IgG4 ANTIBODIES

I. Prolonged Immunization Results in an IgG4-Restricted Response¹

ROB C. AALBERSE, RUTH VAN DER GAAG,² AND JOLANDA VAN LEEUWEN

From the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

Labeled antigen-binding tests were used to determine quantitatively the contribution of IgG4 antibodies to the total IgG antibody response in humans. In agreement with literature, we found no IgG4-restricted antibody responses with tetanus toxoid or streptococcal carbohydrate. In the serum of individuals immunized for several years with phospholipase (PLA) from honey bee venom, grass pollen allergen, or house dust mite allergen, we often found that more than 50% of the total antigen-binding capacity was due to IgG4 antibodies. In the case of beekeepers, it could clearly be shown that during prolonged immunization a shift in the IgG4:IgG1 antibody ratio occurs that finally results in an IgG4-dominated antibody response. Evidence is provided that antigen-binding assays may even underestimate the contribution of IgG4 antibodies, because in contrast to IgG1 antibodies, IgG4 antibodies act as monovalent antibodies in being unable to cross-link immunosorbent-bound antigen and radiolabeled antigen.

In humans, IgG1, IgG2, IgG3, and IgG4, the subclasses of IgG, constitute approximately 65, 25, 6, and 4%, respectively, of the IgG in serum from Caucasian adults. Antibody activity in IgG is not necessarily distributed over the subclasses in this proportion, but may be restricted to some subclass. Well-established examples are anti-carbohydrate antibodies, which are often restricted to the IgG2 subclass, and isoantibodies to red cells, which are mainly IgG1 and IgG3 (for a review, see Reference 1).

In hay fever patients, IgG antibodies to grass pollen were found to belong mainly to the IgG4 subclass (2, 3). In beekeepers, antibodies to phospholipase A2 (PLA)³ from honey bee venom are also restricted to the IgG4 subclass (4); in contrast, as the results presented here will show, in individuals who have been stung only a few times, the IgG antibodies to PLA are predominantly of the IgG1 subclass.

A major part of this investigation is devoted to give a reliable estimate of the contribution of IgG4 antibodies to the total IgG antibody activity. Neither of two commonly used methods to determine antibody activity in a subclass—the indirect hemag-

glutination test and the labeled antiglobulin-binding test, respectively—allow an accurate estimation of the ratio of antibody activity in the IgG4 subclass relative to that in the total IgG. The anti-subclass reagents recognize only a small number of antigenic determinants on the IgG molecule; for this and other reasons ("avidity" differences), the number of IgG4 antibody molecules, required for agglutination by anti-IgG4 antibody, is higher than that required for agglutination by anti-IgG_{total}. The contribution of IgG4 antibody to the total IgG antibody activity, therefore, cannot be deduced from the ratio of the agglutination titer with the two antiglobulin reagents. A similar situation is encountered with the labeled antiglobulin-binding assay. Even if an estimate of the relative sensitivity of the two antiglobulins can be made by chemically coupling known amounts of purified IgG4 protein either to red cells or to Sepharose particles, these estimates require assumptions on the availability for anti-Ig of the coupled Ig.

For a direct comparison of the antibody activity of subclasses, labeled antigen-binding assays are more informative; an excess of antiglobulin reagent will separate all Ig of that particular subclass from the other Ig; the results are thus independent of binding characteristics of the antiglobulin reagent used.

For the interpretation of the results of antigen-binding assays, the valency of the antibody is of relevance. When an antibody that is functionally monovalent (i.e., will bind only one antigen molecule under antigen-excess conditions) is compared with a bivalent antibody, the latter antibody will obviously score twice as high in an antigen-binding assay, whereas both antibodies may give identical responses in antiglobulin-binding assays. The most reliable way of measuring the functional valency would have been to purify IgG4 antibodies and to measure the antigen-binding capacity per milligram of antibody. Unfortunately, we have not succeeded as yet in obtaining a sufficiently pure IgG4 antibody preparation. Therefore, we used another approach: we tested whether the antibodies could cross-link two antigen molecules: a solid-phase-coupled antigen on the one hand and a labeled antigen on the other.

