

SEROLOGIC ASPECTS OF IgG4 ANTIBODIES

II. IgG4 Antibodies Form Small, Nonprecipitating Immune Complexes Due to Functional Monovalency¹

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Human IgG4 antibodies directed against phospholipase A, the P1 antigen from *Dermatophagoides pteronyssinus* extracts, and cat albumin were found unable to cross-link antigen. Previously, it was demonstrated that IgG4 antibodies, in contrast to IgG1 antibodies, did not cross-link Sepharose-bound antigen and antigen added in solution. To eliminate the possibility that this phenomenon was caused by preferential binding of both IgG4 Fab fragments to the solid-phase-bound antigen, cross-linking of antigen was studied in a fluid-phase system. In this test, incapability of IgG4 antibodies to bridge two antigens was also found. As a result of such a phenomenon, it is expected that immune complexes formed by IgG4 antibodies will be considerably smaller than complexes formed by IgG1. This was confirmed by analysis of the molecular size profiles of IgG1- and IgG4-containing immune complexes in sucrose-density gradients. Moreover, IgG1 was able to precipitate antigen in a radioimmunoprecipitation test, whereas precipitation was not demonstrable by the same amount of IgG4 antibodies. Even 3% polyethylene glycol 8000 did not precipitate the small IgG4-containing immune complexes efficiently.

The antibodies studied were of a high-affinity type, and there was no significant difference in association constants between IgG1 and IgG4 antibodies. Therefore, we were not able to confirm observations reported in the literature that the IgG4 subclass is associated with a low-affinity antibody response; probably, the affinity of the IgG4 antibodies was underestimated by other investigators because of the polyethylene glycol precipitation technique used to separate antibody-bound and free antigen.

Our findings stress the point that IgG4 antibodies take a special place in the immune response upon chronic exposure to antigen.

In a previous study, it was shown that after prolonged antigenic stimulation with bee venom the IgG response against phospholipase A (PLA;³ a major component of bee venom) became dominated by IgG4 antibodies (1). IgG4-restricted responses are also reported in the literature in chronic parasitic infections like schistosomiasis (2) and filariasis (3).

Frequently, the IgG response in patients undergoing hyposensitization therapy with grass-pollen or house-dust mite becomes dominated by antibodies of the IgG4 isotype (4, 5). These "blocking antibodies," a term used to designate nonanaphylactic antibodies that compete with anaphylactic antibodies for antigen, do not precipitate antigen well, in contrast to most other human IgG antibodies (6-8). We made a similar observation in the PLA antigen system: precipitation of antigen by IgG1 antibodies was clearly demonstrable, but no precipitation was detectable with the same amount of IgG4 antibodies. Moreover, precipitation that occurred with IgG1 antibodies was even significantly inhibited when IgG4 antibodies were added. Parallel to this phenomenon, there was also inhibition of C1 activation by the IgG4 antibodies (9).

A clue to the cause of this absence of precipitation of the IgG4 isotype was the observation that IgG4 antibodies, in contrast to IgG1 antibodies, were unable to cross-link immunosorbent-bound PLA and radiolabeled PLA in a two-site radioimmunoassay (RIA) (1). This phenomenon, indicative for functional monovalency of IgG4 antibodies, was confirmed with the same procedure in the grass-pollen and the house-dust mite (*Dermatophagoides pteronyssinus*) antigen systems. In this test system, however, it could not be excluded that lack of reaction of IgG4 antibodies was in fact due to a tendency to cross-link two solid-phase-coupled antigens.

Therefore, in the present study, functional valency of IgG4 antibodies directed against PLA, P1 (the major allergen from *D. pteronyssinus*), and cat albumin was evaluated by investigating their ability to cross-link trinitrophenyl (TNP) and ¹²⁵I-labeled antigen in a fluid-phase system. Reduction by dithioerythritol (DTE) and pepsin digestion of the antibodies was applied to study the effect of artificially enhanced segmental flexibility on the ability to cross-link antigen (10, 11). The molecular size distribution of the immune complexes formed by IgG1 and IgG4 antibodies directed to cat albumin antigen was

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³ Abbreviations used in this paper: DTE, dithioerythritol, PLA, phospholipase A; RIP, radioimmunoprecipitation.

evaluated in sucrose-density gradients.

