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Inhibition of complement activation by IgG4 antibodies

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SUMMARY

Prolonged exposure to antigens may result in high IgG4 antibody titres as was shown in a previous paper (Aalberse *et al.*, 1983b). In novice bee keepers, a shift in the IgG1/IgG4 ratio of the response against phospholipase-A (PLA; a major component of bee venom) occurred. This resulted in an IgG4-dominated response after approximately 2 years of bee-keeping experience.

Subject of the present study was the influence of relatively high concentrations of IgG4 antibodies on the biological activity of immune complexes. In the PLA antigen model, it was demonstrated (a) that IgG4-containing immune complexes do not activate complement and (b) that IgG4 antibodies effectively inhibit immune precipitation and complement activation by IgG1 antibodies. Evidence is provided that IgG4 antibodies inhibit binding of C1q to IgG1 in mixed, IgG1- and IgG4-containing complexes.

It is proposed that IgG4 antibodies protect against the biological effects of the complement-fixing IgG subclasses. For this reason, determination of the total IgG response or just determination of antibody activity in the complement-fixing isotypes is insufficient in immune-complex diseases. The modulating effect of the non-complement-fixing isotypes should be taken into account to predict the biological activity of the immune complexes.

Keywords IgG4 complement immune precipitation antigen-binding assay phospholipase-A

INTRODUCTION

Most of the textbooks state that IgG4 antibodies can be distinguished from the other IgG subclasses by lack of interaction with the complement system. The inability to activate the complement cascade was demonstrated by Ishizaka *et al.* (1967) in experiments with native and chemically aggregated myeloma IgG4. However, Isenman, Dorrington & Painter (1975) provided evidence that IgG4-Fc fragments were equally well capable of activating C1 as IgG1-Fc fragments were. For this reason, it has been suggested that IgG4 antibodies might be complement-activating after interaction with antigen (Jeske & Capra, 1984).

The commonly used serological tests for diseases with a suspected immune-complex origin, such as bronchopulmonary aspergillosis, pigeon breeders disease and farmers lung, give no information about subclass distribution of the IgG response. However, we found that with some antigens the contribution of IgG4 antibodies to the total IgG response varies considerably. In a previous paper (Aalberse, van der Gaag & van Leeuwen, 1983b), it was shown that prolonged immunization with phospholipase-A (PLA) from honey-bee venom in novice bee keepers resulted in a shift from an IgG-1-dominated response to an IgG4-dominated response. Sera of individuals that were only

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occasionally stung contained predominantly IgG1 anti-PLA, whereas in sera of individuals that were frequently stung (experienced bee keepers) the anti-PLA antibodies were almost completely of the IgG4 subclass.

In the present study, this PLA model was used to study the interaction between IgG4-containing immune complexes and C1. Furthermore, the influence of IgG4 antibodies on complement activation by the other isotypes was investigated.

The effect of IgG4 antibodies on complement activation by PLA-anti-PLA immune complexes was measured with a radioimmunoassay as described by Hack *et al.* (1981b). This test detects the formation of $C\overline{1r}C\overline{1s}(C\overline{1}-In)_2$ complexes which indicates the amount of C1 activation and provides a simple and reliable method to measure classical-pathway complement activation *in vitro*.

Whenever purified antigen is available, a labelled antigen-binding test is the method of choice for the measurement of IgG-subclass antibodies as was discussed previously (Aalberse & van der Zee, 1983). In the present study, we applied a Sepharose-bound monoclonal antibody that reacts with the IgG1, IgG2 and IgG3 subclasses (anti-IgG123) to measure IgG antibodies in the complement-fixing isotypes; Sepharose-bound anti-IgG4 was used to measure IgG4 antibodies.

MATERIALS AND METHODS

Sera. Serum Elv was obtained from a person who was incidentally stung by honey bees. Serum Ko was obtained from an experienced bee keeper. Normal human serum contained no detectable anti-PLA antibodies and was stored at −70°C. In the radioimmunoprecipitation assay, the C1q solid phase, the rheumatoid factor solid phase and the C1-activation assay serum mixtures, containing equal amounts of fresh normal human serum as a complement source and heat-inactivated (30 min, 56°C) sera containing anti-PLA antibodies, were used. Dilutions of the anti-PLA-containing sera were made in heat-inactivated normal human serum.