MATERIALS AND METHODS

Sera. Sera with IgG antibodies to PLA were in general obtained from asymptomatic beekeepers; most experiments reported here were performed with a typical beekeeper serum, serum Bru. An exception was serum Elv; this was obtained from someone who was accidentally stung. Sera with IgG antibodies to allergens in grass pollen or in cultures of house dust mites (*Dermatophagoides pteronyssinus*) were obtained from allergic patients who had received hyposensitization treatment. These sera were collected by Dr. R. Voorhorst and Dr. V. Knul-Bretlova, Department of Allergology, Academisch Ziekenhuis, Leiden, and by Dr. P. Vooren, Department of Lung Diseases, Academisch Ziekenhuis, Leiden, and Dr. P. H. Dieges, Department of Allergology, Academisch Ziekenhuis, Rotterdam.

Sera with IgG antibodies to tetanus toxoid were obtained from laboratory workers 2 wk after a booster injection of toxoid with AIPO₄ as adjuvant. Sera with IgG antibodies to group A streptococcal carbohydrate were

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¹ This investigation was supported by the Netherlands Asthma Foundation, Grant 267. Correspondence: Publication Secretariat, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, P.O. Box 9406, 1006 AK Amsterdam, The Netherlands.

² Present address: Netherlands Ophthalmic Research Institute, c/o Academic Hospital, Amsterdam, The Netherlands.

³ Abbreviations used in this paper: PLA, phospholipase A2; RIA, radioimmunoassay; RIP, radioimmunoprecipitation; EDTA, ethylenediaminetetraacetic acid.

selected from sera submitted to the rheuma-serology department, or from normal laboratory workers.

Antigens. PLA was obtained from Sigma. For radiolabeling, 50 μ g PLA were incubated with 1 mCi ^{125} I (Amersham) and 20 μ g chloramine-T in phosphate-buffered saline. After 1 min, 48 μ g sodium metabisulfate was added, followed by 6 mg carrier protein (bovine serum albumin [BSA] obtained from Poviet, Amsterdam). The product was gel filtrated over Sephadex G-75, and the fractions corresponding to the main labeled, macromolecular peak were pooled. Tyraminated group A streptococcal carbohydrate was a gift from Prof. Dr. D. Braun, Ciba-Geigy, Basel. This material was labeled with ^{125} I, as described above for PLA. Allergens were isolated from grass pollen (*Phleum pratense*) and house dust mite cultures, respectively (both from Diephuis, Groningen), by gel filtration and electrophoresis on Pevikon C870 (Serva). The allergen preparations were labeled as described for PLA. Tetanus toxoid was a gift from the Rijksinstituut voor Volksgezondheid (RIV), Bilthoven. The toxoid preparation contained 250 limit flocculation (Lf) units/mg protein; 200 μ g protein were labeled with 1 mCi ^{125} I and subsequently gel filtrated over Sephadex G-200.

Anti-IgG reagents. Protein A-Sepharose was obtained from Pharmacia. The anti-IgG_{total} and anti-IgG subclass antisera, prepared as described by Van der Giessen *et al.* (5), were from the Department for the Production of Immune Reagents. Immunosorbent-purified anti-IgG4 antibodies were provided by Dr. A. Vlug from the same department. The results of specificity tests of these antibodies have been described elsewhere (6). Anti-IgG4 Sepharose was prepared by coupling 1 mg purified antibodies to 100 mg CNBr-activated Sepharose (Pharmacia).

Total IgG1 and IgG4 assay. A competitive immunoradiometric assay (7) was used for the determination of total IgG1 and total IgG4. The test sample was added to 0.5 ml of 1 mg/ml suspension of Sepharose-coupled normal human serum (50 μ l serum per 100 mg CNBr-activated Sepharose); to this mixture, 50 μ l of a solution of ^{125} I-labeled anti-IgG1 or ^{125} I-labeled anti-IgG4 were added. After overnight incubation at room temperature under vertical rotation, Sepharose-bound radioactivity was measured.