It was reported recently that the IgG4 subclass is associated with a low-affinity antibody response (12). However, in that study affinity calculations were based on results obtained after separation of antibody-bound antigen and free antigen by polyethylene glycol (PEG) precipitation. As PEG favors precipitation of larger molecules (13), differences in the size of IgG1- and IgG4-containing immune complexes might have influenced the affinity determinations. We compared affinity values of the IgG1 and IgG4 antibodies in the PLA and the cat albumin antigen system obtained after separation of antibody-bound antigen from free antigen by i) absorption to Sepharose-bound protein A; ii) precipitation by ammonium sulfate, and iii) precipitation by PEG 8000.

MATERIALS AND METHODS

Sera. Sera with IgG antibodies to PLA were obtained from asymptomatic bee keepers. Serum Elv, however, was obtained from a healthy person who was accidentally stung. Sera with IgG antibodies to allergens from cultures of *D. pteronyssinus* were selected from sera submitted for a routine serologic follow-up of hyposensitization treatment. Sera with IgG antibodies to cat albumin were obtained from allergic patients who received hyposensitization therapy with cat dander extracts. Serum SA was an exception: this cat-allergic patient did not receive hyposensitization. Fresh normal human serum was obtained from a healthy laboratory worker and contained no detectable antibodies against PLA, cat albumin, or P1 (major allergen of *D. pteronyssinus*). Blood was allowed to clot for 1 hr at 37°C, and serum was stored at -70°C. Hyperimmune antibodies against PLA, cat albumin, and P1 were raised in rabbits by immunization with antigen in complete Freund's adjuvant and booster injections in incomplete Freund's adjuvant. The rabbit antisera against PLA and cat albumin were allotted 1000 and 10,000 U, respectively. Antibody units of the human sera were read from a standard curve of the rabbit antiserum in the antigen-binding test with protein A-Sepharose.

Antigens. PLA was purchased from Sigma Chemical Co. (St. Louis, MO). Cat albumin was purified from cat serum by ion-exchange chromatography on DEAE-Sephacel and gel filtration on G-75 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden). Dried *D. pteronyssinus* cultures were obtained from Commonwealth Serum Laboratories (Melbourne, Australia) and extracted for 12 hr in phosphate-buffered saline (PBS) at 4°C (2% w/v extract). The allergen P1 (14) was purified from the *D. pteronyssinus* extract by affinity chromatography, using immunosorbent-bound murine monoclonal anti-P1; the antigen was eluted with a buffer containing 50% (v/v) ethylene glycol and 5 mM glycine at pH 11.0.

PLA, cat albumin, and P1 were radiolabeled with ¹²⁵I as described (1). TNP haptenization of the antigens was performed with trinitrobenzene sulfonic acid (TNBS; Sigma) in 0.2 M borate buffer, pH 9.0, for 3 hr at room temperature; the reaction was stopped by the addition of crystalline glycine and the mixture was dialyzed against PBS. Typically, in a solution of 0.5 mg/ml antigen, optimal haptenization was achieved with 0.05 to 1 mg/ml TNBS.

Anti-TNP beads. Polystyrene beads, purchased from SpheroTech Kugeln (Fulda, F.R.G.), were washed with water before use. Affinity-purified sheep anti-TNP antibodies (0.04 mg/ml in 0.1 M NaHCO₃, pH 9.0) were added to 1000 beads. Coating was continued for 2 days under rotation. Then the beads were washed with saline containing 0.2% Tween-20 (Baker, Deventer, The Netherlands) and finally with water and were dried by lyophilization.

Anti-IgG reagents. Sepharose-coupled protein A was obtained from Pharmacia. The anti-IgG4 reagent was described earlier (1). Anti-IgG4 Sepharose was prepared by coupling 1 mg purified antibodies to 100 mg CNBr-activated Sepharose (Pharmacia). RIA buffer consisted of 0.15 M PBS with 0.3 mg/ml bovine serum albumin (BSA; Poviet, Amsterdam, The Netherlands), 10 mM EDTA, 5 mM Na₂S₂O₈, and 0.1% Tween-20 at pH 7.4.