Reagents. Purified C1q prepared from a Cohn-1 fraction according to Tenner, Lesaure and Cooper (1981) was a gift from Dr A. Hannema from this Institute. The monoclonal IgM rheumatoid factor (RF) from a type-II cryoglobulin, according to the classification of Brouet *et al.* (1974), was isolated by cryoprecipitation. The precipitate was redissolved at room temperature in 0·01 M sodium-acetate buffer (pH 4·0) containing 0·5 M NaCl in addition, and purified by gelfiltration on ACA-34 (LKB, Bromma, Sweden) in the same buffer. The macromolecular fractions were pooled and contained only IgM in double diffusion analysis. The immunosorbent-purified anti-IgG4 antibodies, prepared as described by van der Giessen, De Lange and van der Lee (1974), and the monoclonal anti-IgG123 reagent were from the Department for the Production of Immune Reagents (Dr A. Vlug). Specificity of the anti-IgG4 reagent was described by van Toorenenbergen and Aalberse (1981). Specificity of the murine monoclonal antibody directed against the IgG1, IgG2 and IgG3 subclasses was assessed by haemagglutination inhibition (van Loghem *et al.*, 1980) and enzyme-linked immunosorbent assay (ELISA) technique. Anti-IgG4 Sepharose and anti-IgG123 Sepharose were prepared by coupling 1 mg purified antibodies to 100 mg CNBr-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). PLA was obtained from Sigma Chemical Co. (St Louis, MO, USA) and was radiolabelled as described by Aalberse *et al.* (1983b). Protein-A Sepharose was obtained from Pharmacia (Uppsala, Sweden). Bovine serum albumin (BSA) was obtained from Poviet (Amsterdam, The Netherlands). RIA buffer consisted of 0·15 M phosphate-buffered saline (PBS) with 0·3 mg/ml BSA, 10 mM EDTA, 5 mM NaN_3 and 0·1% Tween-20 (Baker, Deventer, The Netherlands) at pH 7·4.

Antigen-binding assays. These tests were performed for the quantitation of the total antigen-binding capacity and the antigen-binding capacity in the IgG subclasses. Total antigen-binding capacity in serum was determined by a Farr assay: 100 μl serum diluted in PBS/Tween-20 0·1%, 100 μl ^{125}I-PLA and 300 μl RIA buffer were incubated for 1 h at 37°C. Then, 200 μl were added to 5 ml cold 50%-saturated (1·96 M) $(NH_4)_4SO_4$. The precipitate was allowed to form at 4°C for 30 min. After centrifugation for 15 min at 3000 *g* in a refrigerated centrifuge (4°C), radioactivity was counted in 2·5 ml of the supernatant and the percentage radioactivity bound in the precipitate was calculated. Sepharose-coupled protein-A, anti-IgG4 and anti-IgG123 were applied to measure the

antigen-binding capacity in the IgG1,2,4, IgG4 and IgG1,2,3 subclasses. To 0·5 ml of 1 mg/ml Sepharose in RIA buffer, 50 μl of diluted serum were added, followed by 50 μl ^{125}I-labelled PLA. The mixture was incubated overnight on a vertical rotator at room temperature. The suspension was centrifuged and washed and the radioactivity bound was measured. Controls with only buffer and with human sera without detectable anti-PLA antibodies gave binding of less than 1·5% of the radioactivity added.

Antibody unit. The total PLA-binding capacity of serum Elv was allotted 100 u/ml. One μl of serum Elv will bind 50% of 200 μl 20 ng/ml PLA.