Radioimmunoassays (RIA). RIA were performed in RIA buffer: PBS with 0.3 mg/ml BSA, 10 mM EDTA, 3 mM Na₂SO₄, and 0.1% Tween-20 (Baker), pH 7.2.

Radioimmunoprecipitation (RIP) assay. RIP assay for the quantitation of IgG_{total}-anti-PLA antibody was performed by incubation of a 250- μ l sample (diluted in RIA buffer to contain less than 0.2 mg/ml IgG) with 50 μ l ^{125} I-PLA (approximately 5 ng per test) for 1 hr at room temperature; then 100 μ l sheep anti-human IgG were added, and the mixture was incubated for 1 to 3 days at 4°C. One milliliter of buffer was added, and the tubes were centrifuged for 15 min at 4000 rpm in a refrigerated centrifuge; the precipitate was washed once with 2 ml cold RIA buffer, and then the radioactivity in the precipitate was measured. Nonspecific binding was measured with human serum samples without detectable antibody to the antigen; it was less than 1.5% of added radioactivity.

Sepharose-binding assays. These were performed with Sepharose-coupled staphylococcal protein A (Pharmacia) or with Sepharose-coupled antibodies to IgG4 (see above). To 0.5 ml of 1 mg/ml Sepharose suspension in RIA buffer, a 50- μ l sample was added, followed by 50 μ l labeled antigen. The mixture was incubated overnight on a vertical rotator at room temperature. The suspension was centrifuged and washed, and Sepharose-bound radioactivity was measured. Nonspecific binding was measured both with a buffer control and with human serum samples without detectable antibody to the antigen. In both instances, it was less than 1.5% of added radioactivity. Serum Bru was allotted 100 antibody units/ml in the protein A assay; because approximately 75% of the antigen-binding activity of this serum was found in the IgG4 fraction, we allotted this serum 75 antibody units in the anti-IgG4 assay. The difference between the antibody titer in the protein A assay and the IgG4 antibody titer is indicated as IgG1 antibody; strictly speaking, this difference represents non-IgG4 protein A-binding activity.

Two-site assay. For the two-site assay (8), which requires bivalent or multivalent antibody, the amount of Sepharose-coupled antigen proved to be critical; per 100 mg CNBr-activated Sepharose, we used 100 μ g PLA, or 250 μ g dialyzed, lyophilized timothee pollen extract, or 500 μ g dialyzed, lyophilized house dust mite culture extract. Sepharose-coupled antigen (0.5 ml of a 1-mg/ml suspension in RIA buffer) was incubated with a 50- μ l test sample overnight on a vertical rotator at room temperature. The suspension was centrifuged and washed. Sepharose-bound antibody was then allowed to react with ^{125} I-labeled antigen during a second incubation overnight, then Sepharose-bound radioactivity was measured. Nonspecific binding was measured both with a buffer control and with human serum samples without detectable antibody to the antigen. In both instances, it was less than 1.5% of added radioactivity.

Antigen-binding in agar gel (9, 10). This was performed in a two-step procedure. The human IgG antibody was first precipitated with anti-IgG_{total} (in the radioimmuno-electrophoretic experiments) or with anti-subclass antisera (in double-diffusion experiments). For radioimmuno-electrophoresis, the quantities of the sera tested were adjusted to contain equal antigen-binding activity in the protein A assay; the total serum concentration was kept constant at 2 μ l per analysis by addition of normal serum. After electrophoresis, the troughs were filled first with anti-IgG; after diffusion,

the gel was washed with a buffer that contained 3 mg/ml BSA and 1 mg/ml Na₂SO₄; then the troughs were filled with ^{125} I-PLA. For optimal precipitation in double-diffusion experiments with anti-IgG1, the sera were diluted 1/100 in PBS-BSA. With anti-IgG4, optimal precipitation required more antigen; in this case, the sera were therefore diluted 1/100 in 25% normal serum. The central wells received first the anti-subclass serum, then after the gel had been washed with PBS-BSA, the wells received ^{125} I-PLA.