Reduction of antibodies. For the reduction of antibodies, DTE (Merck, Darmstadt, F.R.G.) was used. Serum was incubated for 1 hr at 25°C in the presence of DTE concentrations ranging from 1.6 to 25 mM. The reaction was stopped by the addition of *N*-ethyl maleimide (BDH Chemicals Ltd., Poole, U.K.) to a final concentration of 50 mM. After 30 min at 4°C, the solution was dialyzed against PBS. Reduction of antibodies in serum was monitored by measuring decrease in the titer of isoagglutinins.

Pepsin digestion of antibodies. The method described by Wernick et al. (15) was applied for the preparation of F(ab')₂ fragments of antibodies in serum. One volume of serum was mixed with 2 vol of a pepsin (Cooper Biomedical, Malvern, PA) solution in acetate buffer 0.1 M, pH 4.0, and the mixture was incubated for 24 hr at 37°C. The final concentrations pepsin ranged from 0.066 to 6.6 mg/ml. Pepsin digestion showed a concentration-dependent decrease in the antigen binding to protein A-Sepharose of both IgG1 and IgG4. In this way, splitting of the protein A-binding Fc portion and the antigen-binding F(ab')₂ portion was monitored.

Total antigen-binding capacity. The total antigen-binding capacity was measured with a modified Farr assay: 100 µl serum or serum dilution, 100 µl radiolabeled antigen, and 300 µl RIA buffer were incubated for 1 hr at 37°C. Then, 5 ml cold 50% saturated ammonium sulfate (1.96 M) were added. The precipitate was allowed to form at 4°C for 30 min. After centrifugation for 15 min at 2500 × G in a refrigerated centrifuge (4°C), radioactivity was counted in 2.5 ml of the supernatant, and the percentage of radioactivity in the precipitate was calculated. Serum dilutions were made in normal human serum.

Isotype-specific antigen-binding capacity. Antigen-binding capacity in the protein A-binding isotypes and in the IgG4 subclass was measured with a Sepharose-binding assay as described (1). Briefly, a 50-µl sample was added to 0.5 ml of 1 mg/ml protein A-Sepharose or anti-IgG4-Sepharose followed by 50 µl radiolabeled antigen. After overnight incubation on a vertical rotator, the suspension was washed and the Sepharose-bound radioactivity was measured.

The results of the Sepharose-binding assays were related to the binding of a standard serial dilution of the relevant rabbit antiserum to protein A-Sepharose and expressed in arbitrary antibody units. The difference between antigen binding onto protein A-Sepharose and anti-IgG4-Sepharose is indicated to be caused by IgG1 antibodies. Strictly speaking, this difference represents non-IgG4, protein A-binding activity.

Radiolabeling (RIP). Typically, RIP was performed by incubation of 0.1 ml serum and 0.1 ml of twofold dilutions of a mixture containing radiolabeled antigen and cold antigen in a ratio of 1:10. Final volume was made up to 0.5 ml with RIA buffer. After incubation for 72 hr at 4°C, 1 ml RIA buffer was added and the tubes were centrifuged for 20 min at 2500 × G in a refrigerated centrifuge (4°C). The precipitate was washed twice with cold buffer and then the radioactivity in the precipitate was measured. In the PLA antigen system, sera with antibodies were heat inactivated (30 min, 56°C) and mixed with an equal volume (50 µl) of fresh normal serum to rule out differences in Clq concentrations between sera tested.

Two-site assays. The direct immunosorbent-bound two-site assay was described in a previous paper (1). This test detects whether antibodies are able to cross-link immunosorbent-bound antigen and radiolabeled antigen that is added in solution.

A variant of this test is the indirect fluid-phase two-site assay. Cross-linking was allowed to occur in a solution containing radiolabeled antigen and TNP-haptenized antigen in a ratio of 1 to 100. Then, TNP antigen, whether free or in immune-complex form, was insolubilized by anti-TNP-coated polystyrene beads. After a wash procedure, solid-phase-bound radioactivity was measured as an indicator for cross-linking that occurred in solution.

The appropriate degree of TNP haptenization was assessed beforehand for each antigen. Diluted serum sample, ¹²⁵I antigen, and TNP antigen were incubated for 2 hr at 20°C. Then, an anti-TNP-coated bead was added and incubation continued overnight under slow horizontal rotation. Subsequently, the beads were washed with PBS and 0.01% Tween-20, and the radioactivity bound was measured. Serum controls without detectable antibodies showed less than 1% binding.