Radioimmunoprecipitation (RIP). RIP and RIP inhibition were performed by incubation of 0·1 ml serum mixture and 0·1 ml of serial dilutions of an PLA mixture containing 4·4 μg/ml PLA and 0·6 μg/ml ^{125}I-PLA. RIA buffer was added to a final volume of 0·6 ml. After incubation for 72 h at 4°C, 1 ml of RIA buffer was added and the tubes were centrifuged for 20 min at 3,000 ***g*** in a refrigerated (4°C) centrifuge. The precipitate was washed twice with cold buffer and then the radioactivity in the precipitate was measured. Non-specific binding with control sera without detectable anti-PLA antibodies was less than 1% of the radioactivity added.

C1q solid phase and RF solid phase. C1q solid phase and RF solid phase were performed as described by Hack *et al.* (1981a) with some minor modifications. Polystyrene tubes were coated with C1q or RF in PBS (10 μg/ml) for 24 h at room temperature. The tubes were washed five times with RIA buffer. Then, 0·05 ml serum mixture and 0·05 ml serial dilutions of the ^{125}I-PLA mixture as described with the RIP were added. RIA buffer was added to a final volume of 0·3 ml. After incubation for 72 h at 4°C, the tubes were washed three times and the percentage radioactivity bound to the tubes was measured. Buffer controls and negative human sera gave less than 1% binding of the radioactivity added.

C1-activation assay. A serum mixture (0·05 ml) was incubated with 0·05 ml PLA diluted in Veronal-buffered saline for 72 h at 4°C. Thereafter, the samples were incubated for 30 min at 37°C to enable the formation of $C\overline{1r}C\overline{1s}(C\overline{1}\text{-In})_2$ complexes. This reaction was stopped by adding 0·05 ml 0·1 M EDTA (pH 7·4). The amount of $C\overline{1r}C\overline{1s}(C\overline{1}\text{-In})_2$ complexes, reflecting the degree of C1 activation, was measured by a radioimmunoassay, as described by Hack *et al.* (1981b). The results of this test are expressed in arbitrary units: 100 units equal maximum activation of the C1 added.

RESULTS

Application of the monoclonal anti-IgG123 reagent in the labelled antigen-binding test

The total antigen-binding capacity of serum Elv was one-third of the antigen-binding capacity of serum Ko (Farr assay). There was no difference between the total antigen-binding capacity as measured in the Farr assay and the antigen-binding capacity in the IgG1, IgG2 and IgG4 subclasses as measured with the protein-A system in serum Elv or serum Ko. This precluded a significant contribution of other isotypes than IgG1, IgG2 and IgG4 to the total antigen-binding capacity in these sera.

The contribution of complement-fixing IgG antibodies (IgG1, IgG2, IgG3) to the total IgG antigen-binding capacity was previously estimated indirectly by subtraction of the binding on anti-IgG4 Sepharose from the binding on protein-A Sepharose. The results of the labelled antigen-binding test with a monoclonal anti-IgG123 reagent that recently became available to us confirmed the results found with the indirect method. In serum Elv, less than 10% of the total PLA-binding capacity was found in the IgG4 subclass. Here, binding to protein-A Sepharose is indistinguishable from binding to anti-IgG123 Sepharose (Fig. 1a). In contrast, in serum Ko over 90% of the antigen binding was caused by IgG4 antibodies. With this serum, binding to anti-IgG123 Sepharose was less than 10% of the binding on protein-A and anti-IgG4 Sepharose (Fig. 1b).

In the following experiments, serum Ko was diluted 1:3 in a negative serum to obtain 100 antibody units/ml as in serum Elv.

C1q-dependent immune precipitation of PLA by human IgG1 antibodies

Immune precipitation of human IgG1 antibodies directed against PLA was found to be completely