DEAE chromatography. Five milliliters of serum were dialyzed against starting buffer (20 mM Tris-HCl, pH 8.4) and applied to a 2.6 x 10-cm column of DEAE-Sephacel (Pharmacia) equilibrated with the same buffer. The column was eluted with 20 mM Tris-HCl, pH 7.4, buffers with stepwise increasing NaCl concentrations. The highest IgG1:IgG4 ratio was found in the fractions that eluted without NaCl; the ratio was more than 300 times the ratio of the unfractionated serum. The lowest IgG1:IgG4 ratio was found in the fraction that eluted with 75 to 100 mM NaCl; this ratio was seven to 10 times less than the ratio in the unfractionated serum.

Chromatofocusing. Serum (2.5 ml) was dialyzed against starting buffer (25 mM ethanolamine-acetic acid, pH 9.4) and applied to a 0.9 x 31-cm column of polybuffer exchanger PBE 94 (Pharmacia). The column was eluted with 235 ml of a 1/10 dilution of polybuffer 96 (Pharmacia), adjusted to pH 6.0 with acetic acid, and then with 1 M NaCl in the same eluent.

Immunoelectrophoresis. Immunoelectrophoresis was performed in 1.3% agar in barbitone buffer, pH 8.6, for 2 hr at 38 V/slide.

RESULTS

The contribution of IgG4 antibodies to the IgG_{total} antigen-binding activity. An indication for the predominance of IgG4 antibodies in a serum can be found by analysis of the electrophoretic behavior of IgG antibodies; IgG4 antibodies have a higher mobility than IgG1 antibodies, and elute a higher salt strength from an anion exchanger. To illustrate this effect, sera with anti-bee venom activity were tested by radioimmuno-electrophoresis (Fig. 1). One serum, serum Elv, was obtained 2 mo after a few incidental bee stings; other sera were from two beekeepers, Bru and VI. The PLA-binding activity of the IgG of serum Elv and of the first sample of serum VI had a distinctly lower electrophoretic mobility than that of serum Bru and the last sample of serum VI.

With precipitating anti-subclass sera, a semi-quantitative analysis was performed by double diffusion in agar and autoradiography (Fig. 2); no activity was found in these sera with anti-IgG2 or anti-IgG3, although the sera were tested five times more concentrated (i.e. diluted 1/20 instead of 1/100).

Quantitation of the contribution of IgG4 antibodies to the total IgG antigen-binding activity was obtained with Sepharose-coupled anti-IgG4 antibodies. Serum was incubated with ^{125} I-PLA and then treated with either Sepharose-coupled anti-IgG4

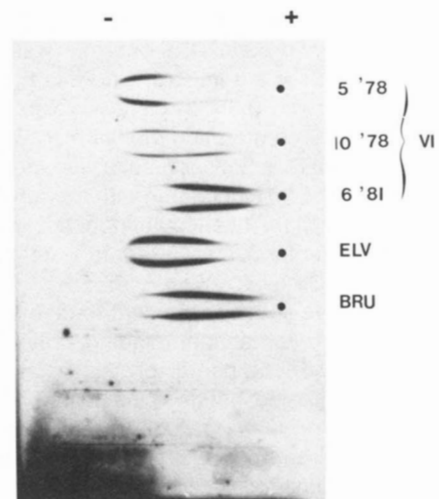


Figure 1. Radioimmuno-electrophoresis of five sera with antibodies to PLA. Serum Elv was from a donor who was incidentally stung by honey bees. Serum Bru was from an experienced beekeeper. Three serum samples of novice beekeeper VI were analyzed; sample 05.78 was obtained 6 wk after the first bee sting; the others were obtained at times indicated.