Determination of antibody affinity. Antibody affinity in unfractionated serum was measured with the method described by Steward and Petty (16). In this technique, antibody-bound antigen and free antigen are separated by precipitation with 50% saturated (1.96 M) ammonium sulfate. In addition, affinity values were calculated after separation of antibody-bound antigen from free antigen by absorption to protein A-Sepharose. Precipitation of complexes with 3% PEG 8000 (m.w. 8000, formerly called PEG 6000; Sigma) was performed as described by Devey et al. (12).

Molecular weight determination. First, a RIP test was performed with 100-µl samples of heat-inactivated serum SA (>95% IgG1 anti-cat albumin) and BL (>95% IgG4 anti-cat albumin) as described above. However, no fresh human serum was added to eliminate the influence of Clq binding on the immune complex size. Molecular size distribution of the immune complexes that were retained in the supernatants of the RIP was determined by zonal centrifugation of isokinetic sucrose-density gradients. The sucrose gradients (5 to

25%) were prepared according to Aarden et al. (17). RIP supernatant (450 μ l) was layered on gradients of 12 ml. The gradients were centrifuged in a Beckman SW-41 rotor at 35,000 rpm for 16 hr at 4°C. Fractions of 0.5 ml were collected. Albumin, IgG, C1q, and IgM were used as a marker. They were measured by turbidimetric immunoassay (18) or Mancini technique.

RESULTS

Antigen-binding assays. The IgG1:IgG4 antibody ratios were calculated from the results of the antigen-binding assays with protein A-Sepharose and anti-IgG4-Sepharose (Table I). There was a close correlation between the results of the Farr assay for total antigen-binding capacity and binding onto protein A-Sepharose with the sera that were studied in the cat albumin and PLA antigen systems. Therefore, a significant contribution of isotypes other than IgG1, IgG2, or IgG4 to the total antigen-binding capacity was unlikely. This was confirmed by the results of antigen-binding tests with Sepharose-coupled monoclonal anti-IgA and anti-IgM. Antibodies in the IgA and IgM isotypes contributed less than 5% to the total antigen-binding capacity of serum SA and serum Elv.

Radioimmunoprecipitation. Native serum SA with an IgG1-dominated response against cat albumin (<1% specific IgG4) precipitated over 80% of the antigen added at the optimal antigen to antibody ratio. Destruction of C1q by heat inactivation of the serum caused reduction of precipitation in the RIP. Nevertheless, 20% of the antigen that was added still precipitated under these circumstances, indicating that precipitation of the antigen was not strictly complement dependent.

Three sera with an IgG4-restricted response against cat albumin (<5% specific IgG1) precipitated less than 5% of the antigen added in the RIP. This result was not affected by heat inactivation of the sera.

In contrast to the cat albumin system, precipitation of the much smaller PLA (16 kDa) by IgG1 antibodies was completely C1q dependent; i.e. after heat inactivation, less than 5% of the antigen was precipitated at the optimal antigen and antibody concentrations. As was the case with cat albumin, no precipitation was detectable

with three sera with an IgG4-restricted response.

It was previously shown in the PLA antigen system (9) that addition of IgG4 antibodies to the same amount of IgG1 antibodies caused a 50% reduction in antigen precipitation and C1 activation of the IgG1 antibodies. This phenomenon was not found in the cat albumin antigen system.

Indirect two-site assay. Cross-linking of soluble ^{125} I antigen and TNP antigen, as detected in the indirect two-site assay, was clearly demonstrable with rabbit antisera and human sera with an IgG1 response. Sera with an IgG4-dominated response showed little activity in this test system (Fig. 1). Reduction by DTE of the IgG4 antibodies directed to PLA or cat albumin did not enhance their ability to cross-link antigen. DTE in the concentrations used for reduction had no effect on IgG1, as indicated by the unaltered results of the antigen-binding assay with protein A-Sepharose and indirect two-site assay.

Only with the highest concentrations of pepsin that were tested did we find a decrease in binding in the indirect two-site assay of IgG1 antibodies. There was no enhancement of binding with IgG4 in this test, indicating that the ability to cross-link antigen was not generated by this treatment (Table II).