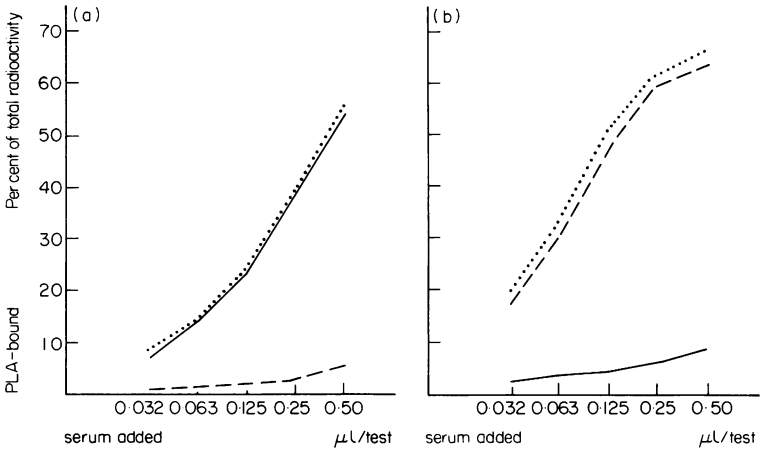

Fig. 1. IgG-subclass distribution of anti-PLA antibodies in serum Elv (a) and serum Ko (b) as detected with the labelled antigen-binding test. In serum Elv, IgG1 antibodies constitute over 90% of the IgG anti-PLA response. In serum Ko, over 90% of the IgG antibodies are of the IgG4 isotype. (· · · ·) protein-A Sepharose; (——) anti-IgG123 Sepharose; (– – –) anti-IgG4 Sepharose.

C1q-dependent. This phenomenon is illustrated in Fig. 2: heat-inactivated (30 min, 56°C) serum Elv and PLA were incubated with increasing amounts of purified C1q. Without C1q less than 2%, the PLA precipitated; however, with a concentration of C1q comparable with the concentration present in native serum, 15% of the PLA added was precipitated. None of the predominantly IgG4 anti-PLA-containing sera precipitated PLA with or without C1q present. In antigen systems with high molecular-weight antigens, for example with tetanus toxoid, we did not find a similar C1q-dependence of immune precipitation by human antibodies.

Influence of IgG4 antibodies on immune precipitation

With 50 and 100 antibody units from serum Elv, approximately 20% of the PLA added is precipitated at the precipitation optimum in the RIP. The same amount of antibody from serum Ko did not precipitate PLA. Addition of 50 units IgG4 anti-PLA to 50 units IgG1 caused a 80% reduction in the amount of PLA precipitated by 50 units IgG1 anti-PLA alone (Fig. 3a).

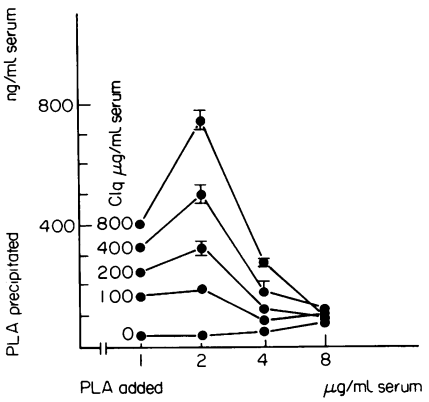

Fig. 2. Influence of C1q on immune precipitation. Various concentrations of purified C1q diluted in RIA buffer were added to heat-inactivated (20 min, 56°C) serum Elv, containing predominantly IgG1 anti-PLA. Final volume and precipitation conditions as described in Materials and Methods.