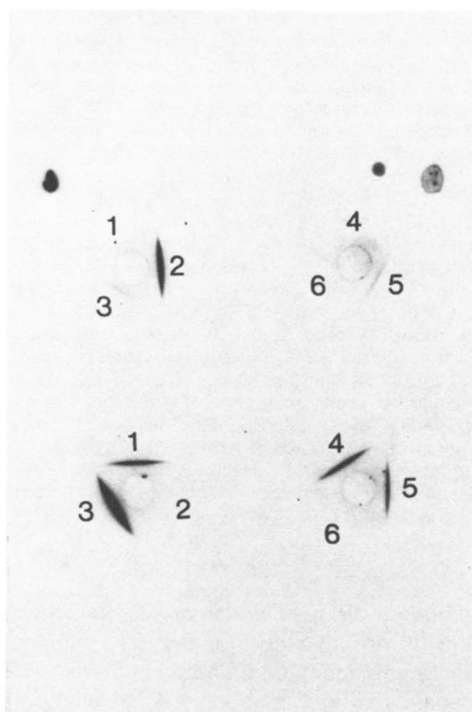


Figure 2. Radioactive indirect double diffusion test. Six serum samples were tested for anti-PLA antibodies of the IgG1 (top) or IgG4 (bottom) subclass. All sera were diluted 1:100. Sera 1, 3, 4, and 5 were from beekeepers; serum 2 was from donor Elv (see Fig. 1); serum 6 was a negative control used as diluent (see Materials and Methods).

or with Sepharose-coupled protein A. When an IgG4-enriched DEAE fraction of serum Bru was tested, virtually identical results were obtained with either protein A-Sepharose or anti-IgG4-Sepharose; when the IgG1-enriched fraction was tested, the protein A-Sepharose was over 10 times more effective in binding the labeled antigen (Fig. 3).

The results with the DEAE fractions indicate the specificity of the anti-IgG4-Sepharose. The results with the unfractionated serum indicate that a very large proportion of the total IgG antibody activity of the serum is of the IgG4 subclass. Therefore, we concluded that the anti-IgG4 Sepharose was sufficiently specific to use it for establishing the contribution of IgG4 antibody to the total PLA-binding activity of serum Elv and of serum Bru. When we tested 0.06 μ l of these two sera, the binding of ^{125} I-PLA to protein A-Sepharose was 14.6% for serum Elv and 16.1% for serum Bru, whereas the binding to anti-IgG4 Sepharose was 0.3% and 12%, respectively. This corresponds to a IgG4 contribution of less than 2% for serum Elv and 75% for serum Bru. Similar results were obtained when the immunosorbent was first incubated with the serum, washed, and then incubated with the labeled antigen; this indicates that the antiglobulin reagents do not interfere with the antigen-binding site of the antibodies.

We measured for five antigens the contribution of antibodies of the IgG4 subclass to the antigen-binding activity of IgG1 + 2 + 4 with anti-IgG4-Sepharose or protein A-Sepharose, respectively, and ^{125} I-labeled antigen; we tested PLA, tyraminated streptococcal group A carbohydrate, tetanus toxoid, grass pollen allergen, and house dust mite allergen. No IgG4 antibody to the streptococcal carbohydrate was found; this was according to the expectations, because these antibodies are predominantly found in the IgG2 subclass (1). The IgG4 contribution was moderate in the case of tetanus toxoid and was high with PLA, grass pollen, and house dust mite (Fig. 4;

the results with house dust mite are not shown).

Longitudinal studies in beekeepers. As a possible explanation for the differences in IgG4/IgG_{total} antigen-binding activity, we considered an effect of repeated antigenic stimulation. This was investigated in a follow-up study of novice beekeepers (Table I). The contribution of IgG4 antibody to the PLA-binding activity of these sera was low at first, and increased with time (and thus with the number of bee stings). The prolonged immunization in these subjects leads to an increase both in total PLA-binding activity and in PLA-binding activity of IgG4; these two do not, however, increase simultaneously: the relative contribution of IgG4 is low at first and increases with time. In view of the apparent monovalency of IgG4 antibodies, the weight ratio of IgG4 antibody to IgG1 antibody might be higher than the ratio of antigen-binding activities.