Antibody-affinity determination of IgG1 and IgG4 directed against cat albumin and PLA. Antibody affinities calculated after separation of antibody-bound antigen from free antigen with 50% saturated ammonium sulfate (Farr assay) tended to be lower than the values calculated after adsorption of antibody-bound antigen to protein A-Sepharose. This phenomenon was also reported by Steward and Petty (16) when they compared ammonium sulfate precipitation with precipitation by anti-IgG. These lower affinities might be caused by dissociation due to high salt conditions in the Farr assay (19). Possibly, contribution of low-affinity antibodies in the Farr assay that are not detected in the protein A assay may play an additional role. It was found with both techniques that affinities of sera that contained predominantly IgG1 antibodies did not differ from sera that contained predominantly IgG4 antibodies (Table I). These results applied for the PLA antigen system as well as for the cat albumin system. The values indicated that both IgG1 and IgG4 antibodies were of a high-affinity type and approximated affinity of antibodies in the hyperimmune rabbit antisera.

Approximately 70% of the IgG4-containing immune complexes that were detected with the Farr method were precipitated by 3% PEG 8000 when antibody was added in excess. However, this percentage gradually decreased to less than 1% when the antibody-antigen ratio was shifted to antigen excess (Fig. 2). PEG and ammonium sulfate precipitated IgG1-containing immune complexes to a similar degree at antigen and antibody concentrations around the precipitation optimum. With these IgG1 antibodies, however, there was also a decrease in the amount of antigen precipitated in the PEG method when antigen was added in excess. This was not detected under the same circumstances in the Farr technique. Because of this incomplete separation of free and antibody-bound antigen, total antigen-binding capacity and, as a consequence, affinity constants could not be calculated reliably from the PEG data.

TABLE I

IgG subclass distribution and affinities of antibodies directed against PLA and cat albumin^a

	Protein A-binding isotypes (antibody units/ml)	IgG4 ^b	Kass (10 log liters/mol)
PLA antigen system			
Rabbit	1,000	—	9.7
Elv	156	7.0 (0.7)	9.4
Bru	32	95.3 (3.6)	9.3
Vi	324	92.8 (2.2)	9.5
Ko	396	93.3 (0.8)	9.7
Cat albumin-antigen system			
Rabbit	10,000	—	9.7
SA	71	<1.0	9.8
BL	147	106 (5.9)	10.0

^a Antibody units in the IgG1, IgG2, and IgG4 isotypes were read from a standard curve of the relevant rabbit antiserum in the antigen-binding test onto protein A. The hyperimmune rabbit antisera against PLA and cat albumin were allotted 1000 and 10,000 U, respectively. The coefficient of variation of this test was 5%. The contribution of IgG4 antibodies was calculated from the results of the antigen-binding test with protein A-Sepharose and anti-IgG4-Sepharose. Association constants were calculated according to the method of Steward and Petty (16) after separation of antibody-bound antigen from free antigen by adsorption onto protein A. The coefficient of variation of the affinity measurements was 1.9%.

^b Values are percent total antigen-binding capacity in protein A-binding isotypes; SEM is in parentheses.