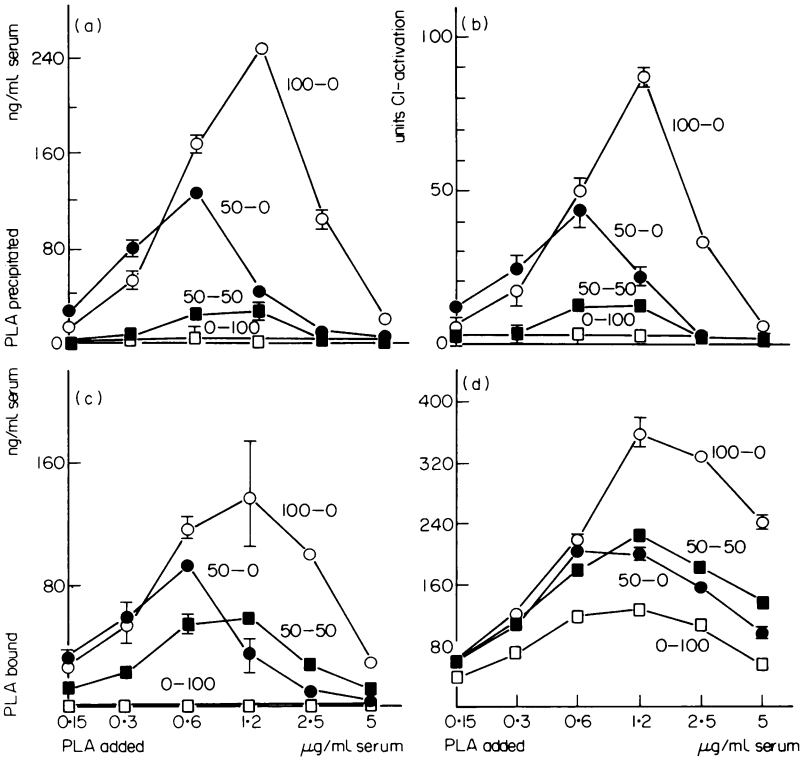

Fig. 3. (a) Radioimmunoprecipitation (RIP), (b) C1-activation assay, (c) C1q solid-phase assay and (d) rheumatoid-factor (RF) solid-phase assay, with serum Elv (> 90% IgG1 anti-PLA) and serum Ko (> 90% IgG4 anti-PLA).
100–0: 100 antibody units of serum Elv.
50–0: 50 antibody units of serum Elv.
0–100: 100 antibody units of serum Ko.
50–50: 50 antibody units of serum Elv plus 50 antibody units of serum Ko.

Influence of IgG4 antibodies on C1 activation

The results of the C1-activation test are almost identical to that of the RIP. As with the RIP, addition of IgG4 antibodies to IgG1 antibodies resulted in a 75% decrease of complement activation (Fig. 3b). Inhibition of immune precipitation and C1 activation was already demonstrable when IgG4 antibodies represented only 20 to 25% of the total PLA-binding capacity (data not shown). Inhibition of complement activation and immune precipitation by IgG4 antibodies as described above was confirmed with three other sera with an IgG4-dominated anti-PLA response.

Solid-phase C1q-binding assay

In agreement with the above-mentioned results, IgG4 antibodies inhibited the binding of IgG1 complexes onto the C1q solid phase (Fig. 3c).

Rheumatoid-factor solid-phase assay

It was investigated whether the inhibition of complement activation and immune precipitation by IgG4 antibodies was caused by inhibition of immune-complex formation. Binding of immune complexes to insolubilized rheumatoid factor was chosen as an indication for Fc aggregation and consequently for immune-complex formation. Binding of PLA-containing complexes was not significantly reduced when IgG4 antibodies were added to IgG1 antibodies (Fig. 3d). Even with IgG4 antibodies alone, a considerable binding to RF was found indicating the presence of complexes containing more than one antibody molecule.

Effect of human IgG4 anti-PLA on immune precipitation by rabbit anti-PLA

Although the human IgG1 anti-PLA investigated showed only precipitation in the presence of C1q, some rabbit antisera precipitated PLA without active complement present. This complement-independent precipitation was also inhibited by addition of human IgG4-anti PLA antibodies, suggesting other inhibition mechanisms of IgG4 antibodies in addition to inhibition of C1q binding.

DISCUSSION

IgG4 represents approximately 4% of the total IgG in adults. Nevertheless, immune responses dominated by IgG4 antibodies do occur. Significant titres of IgG4 are especially found after prolonged antigenic stimulation. For example, IgG4 often dominates the IgG response after hyposensitization therapy with allergens like house-dust mite and grass pollen (Aalberse *et al.*, 1983a). Likewise, in experienced bee keepers with chronic exposure to bee venom, an IgG4-restricted response against PLA is found (Aalberse *et al.*, 1983b).