This finding prompted us to exclude us to exclude the possibility that in sera with a low total PLA-binding activity the contribution of IgG4 might be underestimated, or vice versa, that its contribution in sera with a high total PLA-binding activity might be overestimated. Therefore, we determined the ratio of ^{125} I-PLA binding to Sepharose-coupled anti-IgG4 with that to Sepharose-coupled protein A under various conditions: serum with a high ratio and a high binding activity was diluted either in buffer or in normal serum, or in serum Elv (with a low ratio and a high binding activity); in these three situations, the ratios did not differ significantly from the expected value.

The functional valency of IgG4 antibodies. IgG4 antibodies were found to be unable to cross-link two antigen molecules in a two-site RIA; when serum Elv or the IgG1 fraction of serum Bru were incubated first with Sepharose-coupled PLA, and then with ^{125} I-PLA, binding of radioactivity to the immunosorbent was found; in contrast, the IgG4 fraction of serum Bru had very little activity in this test system. The results with serum Elv, in which virtually all anti-PLA antibody (including the antibody found in the IgG4-enriched fraction) was of the IgG1 subclass, indicates that the effect of the DEAE fractionation found with serum Bru was not due to co-fractionation of an interfering substance with IgG4 (Fig. 5). Similar results were obtained in the grass pollen system with serum Hut (see Table II) and in the *D. pteronyssinus* system. These results might indicate that IgG4 antibodies are not capable of forming Ag₂Ab complexes; therefore, the contribution of IgG4 antibodies is possibly underestimated in a labeled antigen-binding assay.

The results presented in Table II indicate furthermore that the low isoelectric point is not due to a very acid antigen-combining site of these IgG4 antibodies, but is a characteristic of the total IgG4 subclass.

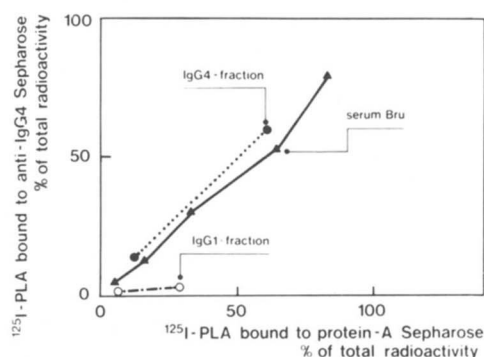


Figure 3. Comparison between the antibody-binding activity of anti-IgG4-Sepharose and protein A-Sepharose. Dilutions of serum Bru or of two DEAE-fractions of this serum were incubated with ^{125} I-PLA and with either anti-IgG4-Sepharose (ordinate) or protein A-Sepharose (abscissa).