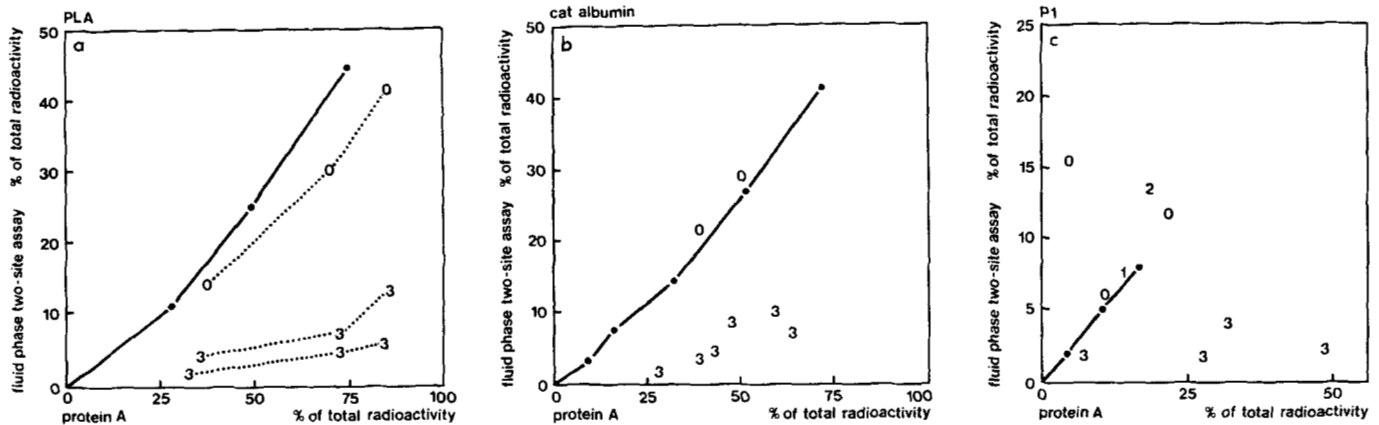


Figure 1. Indirect fluid-phase two-site assay vs protein A binding. Binding of radiolabeled antigen to protein A with 25 μ l of a diluted serum is compared with the binding in the two-site assay caused by 100 μ l of the same serum dilution. Twofold dilutions of the rabbit antisera are connected by solid lines. In the PLA system, twofold dilutions of the three human sera are interconnected by dotted lines. In the other antigen systems, human sera are tested in one serum dilution. Human sera are indicated by: 0, less than 25% specific IgG4; 1, 25 to 50% specific IgG4; 2, 50 to 75% specific IgG4; 3, over 75% specific IgG4.

TABLE II
Pepsin digestion^a

Pepsin (mg/ml)	Percent Radioactivity Bound			
	Antigen binding onto protein A-Sepha- rose	Indirect fluid-phase two-site assay	Antigen binding onto protein A-Sepha- rose	Indirect fluid-phase two-site assay
	Elv		V1	
0	71.5	29.3	68.9	4.7
0.066	26.1	36.5	26.3	5.9
0.66	3.2	26.7	4.1	4.2
6.6	2.1	16.7	2.8	3.8
	SA		BL	
0	47.4	25.0	57.0	5.8
0.066	24.4	24.3	28.7	5.9
0.66	3.3	18.9	10.0	4.4
6.6	2.1	5.2	5.5	3.1

^a Influence of pepsin digestion of antibodies on i) the antigen-binding capacity in the protein A-binding isotypes, and ii) the ability of the antibodies to cross-link antigen, as detected in the fluid-phase two-site assay. There is a progressive reduction in protein A binding when the concentration pepsin is increased. In this respect, there is no difference between sera that contain predominantly IgG1 (Elv, SA) or IgG4 (V1, BL) antibodies. Cross-linking of the antigens by IgG1 antibodies is only affected in the highest pepsin concentrations. No enhancement of cross-linking is observed with the IgG4-containing sera.

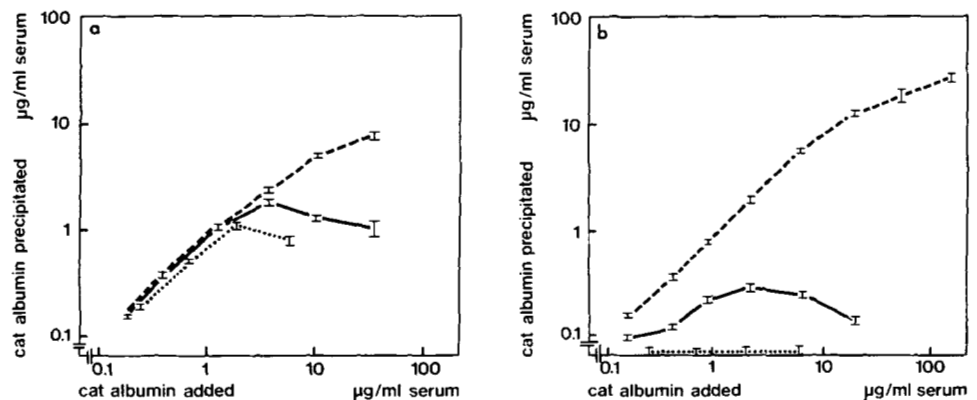
Sucrose-density gradients. To compare the molecular size distribution of the immune complexes formed by IgG1 and IgG4 antibodies directed against cat albumin, sucrose-density centrifugation profiles were made. Serum SA, which contained 71 U IgG1 anti-cat albumin