Classical 'blocking antibodies', a term used to designate all non-anaphylactic antibodies that compete for antigen with anaphylactic antibodies, do not precipitate antigen well in contrast to many other human IgG antibodies (Sherman, 1941; Mansfield *et al.*, 1980). IgG4 is often strongly represented in these blocking antibodies (Aalberse *et al.*, 1983a). Our finding on precipitation inhibition by IgG4 antibodies fits with these observations. Precipitation of PLA with IgG1 was demonstrated to be C1q-dependent. This is probably due to the small molecular size of PLA (17 kD).

We conclude, however, that inhibition of precipitation by IgG4 antibodies is not solely explained by the inhibition of C1q-binding, because inhibition was also found with rabbit antisera against PLA, or with tetanus toxoid as antigen. In these systems, the precipitation was not complement-dependent.

The binding of IgG4-containing complexes to insolubilized RF indicates that Fc aggregation of IgG4 antibodies does occur after interaction with the PLA antigen.

Low affinity of IgG4 antibodies directed against tetanus toxoid was reported in the literature (Devey *et al.*, 1985). The IgG1 and IgG4 antibodies described in our experiments, however, showed no significant difference in avidity (results not shown).

Neither native myeloma IgG4 nor bis-diazo benzidine-aggregated IgG4 activates complement (Ishizaka *et al.*, 1967). The observation of Isenman *et al.* (1975) that IgG4-Fc fragments, in contrast to intact IgG4, do activate C1, prompted the question whether IgG4 antibodies, after interaction with antigen, would activate C1 (Jeske & Capra, 1984; Turner, 1977). In the present study, it is demonstrated that IgG4 antibodies in immune-complex form do not cause complement activation. In contrast, there was a pronounced inhibition of activation when IgG4 antibodies were added to IgG1-anti-PLA antibodies. It is tempting to postulate that the appearance of IgG4 antibodies after prolonged antigenic stimulation is a protective mechanism against complement-induced damage.

Griffis (1983) described a similar inhibition of complement activation by IgA antibodies resulting in a reduction of complement-mediated lysis of bacteria. In the sera used in our experiments, there was no significant antigen binding in the IgA class.

Inhibition of complement activation by a non-complement-fixing IgG subclass was demonstrated in the guinea pig by Minami & Utsumi (1981).

It seems likely that IgG4 antibodies inhibit complement activation by interdigiting between IgG1 antibodies in the immune complex, thereby reducing the degree of IgG1-Fc aggregation, which is necessary for C1 fixation. Inhibition of complement activation by IgG4 antibodies was less pronounced when a model system with cat albumin (mol. wt approximately 68 kD) as an antigen was applied (results not shown here). This may indicate that the molecular size of the antigen is decisive for the degree of inhibition.

Among the mechanisms proposed in the literature (Atsumi *et al.*, 1971; Margni *et al.*, 1977; Klinman & Karush, 1967; Margni & Binaghi, 1972), impaired flexibility in the heavy-chain hinge region, as described by Oi *et al.* (1984) for murine monoclonal antibodies, seems an attractive

explanation for the deviating behaviour of IgG4 with respect to the impaired effector function (i.e. complement activation).

Inhibition of precipitation and complement activation by IgG4 antibodies demonstrates the necessity to measure antibody activity in all subclasses to predict biological effects of the humoral immune responses in immune-complex diseases. Relatively high concentrations of non-complement-fixing isotypes modulate the biological activity of the antibodies in the other isotypes. Measurement of the complement-fixing antibodies is insufficient and should be supplemented by information about the other isotypes.

Whenever purified antigens are available, the ^{125}I-labelled antigen-binding assay is the method of choice for subclass antibody measurements. The anti-IgG123 reagent in combination with anti-IgG4 proved to be useful in this method to make a distinction between complement-fixing and complement-inhibiting IgG subclasses. With complex antigen mixtures, the radiolabelled antigen-binding test is not advantageous. In that case, the reversed method with solid-phase antigens and labelled anti-subclass antibodies is more convenient; however, blocking experiments should be included to prove here whether or not the antibodies in the different subclasses are directed against the same antigen. Alternatively, tests like the complement-fixation test and precipitation techniques may be more appropriate to predict the biological activity of the immune complexes.

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