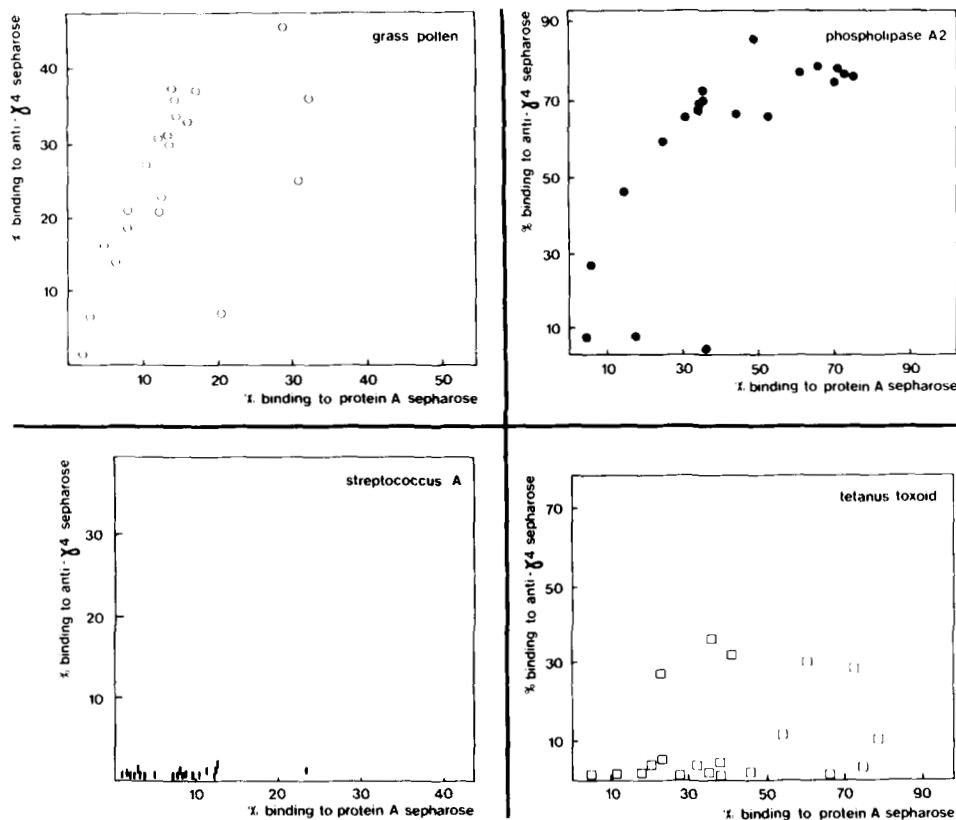


Figure 4. Comparison between the antibody-binding activity of anti-IgG-Sephacrose and protein A-Sephacrose. In contrast to the protocol of the experiment shown in Figure 3, we now tested four times more serum with anti-IgG4 Sephacrose than with protein A-Sephacrose to detect relatively low levels of IgG4 antibodies. For grass pollen and PLA, we used 1.6 and 0.4 μ l serum per test; for streptococcal group A carbohydrate, we used 4 and 1 μ l serum per test; for tetanus toxoid, 0.4 and 0.1 μ l serum per test.

TABLE I
Shift in the IgG4/IgG1 ratio of antibodies to PLA in beekeepers*

		IgG1 (antibody units/ml)	IgG4 (antibody units/ml)	IgG4 (% total antigen-binding activity)
Fok	May '78	7.8	0.1	1
	Oct. '78	11.7	1.0	8
	Apr. '79	5.6	4.4	44
	May '79	9.4	10.6	53
	June '81	14.8	59.0	80
VI	May '78	42.0	0.4	1
	Oct. '78	56.0	22.0	28
	June '81	87.0	240.0	73
Mr. Kön	May '79	<0.1	<0.1	—
	June '79	4.9	0.2	4
	Aug. '79	12.0	4.2	26
	June '81	47.0	294.0	86
Mrs. Kön	May '79	<0.1	<0.1	—
	May '79	1.2	<0.1	<8
	June '79	18.0	0.2	1
	Aug. '79	46.0	12.0	24
	June '81	88.0	559.0	86

* Serum samples from beekeepers were obtained in the first year of beekeeping and later. ^{125} I-PLA-binding activity was measured with either protein A-Sephacrose or with anti-IgG4-Sephacrose; the antibody levels were calculated by using serum Bru as a reference.

DISCUSSION

IgG4 antibodies appear to be exceptional in several respects. In many instances where IgG4 antibodies are formed, the contribution of this subclass to the antibody activity in total IgG is out of proportion to the total subclass levels: IgG4 is in general less than 5% of the total IgG, yet the antibody activity is often over 50% of the total IgG antibody. This seems to be especially common for all allergens that are known to be good inducers of IgE antibodies, such as grass pollen allergen, house dust mite allergen, and PLA. This may reflect a bias of

the investigators, because many other potential antigens have not been investigated as thoroughly. However, tetanus toxoid and diphtheria toxoid do not, as a rule, induce IgG4 antibodies to the same extent.

Another explanation might be that this subclass restriction reflects a dysregulation of the immune response in potentially atopic individuals. The beekeeper data are not consistent with this hypothesis, because virtually all beekeepers with more than 3 yr experience show this IgG4-restricted response; furthermore, in beekeepers with an IgG-restricted anti-PLA response, the anti-tetanus response is predominantly IgG1 and the anti-streptococcal carbohydrate response is predominantly IgG2 (unpublished observation).