and less than 0.7 U IgG4, and serum BL, containing 147 U IgG4 anti-cat albumin and less than 1.5 U IgG1, were tested first in the RIP. Subsequently, supernatants obtained in this test were layered on the gradients. With serum SA, approximately 80% cat albumin was precipitated in the RIP or found in the pellet fraction after ultracentrifugation. This result was not influenced by heat inactivation or Clq depletion of the serum. In contrast, complexes formed by serum BL were not precipitated in the RIP, and less than 15% sedimented to the pellet fraction of the gradients (Fig. 3). Nevertheless, nearly all of the antigen was bound by antibody, as is indicated by the fact that less than 5% free cat albumin was detected after ultracentrifugation. These results demonstrate that IgG1 antibodies form considerably larger immune complexes than IgG4 antibodies. Calculated from the sedimentation data, the IgG4-containing immune complexes are made up of two to five IgG4 antibody molecules. It was shown in the RIP that the largest IgG1-containing complexes were formed at a distinct antibody to antigen ratio. Increase in antibody to antigen ratio resulted in the formation of smaller complexes. The sucrose-density gradients, however, showed that IgG4 antibodies formed the largest complexes in antibody excess. These results fit in with a monovalent behavior of IgG4 antibodies.

DISCUSSION

The IgG4 isotype represents approximately 4% of the total IgG in serum of adults. Nevertheless, in certain

Figure 2. Precipitation of immune complexes by 50% saturated ammonium sulfate (---) or 3% PEG 8000 (—) is compared with precipitation in buffer only (RIP) (···). a, Results obtained with serum SA (over 95% IgG1 antibodies); b, results with serum BL (over 95% IgG4 antibodies).



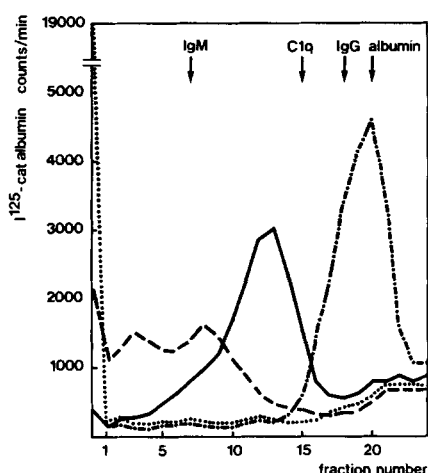


Figure 3. Sucrose-density gradient ultracentrifugation of IgG1- and IgG4-containing immune complexes. Supernatants of the RIP with 100 μ l heat-inactivated SA (>95% IgG1 anti-cat albumin) or 100 μ l BL (>95% IgG4 anti-cat albumin) and various amounts of cat albumin were layered on the gradients. Fraction 0 is the sum of antigen precipitated in the RIP and antigen recovered in the pellet fraction of the gradient. (---), Free cat albumin, no serum added; (—), BL with 600 ng cat albumin; (- - -), BL with 66 ng cat albumin (antibody excess); (···), SA with 200 ng cat albumin.

instances there is a significant contribution of IgG4 antibodies to the IgG response and IgG4 may even dominate this response. As was shown earlier, prolonged antigenic stimulation with PLA, a major component of bee venom, results in an IgG4-dominated antibody response in bee keepers (1). Ottesen et al. (3) reported a prominence of IgG4 antibodies in the antigen-specific IgG response in patients with filariasis. Iskander et al. (2) described high IgG4 antibody titers in patients with schistosomiasis. Adler et al. (20) described high IgG4 anti-thyroglobulin titers in patients with autoimmune thyroiditis. IgG4 antibodies often constitute a major part of the "blocking antibody" response in patients who received hyposensitization therapy for allergic disorders (4, 5). All of these situations where an IgG4-restricted response is found are characterized by prolonged exposure to high antigen concentrations.

Blocking antibodies do not precipitate antigen well (6, 7), and it has even been suggested to make use of this property for quantification of these antibodies (8). This fits in with our results showing that IgG4 antibodies form only small immune complexes that are not precipitated in standard precipitation techniques. In contrast, cat albumin (68 kDa) and even the much smaller PLA antigen (16 kDa), were clearly precipitated by IgG1 antibodies under the same conditions. In the PLA-antigen system, immune precipitation and complement activation by IgG1 were even inhibited by the addition of IgG4 antibodies (9). This phenomenon resembled inhibition of precipitation and complement activation as described for IgA in humans (21) and for noncomplement-fixing IgG isotypes in guinea pigs and rabbits (22, 23).

Margni et al. (24) investigated nonprecipitating antibodies to TNP haptens in rabbits and concluded that the absence of precipitation was due to functional monovalency of these antibodies. Although both Fab fragments could bind antigen, they reported a significant difference in affinity of the two antigen-binding sites in the intact antibody molecule.

The results of the direct and indirect two-site assays

with IgG4 antibodies to cat albumin, PLA, and P1 indicate that the human IgG4 isotype is also unable to cross-link antigen in these systems and therefore behaves as a monovalent antibody.

Sera with a predominant IgG4 response against cat albumin form only small, nonprecipitating immune complexes. In antibody excess, the complexes are of maximum size: up to four or five antibody molecules were bound to a single antigen molecule. Comparable amounts of IgG1 antibodies form much larger complexes, which precipitate antigen in the RIP and in agar gel double diffusion. This observation is consistent with the view that IgG4 antibodies are functionally monovalent and unable to cross-link antigen.

IgG4 is classically described as a non-complement-fixing antibody (25). The observation made by Isenman et al. (26) that the Fc fragment of IgG4 was as potent a C1 activator as the Fc fragment of IgG1 is interesting in this context. Oi et al. (10) and other investigators (27) pointed out that flexibility of the hinge region of antibodies is essential for their effector function. Isotypes that activated complement also showed the highest degree of segmental flexibility. The F(ab')₂ fragments of different isotypes all showed the same maximal segmental flexibility. Furthermore, segmental flexibility of intact immunoglobulins was enhanced by reduction with 2 mM dithiothreitol. Aarden et al. (28) found that DTE reduction of human IgG antibodies directed against DNA caused a sixfold increase in antigen binding.

It is tempting to speculate that the inability of IgG4 to activate complement and the inability to cross-link antigen is the result of rigidity of the hinge region. Attempts made to enhance mobility of the two Fab regions of the IgG4 antibody by reduction or pepsin digestion, however, did not generate the ability to cross-link antigen in the two-site assays. Pepsin digestion was also reported to be unsuccessful in abolishing functional monovalency of nonprecipitating rabbit antibodies (11).

Devey et al. (12) described the IgG4 subclass as associated with a low-affinity antibody response against tetanus toxoid.

All of the IgG1 and IgG4 antibodies that we studied in the cat-albumin and PLA-antigen system were of a high-affinity type, without significant difference in affinity values between these two subclasses. Likewise, we found no indications for the existence of affinity differences between IgG1 and IgG4 antibodies in other antigen systems (results not shown). Devey et al. (12) applied precipitation by 3% PEG 8000 for the separation of antibody-bound antigen and free antigen in their antibody-affinity measurements. This procedure may have mimicked a lower affinity of IgG4 antibodies. PEG is expected to induce a more efficient precipitation of complexes with the highest molecular volume (13). Smeenk and Aarden (19) reported for human antibodies, directed against DNA, that small complexes were only precipitated by 50% saturated ammonium sulfate and not by 3.5% PEG 6000. Indeed, the bulk of the immune complexes in sera with an IgG4-dominated response against cat albumin (i.e., IgG4 over 90% of the total response) were not precipitated by 3% PEG 8000 in our experiments. The IgG1-containing complexes, too, were less efficiently precipitated compared with precipitation by ammonium sulfate. Therefore, we conclude that the PEG precipitation procedure is

unsuitable for comparison of affinities of IgG1 and IgG4 antibodies.

One might expect that a shift in the antibody response after prolonged antigenic exposure to monovalent, non-complement-activating antibodies is favorable for the organism. Complement-induced damage and other consequences of precipitating immune complexes could be avoided. The rarity of immune complex-induced disorders during hyposensitization therapy is possibly a result of this phenomenon.

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