Our hypothesis is that IgG4 antibodies become prominent upon chronic antigenic stimulation. This is based on the observation that IgG1 antibodies are most prominent in individuals with limited antigenic stimulation, such as is the case with prophylactic immunization with tetanus toxoid or after incidental bee stings (donor Elv); the effect of prolonged immunization is most clearly observed in the longitudinal investigation of novice beekeepers (Table II).

Consistent with this idea is our finding that in Schistosoma-infected patients a difference exists between recently infected patients and chronically infected patients: the relative contribution of IgG4 antibodies to the total IgG antibody response toward schistosomal egg antigens was higher in the latter group than in the former (11).

Because of the association between IgG4 antibodies and allergy, it has been suggested that IgG4 antibodies are pathogenic. Because we find, in general, high titers of IgG4 antibodies to PLA in asymptomatic beekeepers, this seems unlikely. We have been unable to demonstrate a basophil-sensitizing activity of IgG4 antibodies either *in vivo* or *in vitro* (6, 12).

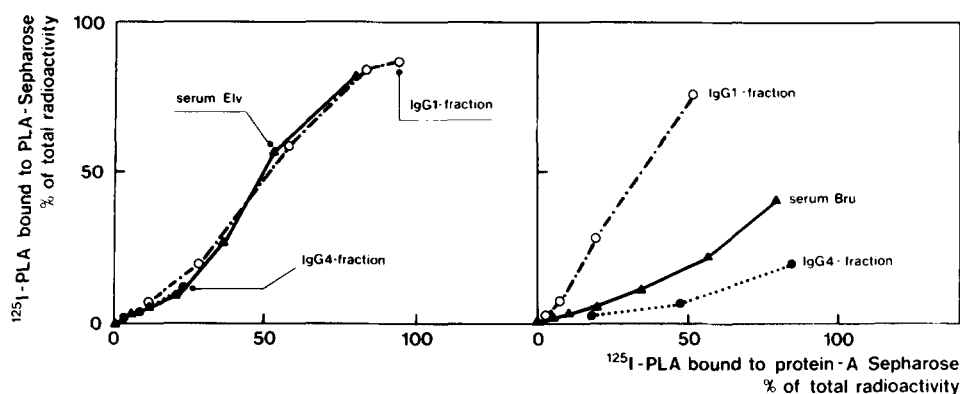


Figure 5. Inability of IgG4 antibody to participate in a two-site antibody assay. Unfractionated serum Elv (at left) or serum Bru (at right) or DEAE-Sephacel fractions of these sera were tested either with protein A-Sepharose and ^{125}I -PLA (abscissa) or with PLA-Sepharose and ^{125}I -PLA in a two-site assay (ordinate).

TABLE II
Chromatofocusing of serum Hut^a

pH Range	OD 280	Total IgG			Antibody	
		IgG	IgG1	IgG4	RIP	Two-site
8.4-7.7	0.33	17	32	<0.3	7	16
7.7-7.3	0.35	20	25	<0.3	6	15
7.3-7.0	0.26	16	10	1	9	17
7.0-6.7	0.12	6	4	3	5	6
6.7-6.4	0.05	3	1	6	6	4
6.4-6.0	0.03	1	1	5	3	1
6.0-1 M NaCl	3.74	9	3	40	24	7
Recovery		73	77	54	60	66

^a Serum Hut, obtained from a hay fever patient who had been treated by hyposensitization for 2 yr, was fractionated by chromatofocusing. The results of the total IgG assays and of the two anti-grass pollen antibody assays are expressed as percentages recovered of the total amount applied to the column.

From an immunopathologic point of view, IgG4 antibodies are presumably harmless, and possibly protective against complement-induced damage. Their role as allergen-blocking antibodies is currently under investigation. If, however, prolonged immunization indeed leads to the predominant formation of IgG4 antibodies, excessive vaccination may in some instances diminish complement-dependent protective immunity.

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